CRISTINA YUMI MIYAKI

**RECONSTRUÇÃO DA HISTÓRIA EVOLUTIVA NEOTROPICAL:
AVES COMO MODELOS DE ESTUDO**

TEXTO APRESENTADO AO INSTITUTO DE BIOCÊNCIAS
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RESUMO

Estudos evolutivo-moleculares de organismos da região neotropical têm revelado que a história dessa região é bastante complexa. Mudanças climáticas e geotectônicas parecem ter influenciado bastante no padrão biogeográfico desses organismos. E as aves tem sido um excelente modelo para reconstruir essa história devido a várias características, como uma taxonomia relativamente bem estabelecida (comparado a outros grupos) e presença em vários biomas e ambientes. Nesse cenário nosso grupo tem contribuído com o aporte de dados filogenéticos e filogeográficos de diversos táxons de aves. Produzimos filogenias entre representantes de diversos gêneros, filogenias de espécies de determinados gêneros e estudos filogeográficos de diversas espécies e complexos de espécies, sempre buscando caracterizar possíveis eventos ou processos relacionados às divergências encontradas. No presente texto, apresento nossos dados relacionados a duas áreas nas quais temos contribuído mais: 1) a sistemática e biogeografia de psitacídeos neotropicais, e 2) a biogeografia da avifauna da Mata Atlântica.

ABSTRACT

Molecular evolutionary studies of Neotropical organisms have been revealing a complex history. Climatic and geotectonic changes seem to have influenced their biogeographical patterns. Birds have proven to be good models to reconstruct such history, as their taxonomy is relatively well established (compared to other organisms) and they occur in various biomes and habitat types. We have been contributing to the growing database of phylogenetic and phylogeographic data of various avian taxa with studies in various taxonomic levels, always trying to identify possible events or processes associated with divergence events. In this document, I present our data on two of the areas where we have been contributing most: 1) systematics and biogeography of Neotropical psitacids, and 2) biogeography of the Atlantic Forest avifauna.

RECONSTRUÇÃO DA HISTÓRIA EVOLUTIVA NEOTROPICAL: AVES COMO MODELOS DE ESTUDO

1. APRESENTAÇÃO

A região neotropical apresenta uma grande diversidade de táxons de animais e de plantas. Como um exemplo, aproximadamente 1.700 das cerca de 10.000 espécies de aves descritas no globo ocorrem no Brasil (Sick 1997). A origem dessa enorme diversidade é intrigante e deve estar relacionada com o complexo histórico da região que inclui processos vicariantes e eventos de dispersão. Oscilações climáticas e eventos geológicos ocorridos em diversos momentos da história da Terra geraram muitas mudanças que podem ter influenciado na origem e extinção de diversos táxons, e também na manutenção dessa alta diversidade. Diversas teorias [ex. vicariância geotectônica (Brown 1987, Cracraft & Prum 1988); hipótese do gradiente ecológico (Endler 1977); hipótese dos rios como barreiras (Wallace 1849); hipótese dos refúgios (Haffer 1969, Vanzolini & Williams 1970)] foram formuladas para explicar a riqueza da diversidade de espécies na região neotropical. Muitas delas podem ser testadas por meio de estudos dos padrões filogenético, biogeográfico e evolutivo dos organismos, uma vez que os resultados destes estudos podem elucidar os processos envolvidos (Marroig & Cerqueira 1997).

Algumas características, como os padrões de endemismo e a correlação entre relações sistemáticas e biogeográficas, tornam as Aves um grupo apropriado para estudos de biogeografia histórica. Além disso, como os táxons de Aves se distribuem nos mais diversos biomas, tais organismos são modelos de trabalho muito apropriados para estudar a origem dessa diversidade. Associado à utilização de técnicas moleculares em estudos filogenéticos e filogeográficos que possibilitam a datação molecular dos eventos de separação entre os táxons (Rheindt *et al.* 2009), os dados gerados permitem contribuir com conhecimentos sobre a origem da diversidade biológica da região neotropical (Ribas *et al.* 2012). Além disso, reconstruções filogenéticas e filogeográficas também podem contribuir na resolução de alguns problemas de sistemática.

Nesse cenário, no presente texto baseado em parte de nossa produção científica, pretendo realizar uma síntese dos nossos estudos filogenéticos e filogeográficos moleculares de Aves, visando agregar informação para compreender melhor a origem e a manutenção da sua diversidade.

2. SISTEMÁTICA E BIOGEOGRAFIA DE PSITACÍDEOS NEOTROPICAIS

Os psitacídeos (ordem Psittaciformes) são popularmente conhecidos como papagaios, araras e periquitos. Atualmente apresentam distribuição predominantemente Gondwânica, com extensa radiação na América do Sul e Austrália e uma radiação em menor escala na África e Ásia tropical (Rowley 1997, Collar 1997). Os registros fósseis mais antigos do grupo foram localizados no hemisfério norte: no final do Cretáceo (65 milhões de anos atrás [Ma.]) da América do Norte (Stidham 1998, mas ver comentário contrário de Dyke & Mayr 1999) e no Eoceno (53-36 Ma.) da Europa (Mayr & Daniels 1998). Tais registros indicariam uma possível origem do grupo no norte, com posterior dispersão para o sul, com a diversificação na Gondwana.

Morfológicamente, os psitacídeos constituem um grupo relativamente homogêneo. Dados de filogenias moleculares multigênicas indicam que, inesperadamente, as ordens Psittaciformes e Passeriformes são irmãos (Ericson *et al.* 2006; Hackett *et al.* 2008). Atualmente, as cerca de 350 espécies de psitacídeos estão

classificadas em duas famílias: Cacatuidae e Psittacidae. No entanto, dados de filogenias moleculares indicam que essa classificação necessita ser revista (de Kloet & de Kloet 2005, Wright *et al.* 2008). A família Psittacidae inclui duas subfamílias: Loriinae e Psittacinae. A tribo Arini, pertencente à subfamília Psittacinae, compreende todas as espécies neotropicais (Collar 1997). No Brasil ocorrem cerca de 70 espécies, constituindo o país de maior riqueza em número de espécies.

Atualmente, temos uma boa quantidade de dados filogenéticos moleculares de psitacídeos do neotrópico, tendo o nosso laboratório contribuído de forma considerável para esse cenário. Em um estudo inicial (Miyaki *et al.* 1998 – Anexo I), sequenciamos e realizamos análises filogenéticas com as sequências de genes mitocondriais de dez espécies de psitacídeos neotropicais pertencentes a dez gêneros. Observamos que as espécies neotropicais estudadas se agrupavam em dois clados: um de cauda longa (oito espécies) e outro de cauda curta (duas espécies). Com o objetivo de explorar melhor as relações filogenéticas entre os gêneros de cauda longa, obtivemos sequências mitocondriais mais longas de 14 espécies de nove gêneros do grupo de cauda longa (Tavares *et al.* 2004 – Anexo II). Observamos uma relação de espécies irmãs nunca antes sugerida e que algumas espécies antes agrupadas no gênero *Ara* parecem pertencer a três gêneros adicionais, conforme havia sido recomendado por Sick (1997). Passamos então a explorar as relações filogenéticas entre representantes de 25 dos 30 gêneros neotropicais baseadas em sequências mitocondriais e nuclear (Tavares *et al.* 2006 – Anexo III). Foram encontradas três grandes linhagens neotropicais com a mistura de táxons com comprimentos de cauda distintos. Além disso, a data estimada de separação entre o clado neotropical e o australiano é anterior à separação da Austrália em relação ao bloco de continentes que restara da antiga Gondwana, sugerindo que as linhagens que originaram os psitacídeos atuais deveriam ocorrer por uma grande extensão nesse antigo continente.

As filogenias moleculares indicam que o grupo neotropical seja monofilético (de Kloet & de Kloet 2005; Wright *et al.* 2008; Schirtzinger *et al.* 2012 – Anexo IV). Nesses estudos representantes de diversos gêneros da ordem Psittaciformes foram analisados baseados em sequências nucleares e em sequências mitocondriais e nucleares, respectivamente.

Em relação a reconstruções filogenéticas de psitacídeos neotropicais no nível infra-genérico, há alguns grupos estudados e nosso laboratório também contribuiu significativamente. Os representantes dos papagaios do gênero *Amazona* (31 espécies) são popularmente conhecidos como papagaios. Em uma análise filogenética baseada em sequências mitocondriais e nucleares de 29 espécies foi observado que: 1) o gênero não é monofilético, 2) há um clado que agrupa uma espécie que ocorre na América Central e as espécies que ocorrem nas Grandes Antilhas e 3) as espécies das Pequenas Antilhas mostram relações próximas com espécies da América Central e/ou da América do Sul (Russello & Amato 2003). Esse artigo contou com nossa colaboração direta uma vez que o primeiro autor passou treze dias no nosso laboratório sequenciando algumas amostras de nosso acervo e que faltavam para completar seu trabalho. A colonização das Antilhas pelas espécies do gênero *Amazona* foi mais explorada em um artigo baseado na análise filogenética baseada na sequência de um gene mitocondrial (Ottens-Wainright *et al.* 2004). Os resultados sugerem que houve dois eventos de colonização a partir da América do Sul em direção às

Pequenas Antilhas e possivelmente dois eventos de dispersão para as Grandes Antilhas. A análise do complexo de espécies *Amazona ochrocephala* baseada sequências mitocondriais revelou que o grupo que ocorre na América Central é monofilético, mas as linhagens da América do Sul não refletem as subespécies descritas e ainda, representantes de outra espécie, *Amazona aestiva*, aparecem dentro desse clado (Eberhard & Bermingham 2004). Como poucos indivíduos dessa segunda espécie foram analisados nesse estudo, ampliamos sua amostragem e re-analisamos esses dados (Ribas *et al.* 2007b – **Anexo V**). Nesse trabalho corroboramos a existência de três linhagens principais, mas encontramos uma subdivisão adicional de linhagens dentro da América do Sul.

A análise filogenética das quatro espécies do complexo de periquitos *Aratinga solstitialis* baseada em sequências mitocondriais confirmou que o gênero *Aratinga* não é monofilético (Tavares *et al.* 2004 – **Anexo II**), que *Nandayus nenday* pertence a esse grupo e que as divergências ocorreram durante o Plioceno e o Pleistoceno (Ribas & Miyaki 2004 – **Anexo VI**). Como esse gênero é composto de 21 espécies, realizamos uma análise de 20 espécies e estamos finalizando o manuscrito para publicação.

Em um estudo filogenético com quase a maioria dos táxons (espécies e subespécies) do gênero *Pionopsitta* baseado em sequências mitocondriais foi sugerido que a primeira divergência corresponde ao táxon da Mata Atlântica (Eberhard & Bermingham 2005). No entanto, nossa análise independente com todas as espécies do gênero também baseada em sequências mitocondriais e mais cinco representantes de gêneros de psitacídeos com cauda curta revelou que o gênero *Pionopsitta* não é monofilético, pois a linhagem da Mata Atlântica aparece fora do grupo interno (Ribas *et al.* 2005 – **Anexo VII**). Adicionalmente, uma espécie então recentemente descrita (Gaban-Lima *et al.* 2002) revelou ser uma linhagem independente dentro do grupo (Ribas *et al.* 2005 – **Anexo VII**).

Outro de nossos estudos com 21 das 24 espécies dos periquitos do gênero *Pyrrhura* baseado em sequências mitocondriais revelou três linhagens principais, duas das espécies analisadas não parecem ser monofiléticas e várias linhagens obtidas coincidem com propostas taxonômicas recentes (Ribas *et al.* 2006 – **Anexo VIII**). Essa diversidade de táxons parece ter se originado em curto intervalo de tempo no Plio-Pleistoceno.

Nosso trabalho com todos os táxons descritos do gênero *Pionus* baseado em sequências mitocondriais indicaram a presença de três linhagens que ocorrem nos Andes que não formam um grupo monofilético, pois se relacionam com linhagens de terras baixas. Esses resultados e dados de datação indicam que possivelmente essas três linhagens de montanhas foram passivamente levadas para altas altitudes e sofreram diversificação posterior (Ribas *et al.* 2007a – **Anexo IX**).

Nosso estudo com todas as espécies dos periquitos do gênero *Brotogeris* baseado em sequências mitocondriais e nuclear revelou que o gênero e suas oito espécies são monofiléticos. No entanto, as espécies amazônicas não formaram um clado monofilético. A diversificação do grupo ocorreu nos últimos 6 milhões de anos, o que coincide com os intervalos de diversificação de outros gêneros de psitacídeos (Ribas *et al.* 2009 – **Anexo X**).

Já estudos filogeográficos e populacionais de psitacídeos neotropicais não são tão frequentes, principalmente devido à complexidade de realizar sua amostragem. Como os psitacídeos são aves que

raramente podem ser capturadas em redes de neblina, geralmente somente os filhotes são amostrados. Assim, é necessário encontrar ninhos com filhotes. Nesse contexto, realizamos estudos de seleção de marcadores moleculares. Em um trabalho testamos marcadores heterólogos em cinco espécies de psitacídeos neotropicais, sendo que alguns foram polimórficos e os níveis de variabilidade encontrados parecem ser congruentes com o nível de ameaça das espécies testadas (Faria & Miyaki 2006 – **Anexo XI**). Em outro estudo descrevemos *primers* específicos desenvolvidos para a amplificação de microssatélites da arara-canindé *Ara ararauna* (Caparroz *et al.* 2003 – **Anexo XII**). Outro trabalho descreve o sucesso de amplificação utilizando *primers* heterólogos para microssatélites em três espécies de papagaios do gênero *Amazona* (Caparroz *et al.* 2007 – **Anexo XIII**). Em um estudo populacional da arara-azul-grande (*Anodorhynchus hyacinthinus*) baseado em dados nucleares e mitocondriais foi encontrada diferenciação genética entre indivíduos do Pantanal e do Piauí (Faria *et al.* 2008 – **Anexo XIV**). Além disso, a análise da composição genética de cinco indivíduos apreendidos sugeriu que eles não deveriam ser do Pantanal. No nosso estudo de arara-canindé (*Ara ararauna*), os microssatélites revelaram ausência de estrutura populacional, já sequências do DNAm indicaram a existência de dois grandes grupos distribuídos a leste e a oeste da área de ocorrência da espécie. Tais resultados sugerem que machos tendem a se dispersar (migrar) enquanto fêmeas tendem a ser filopátricas (Caparroz *et al.* 2009 – **Anexo XV**).

3. BIOGEOGRAFIA DA AVIFAUNA DA MATA ATLÂNTICA

Mais recentemente, temos realizado diversos estudos de aves da Mata Atlântica visando compreender a história desse bioma. A Mata Atlântica é o segundo maior bioma florestal da região neotropical (cerca de 1.477.500 km²) abrangendo parte dos territórios do Brasil, do Paraguai e da Argentina. Em função do processo de acentuada antropização, a Mata Atlântica foi reduzida em 11,4% a 16% de sua extensão original (Ribeiro *et al.* 2009). Esse bioma destaca-se por sua extensão e sua alta diversidade e endemismo. Dentre as 22 regiões biogeográficas definidas para o neotrópico, a Mata Atlântica é uma das que apresenta maior nível de endemismo, sendo registrada a ocorrência de 682 espécies de aves, das quais 199 são endêmicas (Stotz *et al.* 1996). Em razão dessas características e de seu estado de conservação, é considerada uma das cinco áreas de maior prioridade para conservação do planeta (Mittermeier *et al.* 2000).

Sob uma perspectiva continental, a Mata Atlântica pode ser considerada uma ilha (Silva *et al.* 2004), pois encontra-se isolada das demais regiões florestais da América do Sul por formações vegetais predominantemente abertas (Chaco, Cerrado e Caatinga) denominadas como "diagonal de formações abertas" (Ab'Saber 1977). Tal diagonal pode ser uma barreira intransponível para algumas espécies típicas de ambientes florestais, mas em alguns casos a vegetação associada aos rios pode permitir alguma conexão (Costa 2003).

Os padrões atuais de distribuição geográfica e de variação morfológica que as aves exibem ao longo da Mata Atlântica sugerem uma história biogeográfica bastante complexa, que envolve fluxos pretéritos entre essa e outras regiões florestais do neotrópico e processos de diferenciação ao longo da mesma. Possivelmente vários fatores históricos e ecológicos determinaram tais padrões. Podemos identificar três grupos de aves típicas de ambientes florestais registradas para a Mata Atlântica: as que apresentam ampla

distribuição por florestas tropicais úmidas da América do Sul (Haffer 1974, Sick 1997, Stotz *et al.* 1996); as que também ocorrem nas florestas andinas (Haffer 1974, Sick 1997); e grupos endêmicos da Mata Atlântica.

Apesar de a Mata Atlântica encontrar-se isolada das demais formações florestais sul-americanas, evidências vêm mostrando que em diversos momentos no passado áreas atualmente ocupadas por formações vegetais mais abertas (Cerrado e Caatinga), foram cobertas por formações florestais (Auler *et al.* 2004, Bigarella *et al.* 1975, Ledru 1993, Ledru *et al.* 1996, Prado & Gibbs 1993). As evidências sugerem que a Amazônia e Mata Atlântica foram provavelmente conectadas no passado, vindo a se isolarem com o aumento da aridez no Terciário (Bigarella *et al.* 1975). Adicionalmente, outros estudos vêm indicando que mesmo durante o Quaternário mudanças ambientais induzidas por ciclos de alterações climáticas foram capazes de promover o contato entre estes ecossistemas. O registro palinológico do Quaternário indica que mudanças climáticas tiveram impacto considerável na cobertura vegetal da região central do Brasil. Entre 50.000 e 40.000 anos atrás houve uma fase intensamente árida, substituída entre 40.000 e 27.000 anos por um período de grande umidade, aumentada gradualmente durante o final do Pleistoceno (Ledru *et al.* 1996). Ao longo deste período o Brasil Central, atualmente ocupado pelo Cerrado, era mais úmido e coberto por floresta pluvial (Ledru 1993).

Adicionalmente, estudos desenvolvidos na região da Caatinga vêm contribuindo para a compreensão dos cenários paleoambientais na região compreendida entre as maiores formações florestais da América do Sul. Vários registros de aspectos geomorfológicos e paleobióticos no semi-árido brasileiro indicam grandes mudanças paleoambientais durante períodos de aumento da pluviosidade ao longo de grande parte do Pleistoceno (Auler *et al.* 2004). Padrões atuais de distribuição de espécies de plantas e de animais apontam para a existência no passado de extensivo fluxo entre a Mata Atlântica e o leste da Amazônia, através da região atualmente ocupada pela Caatinga (Vivo 1997, Sick 1997). Formações conhecidas como “brejos de altitude” (encaves de floresta úmida em meio à caatinga) somam-se às evidências a favor da existência de conexões passadas entre Mata Atlântica e Amazônia, uma vez que revelam importantes disjunções florísticas entre estes biomas (Rizzini 1963, Bigarella *et al.* 1975).

Dentro desse cenário, o conhecimento dos padrões de distribuição geográfica dos táxons e de suas relações filogenéticas (e filogeográficas) é fundamental para permitir inferir os processos que têm causado a diversificação da biota (Bates *et al.* 1998). Além disso, a compreensão desses processos evolutivos geradores e mantenedores da diversidade da fauna tropical são fundamentais para o delineamento de estratégias e ações de conservação (Moritz *et al.* 2000). No entanto, em função da sobreposição de eventos de diversificação ao longo da Mata Atlântica e das alternâncias climáticas que determinaram ciclos de isolamento e contato entre esse e outros biomas florestais, é esperado encontrar padrões complexos de relações filogenéticas e filogeográficas. Por exemplo, Vanzolini (1988), Bates *et al.* (1998), Costa *et al.* (2000) e Moraes-Barros *et al.* (2006), analisando répteis, aves e mamíferos, respectivamente, destacam a existência de uma quebra latitudinal na distribuição de vertebrados da Mata Atlântica. Os dois primeiros estudos apontaram forte relação entre grupos irmãos oriundos do norte e sul da Mata Atlântica. No entanto, Costa (2003) observou que táxons encontrados no norte e no sul da Mata Atlântica frequentemente não são grupos irmãos. Dado que a representatividade da diversidade de organismos do bioma estudada até o momento é pequena, esse

problema ainda necessita ser muito explorado.

Essa heterogeneidade verificada ao longo da distribuição da Mata Atlântica pode estar associada à grande variação de dois fatores, o gradiente latitudinal (de 5° S a 30° S) e o altitudinal (do nível do mar à cerca de 1700 m). Somados aos processos de transformação da paisagem já descritos (ex. ciclos de mudanças climáticas), esses fatores podem ter atuado na diversificação ao longo do bioma, criando cenários para que processos como vicariância e dispersão ocorressem. Como exemplo da complexidade da história deste bioma, um estudo de distribuição de aves identificou quatro áreas de endemismo ao longo do bioma (Silva *et al.* 2004). Essas áreas de endemismo são consideradas produtos de eventos vicariantes que tiveram um importante papel na geração da alta diversidade de organismos na Mata Atlântica. A existência de padrões congruentes entre múltiplos táxons é uma forte evidência a favor de uma história comum de resposta a eventos vicariantes (Cracraft 1985).

Dentro desse complexo cenário biogeográfico da Mata Atlântica, temos estudado espécies endêmicas desse bioma, bem como buscamos compreender como táxons da Mata Atlântica se relacionam com táxons distribuídos em outros biomas. Iniciamos essa linha de pesquisa com uma espécie de ave florestal e endêmica do bioma (*Xiphorhynchus fuscus*) como modelo de estudo. Assim como para outros organismos (pequenos mamíferos, Costa *et al.* 2000; lagarto, Pellegrino *et al.* 2005; bicho-preguiça, Moraes-Barros *et al.* 2006; serpente, Grazziotin *et al.* 2006; abelha, Batalha-Filho *et al.* 2010), encontramos clara estrutura genética populacional, com componentes distribuídos ao longo do gradiente latitudinal da Mata Atlântica (Cabanne *et al.* 2007, 2008 – **Anexos XVI e XVII**). Uma das disjunções encontradas ocorre no estado de São Paulo, em uma área que também foi identificada em outros organismos (Grazziotin *et al.* 2006, Musturangi & Patton 1997, Pellegrino *et al.* 2005, Grazziotin *et al.* 2006, Batalha-Filho *et al.* 2010). Além disso, a subespécie que ocorre nos brejos de altitude constitui um clado monofilético e mereceria ter avaliada a possibilidade de ser elevada à categoria de espécie plena (Cabanne *et al.* 2008 – **Anexo XVII**). Ainda nesse estudo, foi realizada uma análise filogenética para confirmar o monofiletismo da espécie *X. fuscus* (Cabanne *et al.* 2008 – **Anexo XVII**).

Outra espécie florestal de Mata Atlântica que apresentou estrutura genética populacional com componentes diferenciados segundo a latitude foi *Sclerurus scansor* (d'Horta *et al.* 2011 – **Anexo XVIII**). Esse estudo indicou que aves de regiões diferentes do bioma responderam de forma distinta, mesmo durante o mesmo período climático, promovendo contatos e isolamentos simultâneos. Também avaliamos se essa espécie é monofilética por meio de uma análise filogenética que incluiu as seis espécies do gênero *Sclerurus* (d'Horta *et al.* 2013 – **Anexo XIX**). A filogenia baseada em dados mitocondriais indicou que todas as espécies são reciprocamente monofiléticas, com exceção de *S. mexicanus*. Apesar de esse trabalho ter focado nos táxons amazônicos, podemos destacar que a espécie da Mata Atlântica, *S. scansor*, foi recuperada como irmã de uma espécie que ocorre nas florestas da base dos Andes e na Amazônia. No entanto, baseado em dados nucleares, não foi encontrada monofilia recíproca; o que não é um resultado inesperado dado a diferença no tempo de coalescência desses tipos de marcadores.

No caso do estudo das espécies florestais e irmãs *Myrmeciza squamosa* e *M. loricata*, inicialmente foram desenvolvidos *primers* para a amplificação de vários locos anônimos (Amaral *et al.* 2012 – **Anexo XX**). Essas

espécies se distribuem de forma parapátrica na Mata Atlântica. Os resultados baseados em uma análise multilocus (oito anônimos e um mitocondrial) indicaram que: o fluxo gênico entre essas espécies é ausente ou muito pequeno e houve maior estabilidade ao norte do bioma (Amaral *et al.* 2013 – **Anexo XXI**). Esse trabalho recebeu destaque no volume onde foi publicado com a foto de uma das espécies na capa do mesmo.

Um estudo inicial do complexo da Mata Atlântica *Synallaxis ruficapilla* incluindo o grupo irmão (complexo *S. moesta* que ocorre nos Andes, Tepuis e Escudo da Guiana) indicou que as espécies analisadas são reciprocamente monofiléticas. No entanto, os dados sugeriram que o complexo da Mata Atlântica não compõe um grupo monofilético, sendo que a espécie que ocorre no norte do bioma formou um clado com uma espécie andina. Isso indicaria dois eventos de colonização da Mata Atlântica. A diversificação observada dentro desse bioma foi congruente no tempo e no espaço com outros organismos (componentes diferentes associados à latitude) e poderiam ter sido gerados por mudanças climáticas e atividades tectônicas do passado (Batalha Filho *et al.* 2013b – **Anexo XXII**).

Em relação à compreensão de como os táxons da Mata Atlântica se relacionam com táxons irmãos em outros biomas, temos ainda o estudo de *Dendrocolaptes platyrostris*, que ocorre tanto na Mata Atlântica quando em áreas de vegetação aberta (cerrado e caatinga). Esse trabalho revelou que a distribuição geográfica da diversidade genética não é congruente com a diferenciação morfológica das duas subespécies dessa ave. Assim, possivelmente essa divergência ocorreu ainda com fluxo gênico. Ainda, dentro da Mata Atlântica, foi encontrada estrutura com diferenciação latitudinal (Cabanne *et al.* 2011 – **Anexo XXI**).

Por outro lado, também foram encontradas espécies de aves da Mata Atlântica sem estrutura genética populacional. *Basileuterus leucoblepharus* apresentou sinal de expansão populacional durante o Pleistoceno e, ainda, não foi encontrada evidência de redução demográfica no último máximo glacial (Batalha-Filho *et al.* 2012 – **Anexo XXIV**). No caso de *Schiffornis virescens*, também não foi encontrada estrutura genética, mas há evidências de um gargalo populacional seguido de expansão demográfica no final do Pleistoceno (Cabanne *et al.* 2013 – **Anexo XXV**).

Um estudo baseado na compilação de dados filogenéticos e de distribuição geográfica de táxons irmãos de Suboscines (Passeriformes) que ocorrem na Mata Atlântica e Amazônia indicou duas conexões entre essas florestas úmidas: 1) a mais antiga (Mioceno médio e tardio) mais ao sul, e 2) a mais recente (Plio-Pleistoceno) mais ao norte. Essas conexões antigas e recentes poderiam estar associadas, respectivamente, a eventos geotectônicos do final do Terciário relacionados ao soerguimento dos Andes e a mudanças climáticas do Quaternário (Batalha-Filho *et al.* 2013a – **Anexo XXVI**).

Assim, o cenário atual indica que há espécies de aves da Mata Atlântica com e outras sem estrutura genética populacional. Possivelmente isso é resultado da resposta diferencial das espécies aos processos que ocorreram no bioma. É interessante que, quando há estrutura, as quebras filogeográficas encontradas são congruentes quanto à presença de componentes diferenciados latitudinalmente. Além disso, alguns táxons da Mata Atlântica podem ter relação bastante próxima com táxons de outros biomas. Finalmente, no caso de táxons irmãos na Amazônia, essa relação pode ter variado no espaço e no tempo.

4. PERSPECTIVAS

Ainda temos muito a investigar sobre a biogeografia histórica da região neotropical e, em especial, da América do Sul e as aves têm sido um modelo de estudo muito apropriado. Seguimos realizando estudos filogenéticos e filogeográficos de diversos táxons de aves visando agregar mais dados e também vamos iniciar o uso de metodologias que permitirão avaliar um conjunto de dados mais abrangente (sequenciamento de nova geração, McCormack *et al.* 2011). Além disso, iniciamos estudos de zonas de contato de linhagens (táxons) diferenciados, sendo na maioria dos casos, zonas onde ocorre hibridação. Essas áreas permitem analisar diversos processos evolutivos como mecanismos de isolamento reprodutivo, seleção, estimativas de dispersão e número de genes envolvidos na diferenciação (Hewitt 1988, Barton & Hewitt 1989, Kawaki & Butlin 2012).

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Anexo I

Parrot Evolution and Paleogeographical Events: Mitochondrial DNA Evidence

Cristina Yumi Miyaki,* Sergio Russo Matioli,* Terry Burke,† and Anita Wajntal*

*Departamento de Biologia, Instituto de Biociências—Universidade de São Paulo, São Paulo, Brazil; and †Department of Zoology, University of Leicester, Leicester, England

We sequenced 1,771 bp of three mitochondrial genes (12S rDNA, 16S rDNA, and cytochrome *b*) of nine New World parrot genera (*Amazona aestiva*, *Anodorhynchus hyacinthinus*, *Ara ararauna*, *Aratinga aurea*, *Cyanopsitta spixii*, *Derophtus accipitrinus*, *Guaruba guarouba*, *Pionus menstruus*, and *Pyrrhura picta*) and compared them with the corresponding sequence of Australian parakeet (*Melopsittacus undulatus*). Phylogenetic analyses (maximum parsimony and maximum likelihood) showed that the Neotropical species we studied constitute two monophyletic groups: the long-tailed and the short-tailed species. The separation within the long-tailed species could be assigned to the late Oligocene–early Miocene, when paleoenvironmental changes might have influenced this radiation. The long-tailed Neotropical and Australasian species diverged during the Cretaceous–Tertiary boundary, when South America and Australia were moving away from Antarctica (Gondwanaland fission). We also compared our data with the cytochrome *b* sequences of seven different genera of Australasian parrots obtained by other investigators, and these comparisons also support the independent evolution of the Neotropical and Australasian species. Analyses performed with 567 bp of partial sequences of 12S rDNA and cytochrome *b* did not support or refute the hypothesis of monophyly of the Neotropical parrots with respect to an African species whose sequences were available. However, this analysis supported the view that the divergence between Neotropical short- and long-tailed taxa was older than the Oligocene–Miocene divergence among the long-tailed genera.

Introduction

According to Forshaw (1989), there are more than 330 living species of parrots, mainly distributed in the Southern Hemisphere. One hundred species occur in South America, 69 of them in Brazil (Sick 1993, p. 264). Cracraft (1973) considers that the amount of evolution among parrots in Australia and South America "... suggests that the early history of the family was Gondwanaland, and therefore that paleogeography of the Southern continents was probably important."

Parrot fossils are scarce; the oldest known is *Archaeopsittacus verreauxi* (Upper Oligocene or Lower Miocene, about 30 MYA), found in France. The earliest modern genus representative is *Conuropsis fratercula* (Upper Miocene, around 20 MYA), found in the U.S.A. In South America, only Pleistocene (less than 2 MYA) fossils have been found, in Argentina, Ecuador, and Brazil (Forshaw 1989; Sick 1993, p. 251). The New and Old Worlds share no genera. Karyotype studies show the homogeneity among the South American species compared to the diversity among those found in Australasia (Lucca, Shirley, and Lanier 1991). DNA hybridization data (Sibley and Alquist 1990) suggest that there are three subgroups of parrots: Australasian, African, and American species. These results support the suggestion that New World parrots diverged from the other groups (Smith 1975; Sick 1993, p. 251).

Parrot phylogeny is better known among the Australasian species. Analysis of mitochondrial DNA (mtDNA) restriction fragment polymorphism carried out

on six species of the Australasian genus *Platycercus* revealed the presence of two groups and suggested the existence of a third (Overden, Mackinlay, and Crozier 1987). Leeton et al. (1994) sequenced 924 bp of the mitochondrial cytochrome *b* gene of eight Australasian parrots, including one considered extinct (*Geopsittacus occidentalis*), and established phylogenetic relationships among them. A shorter sequence of the same gene (307 bp) was compared among two African (*Poicephalus senegalus* and *Psittacus erithacus*), four Australasian (*Cacatua goffini*, *Melopsittacus undulatus*, *Nymphicus hollandicus*, and *Purpureicephalus spurius*), and six American species (*Amazona autumnalis autumnalis*, *A. oratrix*, *A. tucumana*, *A. xanthops*, *Graydidascalus brachyurus*, and *Pionus senilis*) (Birt et al. 1992). In this study, two groups were analyzed separately: the first included all the African and Australasian species, one American species (*Amazona ochrocephala*), and the common pigeon (which was considered as the out-group), while the second included all the American and one Australian species. Some consistent results were obtained: the African species were placed in one group, and three species of the American genus *Amazona* also formed a monophyletic group, while the fourth species (*Amazona xanthops*) seemed to diverge from the other species in the genus.

The information on the relationships among New World parrot species is scarce; the species can be separated according to their tail morphologies as long or short (Sick 1993, p. 703). These two groups can also be recognized by their peculiar behavior: long-tailed parrots generally live in the midstrata of trees and usually fly down to feed on the ground, while short-tailed parrots inhabit the canopy and are rarely seen on the ground (Montón 1977). The karyotypic characteristics of some long-tailed species also separate them from short-tailed ones (Valentine 1990). Another distinguishing feature is

Key words: parrots (Psittaciformes, Aves), mitochondrial DNA, evolution of parrots, paleogeography.

Address for correspondence and reprints: Cristina Yumi Miyaki, Departamento de Biologia, Instituto de Biociências—Universidade de São Paulo, R. do Matão, 277, 05508-900, São Paulo, SP, Brazil. E-mail: cymiyaki@usp.br.

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Table 1
Primers Used

Gene ^a	Location ^b	Sequence	Reference
12S rDNA L	1735	GGATTAGATACCCCACTATGC	c
12S rDNA H	2170	AGGGTGACGGGCGGTATGTACG	c
16S rDNA L	2702	CCTACCGAGCTGGGTGATAGCTGGTT	c
16S rDNA H	3309	TGCGCTACCTTCGCACGGT	c
Cytochrome <i>b</i> L (universal)	14957	AAAGTAGTACGACTCTACAACCTACCTTCGAAAAA	Kocher et al. (1989)
Cytochrome <i>b</i> H (universal)	15331	AACTGCAGCCCTCAGAATGATATTTGTCCTCA	Kocher et al. (1989)
Cytochrome <i>b</i> H (long)	16092	AACTGCAGTCATCTCCGGTTTACAAGAC	Kornegay et al. (1993)
Cytochrome <i>b</i> L (reverse)	15298	TGAGGCCAAATATCATTCTGAGGGGC	Cheng, Higuchi, and Stoneking (1994)
Cytochrome <i>b</i> L (internal)	15637	AACCTCCTAGGAGACCCAGAAAACCTC	This work
Cytochrome <i>b</i> H (internal 2)	15764	CCTCCTAGTTTGTGGGGATTGA	This work
Cytochrome <i>b</i> H (internal 3)	15417	GTCCGGTTGTCTACGGAGA	This work

^a L and H stand for the light and heavy chains, respectively.^b Location in the chicken map (Desjardins and Morais 1990).^c Designed by T.B. for another work.

the presence of minisatellite sequences detected by probe 33.15 (Jeffreys, Wilson, and Thein 1985) and linked to the W chromosome that are observed exclusively in long-tailed species (Miyaki et al. 1992, 1993, 1995, 1997).

In the present work, we sequenced regions of three mitochondrial genes (12S rDNA, 16S rDNA, and cytochrome *b*) from nine species of Neotropical parrots belonging to nine different genera. These data, along with previously published sequences (Birt et al. 1992; Leeton et al. 1994; Cooper and Penny 1997; Moore and DeFilippis 1997), were used to assess the time of divergence of these genera by applying substitution rate tests and maximum-likelihood methods.

Materials and Methods

The Neotropical species studied belong to aviculturists and official institutions in Brazil and can be morphologically distinguished as short-tailed (*Amazona aestiva* and *Pionus menstruus*) and long-tailed (*Anodorhynchus hyacinthinus*, *Ara ararauna*, *Aratinga aurea*, *Cyanopsitta spixii*, *Deroptyus accipitrinus*, *Guaruba guarouba*, *Nandayus nenday*, and *Pyrrhura picta*). Blood samples were collected by venipuncture. Budgerigar (*Melopsittacus undulatus*) feathers were obtained from an aviculturist in England. Total DNA of two individuals of each species was extracted (Bruford et al. 1992).

Polymerase chain reaction (PCR) was used to amplify parts of three mitochondrial genes: 12S rDNA (436 bp), 16S rDNA (608 bp), and cytochrome *b* (1.1 kb). The amplifications were performed in 10- μ l volumes of 67 mM Tris (pH 8.8), 16.6 mM (NH₄)₂SO₄, 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, 10 mM EDTA (pH 8.8), 1.5 mM of each dNTP, 0.1 mg of BSA, 1–20 ng genomic DNA, 1 μ M of each primer, and 0.2–2.5 U of *Taq* polymerase (Advanced Biotechnologies Ltd.).

Primers used are listed in table 1. The products were isolated in 1% agarose in TBE buffer, PEG-recovered (Zhen and Swank 1993), and purified by phenol:chloroform extraction. This DNA was used as template, and the sequencing reaction was prepared according to the *Taq* dye deoxy terminator cycle sequencing kit man-

ufacturer (Applied Biosystems). Cytochrome *b* internal primers (table 1) were designed to cover both strands of the amplified region. After the sequencing reaction was carried out, the products were purified with phenol:chloroform:isoamyl alcohol (Applied Biosystems) and run in an automatic sequencer (ABI 373).

Since the partial *M. undulatus* cytochrome *b* sequence has already been published (Leeton et al. 1994), we sequenced only 116 bp of this gene and the 12S and 16S rDNA regions to adjust the alignment with the other New World species and *Gallus gallus* (outgroup; Desjardins and Morais 1990).

The DNA sequences obtained were visually aligned with multiple sequence editors (Sequence Navigator, Applied Biosystems and SeqPup; Gilbert 1996). The sequences of the genes (12S and 16S rDNAs and cytochrome *b*) were phylogenetically analyzed contiguously, as if they were a single gene. The phylogenetic contribution of each gene separately was also evaluated.

Cytochrome *b* sequences (885 bp) of the Neotropical parrots listed above were compared with those of Australasian parrots (*Geopsittacus occidentalis*, *Melopsittacus undulatus*, *Neophema petrophila petrophila*, *Pezoporus wallicus wallicus*, *Platycercus icterotis xanthogenys*, *Polytelis anthopeplus westralis*, and *Strigops habroptilis*; Leeton et al. 1994) and a woodpecker (*Colaptes rupicola*) (outgroup; Moore and DeFilippis 1997).

We also analyzed the partial sequences of 12S rDNA and cytochrome *b* (567 bp in total) of the Neotropical parrots, two Australasian parrots (*M. undulatus* and *S. habroptilis*; Leeton et al. 1994; Cooper and Penny 1997), one African parrot (*Psittacus erithacus*; Birt et al. 1992; Cooper and Penny 1997), and our outgroup (*G. gallus*; Desjardins and Morais 1990).

The final analysis comprised a comparison of 267 bp from cytochrome *b* of 2 African, 10 Australasian, and 15 Neotropical parrot species (Birt et al. 1992; Leeton et al. 1994; this work), chicken (Desjardins and Morais 1990), and *Columba livea* (Birt et al. 1992).

Phylogenetic analyses of the sequences were performed according to the following strategy: the preliminary search for the most-parsimonious tree was done using PAUP, version 3.1.1 (Swofford 1993). The same

program then calculated the strict consensus. A bootstrap test was performed (resampling of the original sequences' sites 100 times with reposition; for each sample, a most-parsimonious tree was estimated by heuristic search with 10 different starting orders to avoid spurious trees). The decay index of Bremer (1994) was calculated for each node of the consensus tree using the Autodecay program by Eriksson and Wikström (1995), together with PAUP.

The same data were used for a maximum-likelihood search using PAML (Yang 1997). This method uses an explicit model of nucleotide evolution that allows the comparison of parameter estimation among different sets of analyses. We applied the Tamura and Nei (1993) model, which was developed for mitochondrial DNA evolution that is characterized by its high A-T content and high transition/transversion rate. The heterogeneity of substitution rates (as that for synonymous vs. nonsynonymous substitution rates in the case of cytochrome *b* and that for conserved vs. variable sites, observed in almost any DNA sequence) was considered by distributing the rates in a discrete gamma distribution within eight categories. The alpha parameter, which is related to the shape of the gamma distribution, and the transition/transversion parameters were estimated from the data.

Rate constancy tests were done following the method described by Takezaki, Rzhetsky, and Nei (1995), using Takezaki's LINTREE programs. The taxa that presented significantly different rates (at the 5% level) were excluded from further analyses. The remaining taxa were reanalyzed by the maximum-likelihood method to obtain a tree that was constrained to behave as if it were directed by a molecular clock (in which all root-to-tip lengths have the same values). The analyses were performed in the group of species with the 1,771-bp sequences (12S and 16S rDNAs and cytochrome *b*) and the one with 885 bp of cytochrome *b* sequence.

Results

We obtained 342 bp of DNA sequence from the 12S rRNA genes and 428 bp from the 16S rRNA genes (GenBank accession numbers are presented after species names) from 10 species of Neotropical parrots (*A. aestiva*—U70738 and U70749, *A. hyacinthinus*—U70741 and U70752, *A. ararauna*—U70739 and U70750, *A. aurea*—U70740 and U70751, *C. spixii*—U70742 and U70753, *D. accipitrinus*—U70743 and U70754, *G. guarouba*—U70744 and U70755, *N. nenday*—U70746 and U70757, *P. menstruus*—U70747 and U70758, and *P. picta*—U70748 and U70759, respectively) and from the Australian parakeet (*M. undulatus*—U70745 and U70756). We also sequenced 1,005 bp of the cytochrome *b* genes of nine species of New World parrots (*A. aestiva*—U70760, *A. hyacinthinus*—U70763, *A. ararauna*—U70761, *A. aurea*—U70762, *C. spixii*—U70764, *D. accipitrinus*—U70765, *G. guarouba*—U70766, *P. menstruus*—U70767, and *P. picta*—U70768). Two individuals per species were studied.

Table 2
Frequencies of Transitions and Transversions Among Variable Sites

Gene ^a	Transitions	Transversions	Total	Ratio ^b
12S rDNA	1,270 (80%)	316 (20%)	1,584	4:1
16S rDNA	2,415 (46%)	2,840 (54%)	5,255	1:1
Cytochrome <i>b</i> (N) ..	5,554 (66%)	2,892 (34%)	8,446	2:1
Cytochrome <i>b</i> (N-A)	11,581 (57%)	8,826 (43%)	20,407	1.3:1

^a N and A stand for the New World and Australasian genera, respectively.
^b Transitions : transversions.

Since the first tree constructions always showed individuals of the same species grouped together, we considered just one sequence per species in the phylogenetic analyses to speed up the computer analyses.

The alignment showed that insertions and/or deletions ("indels") were present only in 12S and 16S rDNA genes (ranging from 20 indels in *D. accipitrinus* to 34 in *P. picta*); the cytochrome *b* fully aligned. The composition of the sequences showed a higher frequency of cytosine and adenine. The number of transitions observed was higher than the number of transversions at the variable sites studied (table 2). As expected, the cytochrome *b* third position was the most variable position.

The pairwise distances among the 1,771-bp sequences were calculated according to the model of Tamura and Nei (1993) and are presented in table 3. The average kappa parameters (reflecting the overall transition/transversion substitution rates) are also presented in table 3. The phylogenetic trees resulting from the analyses of each gene separately for the Neotropical parrots plus *M. undulatus* and *G. gallus* showed that the relationships (associations and/or divergences) obtained using the 12S rDNA sequences were not always totally in accordance with the relationships obtained using the 16S rDNA and cytochrome *b* sequences, which resulted in similar trees (data not shown).

Two shortest trees of equal length (1,561 steps) were obtained in parsimony analyses of the 1,771 bp involving the nine Neotropical species, *M. undulatus*, and *G. gallus*. The strict-consensus and maximum-likelihood trees are presented in figure 1. These trees show that the polytomy obtained in the parsimony analysis corresponds to very short branch lengths in the maximum-likelihood analysis. We also performed neighbor-joining analyses using a distance matrix, and these showed almost the same tree patterns as those obtained with the maximum-likelihood analyses (data not shown).

The parsimony and maximum-likelihood trees of the 885-bp cytochrome *b* sequence from the Neotropical and Australasian species are shown in figure 2. The analyses using the combined sequences of 12S rDNA and cytochrome *b* resulted in the parsimony and maximum-likelihood trees presented in figure 3. The phylogeny obtained by a maximum-parsimony heuristic search of 267 bp of the cytochrome *b* gene is shown in figure 4.

We tested the constancy of evolutionary rate of the three mitochondrial gene sequences of each New World

Table 3
Pairwise Distances Among the 1,771-bp Sequences

	<i>Gallus</i> <i>gallus</i>	<i>Melop-</i> <i>sitt-</i> <i>acus</i> <i>undulatus</i>	<i>Amazona</i> <i>aestiva</i>	<i>Anodor-</i> <i>hynchus</i> <i>hyacinth-</i> <i>inus</i>	<i>Ara</i> <i>ararauna</i>	<i>Aratinga</i> <i>aurea</i>	<i>Cyanopsit-</i> <i>ta spixii</i>	<i>Derop-</i> <i>tytus</i> <i>accipitrinus</i>	<i>Guaruba</i> <i>guarouba</i>	<i>Pionus</i> <i>menstruus</i>	<i>Pyrrhura</i> <i>picta</i>
<i>G. gallus</i>		0.332	0.292	0.321	0.324	0.300	0.362	0.302	0.316	0.280	0.305
<i>M. undulatus</i>	2.02		0.262	0.261	0.296	0.283	0.317	0.268	0.288	0.243	0.266
<i>A. aestiva</i>	2.17	2.85		0.184	0.179	0.179	0.251	0.174	0.186	0.112	0.182
<i>A. hyacinthinus</i> ..	2.03	2.59	5.87		0.122	0.112	0.199	0.131	0.121	0.173	0.140
<i>A. ararauna</i>	2.18	3.43	5.65	10.60		0.114	0.201	0.134	0.121	0.175	0.145
<i>A. aurea</i>	2.06	3.20	6.80	11.10	11.78		0.181	0.126	0.102	0.164	0.131
<i>C. spixii</i>	1.96	3.08	3.56	4.49	4.54	4.56		0.214	0.203	0.250	0.232
<i>D. accipitrinus</i> ...	2.32	3.19	6.41	9.08	9.65	10.99	4.53		0.128	0.158	0.130
<i>G. guarouba</i>	2.30	3.35	6.11	10.29	9.75	12.13	4.54	11.01		0.157	0.138
<i>P. menstruus</i>	1.97	2.94	10.72	5.61	5.69	5.73	3.75	5.69	5.33		0.156
<i>P. picta</i>	2.19	2.68	5.82	9.48	11.34	10.69	4.86	8.10	11.23	4.84	

NOTE.—The distances were calculated according to the Tamura and Nei (1993) correction, with alpha set to 1.0. The distances are presented above the diagonal. The kappa parameters are presented below the diagonal.

species plus *M. undulatus* and *G. gallus* using the two-cluster and branch length tests in LINTREE with the Tamura and Nei (1993) distance. *Cyanopsitta spixii* had a faster rate and *A. aestiva* and *P. menstruus* had slower ones than did the other species. None of these species were included in the time-constrained tree. Assuming that bird orders diverged around 100 MYA (Hedges et al. 1996; Cooper and Penny 1997) and using the time-constrained tree (fig. 5), we estimate the date of the split between the Neotropical long-tailed parrot genera and *M. undulatus* to be around 76 MYA (table 4) and the separation of these New World genera to be between 27 and 20 MYA. The standard errors in table 4 were calculated using the time-constrained maximum-likelihood data.

The 885-bp cytochrome *b* time-constrained tree was also obtained through the PAML program. The evolutionary rates of *A. ararauna*, *D. accipitrinus*, *G. guarouba*, *P. menstruus*, *P. icterotis*, *P. anthoepplus*, and *S. habroptilis* differed too much from those of the remaining 10 genera for them to be included in the time-constrained tree (fig. 6). If the separation of the Neotropical

and Australasian species occurred around 76 MYA (table 4), the split among the New World long-tailed parrots can be dated at between 51 and 24 MYA (table 4).

Discussion

The high frequency of cytosine and adenine in the composition of the three genes studied is similar to that found in the total mitochondrial sequence of chicken (*G. gallus*; Desjardins and Morais 1990). The cytochrome *b* sequences obtained showed a higher frequency of cytosine at the first position. At the second position, there was more thymidine and less guanine, while in the third position, adenine and cytosine were more highly represented. In the Neotropical parrots, transitions are responsible for most of the nucleotide variation in the three genes studied. Other authors observed the same trend in other organisms (Brown et al. 1982; Desjardins and Morais 1990). The highest variability at the third position of a mitochondrial coding gene has been observed in a variety of other organisms (Edwards, Arcander, and Wilson 1991; Ramirez, Savoie, and Morais 1993; Krajewski and Fetzner 1994) and can be explained by the degenerated coding system (silent substitutions). For this reason, some authors have chosen

FIG. 1.—Trees obtained using 1,771 bp of cytochrome *b*, 12S rDNA, and 16S rDNA sequences. Maximum-likelihood tree on the left. Strict-consensus maximum-parsimony tree on the right, showing Bremer decay indices at each branch and bootstrap values in parentheses.

FIG. 2.—Trees obtained using 885 bp of cytochrome *b* sequence. Maximum-likelihood tree on the left. Maximum-parsimony tree on the right, showing Bremer decay indices at each branch and bootstrap values in parentheses.

FIG. 3.—Trees obtained using 567 bp of 12S rDNA and cytochrome *b* sequences. Maximum-likelihood tree on the left. Maximum-parsimony tree on the right, showing Bremer decay indices at each branch and bootstrap values in parentheses.

not to include this position in their phylogenetic analyses (Avice, Nelson, and Sibley 1994). Our results showed that there are some differences among the trees if the third position is not considered, but the most consistent relationships (associations and/or divergences) are not affected (data not shown).

The trees based on the 12S rDNA sequence did not agree very well with the trees based on the other two sequences. This could be due to the small size of the sequence, since the analyses based on a longer sequence of the 16S rDNA gene resulted in trees similar to the ones obtained with cytochrome *b*.

The trees resulting from analyses of the 1,771-, 885-, and 638-bp mitochondrial sequences presented some high values of bootstrap and Bremer decay. However, the 267-bp cytochrome *b* comparisons produced very low values, indicating that relationships obtained

FIG. 5.—Time-constrained tree obtained using 1,771 bp of cytochrome *b*, 12S rDNA, and 16S rDNA sequences.

using this sequence are not reliable. Thus, the data set with the smaller sequences was not analyzed by maximum likelihood.

The monophyly of Neotropical parrots with respect to the Australasian ones is supported by the analyses using all four sequences (the 1,771-bp sequences of the three mitochondrial genes, the 885-bp cytochrome *b* sequences, the 567-bp 12S rDNA and cytochrome *b* comparisons, and the 267-bp of cytochrome *b* sequences). Allozyme (Randi and Bertagnolio 1990) and DNA hybridization (Sibley and Alquist 1990) studies also support separation of the parrots from Australasia, Africa, and South America. Cracraft (1974) suggested that parrots and other groups of birds may have had their dispersal influenced by the Gondwanaland connections since: (1) there is evidence that a flightless ratite ancestor population was present in Gondwanaland, possibly

FIG. 4.—Maximum-parsimony tree obtained using 267 bp of cytochrome *b* sequence. Bremer decay indices are shown over branches, and bootstrap values that were higher than 50 out of 100 replicates are shown in parentheses.

Table 4
Estimated Dates of Separation of Parrot Species

Species	Date $\pm$ SE (MYA)
1,771 bp of 12S rDNA, 16S rDNA, and cytochrome <i>b</i> sequences	
M $\times$ P, D, G, Au, Ah, Ar	76.2 $\pm$ 7.8
P $\times$ D, G, Au, Ah, Ar	27.4 $\pm$ 2.2
D $\times$ G, Au, Ah, Ar	25.7 $\pm$ 2.0
G $\times$ Au, Ah, Ar	22.0 $\pm$ 1.7
Au $\times$ Ah $\times$ Ar	21.2 $\pm$ 1.7
Ah $\times$ Ar	19.7 $\pm$ 1.7
885 bp of cytochrome <i>b</i> sequences	
M $\times$ N, Go, Pw	36.2 $\pm$ 3.5
N $\times$ Go, Pw	34.5 $\pm$ 3.4
Go $\times$ Pw	23.8 $\pm$ 2.9
A $\times$ P, Ah, Au, C	50.8 $\pm$ 4.6
P $\times$ Ah, Au, C	37.2 $\pm$ 3.4
Ah $\times$ Au, C	32.5 $\pm$ 3.2
C $\times$ Au	24.0 $\pm$ 2.9

NOTE.—M, *Melopsittacus undulatus*; P, *Pyrrhura pieta*; D, *Derophtus acipitrinus*; G, *Guaruba guarouba*; Au, *Aratinga aurea*; Ah, *Anodorhynchus hyacinthinus*; Ar, *Ara ararauna*; A, *Amazona aestiva*; C, *Cyanopsitta spixii*; N, *Neophema petrophila*; Pw, *Pezoporos wallicus*; Go, *Geopsittacus occidentalis*, respectively.

FIG. 6.—Time-constrained tree obtained using 885 bp of cytochrome *b* sequences.

isolated into a cassowary/emu group in Australia, rhea in South America, and ostriches in Africa and Eurasia (before the Quaternary); (2) it is possible that galliforms occurred in Australia, East Antarctica, and South America, from where they colonized Laurasia; and (3) the presence of five genera of penguins on Seymour Island (Antarctic Peninsula) related (in various degrees) to New Zealand genera could be explained by the presence of a common ancestor when New Zealand and West Antarctica were close or in contact. Gondwanaland was an antique continent that split during the late Mesozoic and early Tertiary into South America, Africa, Australia, New Zealand, Antarctica, and India. Glenny (1954) even suggested that the origin and center of dispersion of parrots might be assigned to the Antarctic region.

The date of the separation of *M. undulatus* (Australian parakeet) and the long-tailed Neotropical species we estimated using the 1,771-bp comparisons (76 MYA) is in accord with the hypothesis that continental drift played an important role in the evolution of many animals and plants. These paleogeographic continental movements could have been responsible for the isolation of groups of parrots that then evolved independently.

The datings we obtained were based on estimates of the divergence of bird orders suggested by Hedges et al. (1996) and Cooper and Penny (1997). We considered 100 MYA as a conservative estimate for the separation of Galliformes and Psittaciformes (if more accurate dating becomes available in the future, our analyses can be linearly corrected). We observed that the 885-bp comparisons gave older dates than those obtained using the 1,771-bp sequences (table 4). A comparison of kappa parameters for the different data sets (table 5) showed that the analysis involving only the cytochrome *b* se-

quences underestimated the transition/transversion rates. As can be seen in table 3, the smaller distances between sequences correspond to higher values of the transition/transversion rate parameters, because they are estimates closer to the instantaneous rates. The rate parameters obtained in the cytochrome *b* analyses are lower than those obtained in other analyses, which leads to an overestimation of the separation dates. Thus, the datings obtained with the larger data set seem more reliable.

The separation of the long- and short-tailed Neotropical parrots observed in the trees shown in figures 1–3 is also supported by other data: morphology (Sick 1993, p. 264), minisatellites (Miyaki et al. 1992, 1993, 1995, 1997), karyotype (Valentine 1990), and behavior (Montón 1977). The mitochondrial sequences of these short-tailed species evolved more slowly than did those of the long-tailed ones. This suggests that these two groups of Neotropical parrots evolved independently. The divergence between the long- and short-tailed parrots could not be geographically assigned to South America or Africa, since in the 567-bp analyses that included parrots from the three continents (fig. 3), the Neotropical genera were not monophyletic with respect to the African taxa.

Among the long-tailed New World species, *P. picta* and *D. accipitrinus* probably diverged earlier from the others in the group. These species are exceptions among the long-tailed Neotropical parrots regarding the existence of W-chromosome-linked minisatellite sequences (Miyaki et al. 1997). This result adds more data supporting the lack of close relationships between these two species and the rest of the long-tailed parrot taxa. In this context, the W-chromosome-linked minisatellite sequence was acquired after the separation of the monophyletic branch of the long-tailed parrots from *P. picta* and *D. accipitrinus*.

The relationships among the long-tailed New World parrots could not be well established. The association of *C. spixii* and *A. aurea* was the only one observed in all trees (figs. 1–4) except one of the two shortest maximum-parsimony trees. This suggests that the Spixii's macaw is more related to a conure than to a macaw. Based on morphological studies, Sick (1993, p. 261) also suggested that *C. spixii* is not a true macaw.

The lack of resolution in relationships among the long-tailed Neotropical genera could be an inherent consequence of the nature of our data, whereby the phylogenetic signal has been lost by saturation. In this case, a slow-evolving gene would be more suitable for comparisons. Alternatively, the diversification of these taxa might have occurred almost simultaneously. The esti-

Table 5
Estimated Parameters from the Maximum-Likelihood Analyses

Analysis	K1	K2	Alpha
1,771 bp (12S and 16S rDNAs, cytochrome <i>b</i>)	9.02244 (0.88942)	5.17000 (0.55864)	0.33938 (0.02408)
885 bp (cytochrome <i>b</i>)	4.89729 (0.40380)	4.19789 (0.43546)	0.42362 (0.03315)
567 bp (12S rDNA, cytochrome <i>b</i>)	12.67598 (2.01750)	4.25987 (0.69954)	0.23321 (0.02723)

NOTE.—K1 and K2 are parameters from the Tamura and Nei (1993) model related to transition rates. K1 estimates the purine transitions, and K2 estimates the pyrimidine transitions. The standard errors of the estimates are in parentheses.

mated date for this diversification event was between 27 and 20 MYA based on 1,771 bp of 12S and 16S rDNAs and cytochrome *b*. It is clear that these radiation events occurred during the late Oligocene–early Miocene. Unfortunately, studies on the South American paleoenvironment during this period are not very detailed.

It is known that during the Oligocene (ca. 30 MYA), there was a regression of the sea level, and at the transition of this period to the Miocene, there was a huge transgression (Petri and Fúlvaro 1983). Orogenic movements were most active around 30 MYA and characterized the division of the Paleogene and Neogene (Salgado-Labouriau 1994). The Oligocene–Miocene boundary is associated with the beginning of a South American transcendental biotic change which led to the vanishing or disappearance of most “primitive” Paleogene mammals (Pascual and Jaureguizar 1990). Fossil records of immigrant primates and rodents from Africa are also dated to this time (Poirier, Stini, and Wreden 1994). Also based on fossil data, it was suggested that the autochthonous platyrrhini monkeys radiated in the pre-middle Miocene, which may be related to an early–middle-Miocene climatic amelioration and expansion of the forest/riparian habitat (Flynn et al. 1995). The congruence of the dating for the long-tailed parrots’ divergence and the radiations of primates and other mammals suggest that changes in the paleoenvironment may have influenced the diversification of these groups of animals.

Most of the Neotropical parrot genera that we studied are distributed over large ranges. Only *Guaruba* and *Cyanopsitta* can be considered endemic in a strict sense and their differentiation probably occurred independent of (or prior to) the physiographic or climatic barriers as we know them today. Thus, if the nearly simultaneous differentiation of various long-tailed genera detected here is not a consequence of technical limitations, it should provide evidence supporting abrupt climatic changes of continental range which could have expanded and contracted forests.

The final conclusions of the present work are that the divergence of Neotropical and Australasian species in the Cretaceous–Tertiary boundary and the separation among the long-tailed parrots during the late Oligocene–early Miocene can be explained by paleogeographic events.

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Anexo II

PHYLOGENETIC RELATIONSHIPS AMONG SOME NEOTROPICAL PARROT GENERA (PSITTACIDAE) BASED ON MITOCHONDRIAL SEQUENCES

ERIKA S. TAVARES,¹ CARLOS YAMASHITA,² AND CRISTINA Y. MIYAKI^{1,3}

¹Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277,
05508-090, São Paulo, SP, Brazil; and

²Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis, Alameda Tietê 637, 01417-020,
São Paulo, SP, Brazil

ABSTRACT.—The New World tribe Arini includes 30 genera and represents 148 known species of parrots. A previous phylogenetic study examined nine of those genera and suggested the existence of two groups. Our objective was to better understand the relationships among 14 species from 9 genera belonging to one of those groups. We partially sequenced the 12S and 16S ribosomal DNAs, cytochrome *b*, cytochrome oxidase I, and control region. We improved our understanding of the phylogenetic relationships among Neotropical parrots by adding both taxa and sequences, but the relationships among the deeper lineages were not well resolved. Our results agree with present classifications that place some species formerly in the genus *Ara* into three additional genera (*Primolius*, *Orthopsittaca*, and *Diopsittaca*). Additionally, we suggest that (1) *D. nobilis* and *Guarouba guarouba* are closely related and the genus *Aratinga* is not monophyletic; (2) diversification of genera may have occurred during the Miocene, and of species within genera during the Pliocene and Pleistocene; and (3) geologic, climatic, and environmental changes in South America may have been related to that diversification. Received 3 May 2002, accepted 7 November 2003.

RESUMEN.—A tribo Arini, que ocorre exclusivamente no Novo Mundo, agrega 140 espécies de psitacídeos organizadas em 30 gêneros. Em um estudo filogenético prévio foram analisados nove desses gêneros e foi sugerida a existência de dois grupos. Nosso objetivo foi estudar melhor as relações entre 14 espécies de nove gêneros pertencentes a um desses grupos. Nós sequenciamos parcialmente os rDNAs 12S e 16S, o citocromo *b*, o citocromo oxidase I e a região controle. Nossos resultados indicam que a adição de táxons e de seqüências melhorou a compreensão das relações filogenéticas entre psitacídeos Neotropicals, mas as relações entre as linhagens mais antigas continuaram mal resolvidas. Nossos resultados são concordantes com a classificação atual que considera que algumas das espécies que pertenciam ao gênero *Ara* devem estar organizadas em três gêneros adicionais (*Primolius*, *Orthopsittaca* e *Diopsittaca*). Além disso, sugerimos que *Diopsittaca nobilis* e *Guarouba guarouba* são proximamente relacionadas e que o gênero *Aratinga* não é monofilético. A diversificação entre gêneros deve ter ocorrido durante o Mioceno e a divergência dentro dos gêneros, durante o Plioceno e Pleistoceno. Mudanças geológicas, climáticas e ambientais na América do Sul podem estar relacionadas com tal diversificação.

THE ORDER PSITTACIFORMES comprises ~350 species distributed in 84 genera (Collar 1997, Rowley 1997), and ~27% of them are endangered to varying degrees (BirdLife International 2000). They are distributed throughout the southern portion of North America, Central and South America, the Caribbean Islands, Africa, Indian and Pacific islands, the Australasian region, and southern Asia (Forshaw 1989).

The phylogeny of the group is still poorly understood at almost all taxonomic levels.

Using DNA-DNA hybridization data, Sibley and Alquist (1990) suggested that the order descended from an ancient lineage and has no close living relatives. The morphological homogeneity of parrots makes them distinct from other avian orders, while rendering further subdivision difficult (Smith 1975). However, Smith (1975) suggested that New World parrots should be set apart from Old World parrots; he grouped all the New World parrots in the tribe Arini on the basis of some exclusive characters, such as chicks hatching with imperforate ear canal and the copulation posture on one leg. The tribe Arini is also recognized by other authors

³Address correspondence to this author. E-mail: cymiyaki@usp.br

(Forshaw 1989, Sick 1997, Collar 1997). Sibley and Alquist (1990) also suggested that parrots could be separated by their geographical location (i.e. Americas, Africa, and Australasia); but given the limited number of samples studied, they argued that this conclusion may be premature.

Our present study focuses on the tribe Arini. This group belongs to the subfamily Psittacinae, family Psittacidae, and contains the majority of the species of the order (148 of 353; Collar 1997, Rowley 1997). Some authors have attempted to understand the systematics of this tribe on the basis of morphology (Miranda Ribeiro 1920, Smith 1975, Sick 1990) and behavior (Smith 1975, Sick 1990). However, those studies did not try to recover the phylogeny of the taxa studied, and relationships still remain poorly understood.

A phylogeny of nine Arini genera based on 1.7 kilobases (kb) of mitochondrial DNA sequence data (12S rDNA, 16S rDNA, and cytochrome *b*) supports the presence of two groups: (1) *Anodorhynchus hyacinthinus*, *Ara ararauna*, *Aratinga aurea*, *Cyanopsitta spixii*, *Deroptyus accipitrinus*, *Guarouba guarouba*, and *Pyrrhura picta*; and (2) *Amazona aestiva* and *Pionus menstruus* (Miyaki et al. 1998). Relationships among the seven species in the first group were poorly resolved. Therefore, we expanded the taxon sampling and increased the number of mitochondrial genes sequenced to answer the following questions: (1) Are those groups supported even with the addition of more taxa? (2) Are the nonmonotypic genera monophyletic? and (3) are there other species more closely related to the species studied by Miyaki et al. (1998)? We also estimate and discuss the dates of divergence of the taxa studied. The scientific names of the species analyzed here follow Collar (1997), with modifications (Penhallurick 2001).

METHODS

Taxon sampling and outgroup choice.—Samples from the same individuals analyzed by Miyaki et al. (1998) were used in the present work. Eight other taxa were selected: species from previously studied genera (*Anodorhynchus*, *Aratinga*, and *Ara*), and species from the genera *Diopsittaca* and *Primolius*, once in the genus *Ara* (Forshaw 1989) (Table 1). *Falco peregrinus* (GenBank accession number NC_000878; Mindell et al. 1997) was selected as an outgroup representative of another Neoaves, and *Gallus gallus* (GenBank accession number X52392; Desjardins and Morais 1990) was

included in some analyses to calibrate our molecular clock using the divergence time between Galloanserae and Neoaves (Hedges et al. 1996, Cooper and Penny 1997, van Tuinen and Hedges 2001, Paton et al. 2002).

DNA sequencing.—Blood samples of Neotropical parrots were obtained from official institutions and aviculturists in Brazil and stored at the Department of Biology, University of São Paulo. Total DNA from two individuals of each species was extracted according to Bruford et al. (1992), with the exception of *Pr. coultoni*, for which only one sample from one individual was available.

Five mitochondrial genome regions were partially sequenced: 12S ribosomal DNA (rDNA) (328 base pairs [bp]), 16S rDNA (471 bp), cytochrome *b* (1,020 bp), cytochrome oxidase I (461 bp), and control region (966 bp). The following primer pairs were used via polymerase chain reaction (PCR) to amplify specific regions: 12SL1735/12SH2170 for 12S rDNA; 16SL2702/16SH3309 for 16SrDNA; CBL14957/CBH15331, CBL15298/CBH 15764, CBL15637/HThr16092 for cytochrome *b*; COIA7988/COIF7308 for cytochrome oxidase I; and Lglu/CR522Rb, CRL380/CRH1028, CRL456/CRH1028, CRL380/H1250, CRL456/H1250 for the control region (Table 2). Amplifications were performed in 10 μ L of 1 $\times$ buffer (Amersham Pharmacia, Buckinghamshire, United Kingdom), 200 μ M of each dNTP, 1 μ M of each primer, and 0.5 unit (U) of *Taq* polymerase (Pharmacia). The PCR conditions were initial denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 50–54°C for 25 s, 72°C for 40 s, and a final extension of 72°C for 5 min. Agarose gel electrophoresis was conducted on an aliquot of 2 μ L of the amplified products to confirm the presence of a single clean band. Products were purified by incubation at 37°C for 1 h with 5 U of exonuclease I (USB) and 0.5 U of shrimp alkaline phosphatase (USB). Sequencing reactions were prepared using the same amplification primers and the Big Dye Terminator kit (Applied Biosystems, Foster, California)—manufacturer's protocol was followed—and loaded in an automated DNA sequencer (ABI 377). Both strands were sequenced.

Alignment and other data.—The SEQUENCE NAVIGATOR software (Applied Biosystems, Foster City, California) was used to compare heavy and light strands and to correct for ambiguities. Alignments were performed in CLUSTAL X (Thompson et al. 1997) and manually corrected in MACCLADE 4.0 (Maddison and Maddison 2000).

Sequence statistics were obtained using MEGA (version 2.0; Kumar et al. 2001) and PAUP* (version 4.0b8; Swofford 2001). A chi-square test was used to examine homogeneity of base frequencies across taxa for all studied regions (Foster and Hickey 1999). Tests for nonvariable sites were performed in PAUP*. Program MODELTEST (version 3.06; Posada and Crandall 1998) was used to choose the most

TABLE 1. Species of Neotropical parrots, registration number of the Avian Molecular Genetics and Evolution Laboratory (São Paulo, Brazil), and GenBank accession numbers of the mitochondrial sequences.

Species	Registration ^a	12S rDNA	16S rDNA	Cytochrome <i>b</i>	Cytochrome oxidase I	Control region
<i>Anodorhynchus hyacinthinus</i>	114	U70741 ^b	U70752 ^b	U70763 ^b	AF370737	AF430809
	117	AY286197	AY286214	AY286205	AF370738	AF430810
<i>Anodorhynchus leari</i>	410	AF362914	AF365407	AF370763	AF370736	AF430808
	620	AF362915	AF365408	AF370764	—	AF365432 ^c
<i>Ara ararauna</i>	13	U70739 ^b	U70750 ^b	U70761 ^b	AF370739	AF430811
	246	AY286196	AY286213	AY286204	AF370740	AF430812
<i>Ara severa</i>	1629	AF362916	AF365409	AF370765	AF370747	AF430820
	2407	AF362917	AF365410	AF370766	AF370748	AF430821
<i>Aratinga aurea</i>	346	U70740 ^b	U70751 ^b	U70762 ^b	AF370746	AF430818
	461	AY286200	AY286217	AY286208	—	AF430819
<i>Aratinga cactorum</i>	837	AF362920	AF365413	AF370768	AF370750	AF430823
	992	AF362921	AF365414	AY281254	—	AF430824
<i>Aratinga leucophthalmus</i>	1625	AF362918	AF365411	AF370767	AF370749	AF430822
	2090	AF362919	AF365412	AY281253	—	AF365434 ^c
<i>Cyanopsitta spixii</i>	409	U70742 ^b	AF490351	U70764 ^b	AF370744	AF430816
	498	AY286201	AF490350	AY286209	AF370745	AF430817
<i>Diopsittaca nobilis</i>	973	AF362922	AF365415	AF370769	AF370751	AF430825
	1126	AF362923	AF365416	—	AF370752	AF430826
<i>Guarouba guarouba</i>	4	U70744 ^b	U70755 ^b	U70766 ^b	AF370741	AF430813
	69	AY286198	AY286215	AY286206	—	AF430814
<i>Primolius maracana</i>	1290	AF362928	AF365421	AF370774	AF370754	AF430831
	1006	AF362929	AF365422	AF370775	—	AF430832
<i>Primolius auricollis</i>	1742	AF362926	AF365419	AF370772	AF370753	AF430829
	2345	AF362927	AF365420	AF370773	—	AF430830
<i>Primolius couloni</i>	3142	AF362940	AF365431	AF370780	AF370762	AF430835
<i>Pyrrhura picta</i>	397	U70748 ^b	U70759 ^b	U70768 ^b	AF370760	AF365438 ^c
	399	AF362939	AF365430	AY286211	AF370761	AF365439 ^c
<i>Amazona aestiva</i>	385	U70738 ^b	U70749 ^b	U70760 ^b	AF370742	AF430815
	486	AY286195	AY286212	AY286203	AF370743	AF365433 ^c

^a Registration number in the Avian Molecular Genetics and Evolution Laboratory (LGEMA) at the Department of Biology, University of São Paulo, Brazil.

^b Miyaki et al. (1998).

^c Approximately 510 bp; the others have 940 bp.

TABLE 2. Primers used for PCR amplification and sequencing of mitochondrial regions of Neotropical parrots.

Primer name ^a	Location	Sequence (5'–3')	Reference
12S rDNA L (12SL1735)	1735 ^b	GGATTAGATACCCCACTATGC	Miyaki et al. (1998)
12S rDNA H (12SH2170)	2170 ^b	AGGGTGACGGGCGGTATGTACG	Miyaki et al. (1998)
16S rDNA L (16SL2702)	2702 ^b	CCTACCGAGCTGGGTGATAGCTGGTT	Miyaki et al. (1998)
16S rDNA H (16SH3309)	3309 ^b	TGCGCTACCTTCGCACGGT	Miyaki et al. (1998)
Cytochrome-b L (CBL14957)	14957 ^b	AAAAAGCTTCCATCCAAACATCTCAGCATGATGAAA	Kocher et al. (1989)
Cytochrome-b H (CBH15331)	15331 ^b	AAACTGCAGCCCTCAGAATGATATTGTCTCTCA	Kocher et al. (1989)
Cytochrome-b H (HThr16092)	16092 ^b	AACTGCAGTCATCTCCGGTTTACAAGAC	Kornegay et al. (1993)
Cytochrome-b L (CBL15298)	15298 ^b	TGAGGCCAAATATCATCTCTGAGGGGC	Cheng et al. (1994)
Cytochrome-b L (CBL15637)	15637 ^b	AACCTCTAGGAGACCCAGAAAACTTC	Miyaki et al. (1998)
Cytochrome-b L (CBL15764)	15764 ^b	CCTCCTAGTTTGTGGGGATTGA	Miyaki et al. (1998)
Cytochrome oxidase I A (COIA7988)	7988 ^b	AGTATAAGCGTCTGGGTAGTC	Palumbi (1996)
Cytochrome oxidase I F (COIF7308)	7308 ^b	CCTGCAGGAGGAGGAGAYCC	Palumbi (1996)
tDNA ^{Thr} (LThr 16063)	16063 ^b	TGGTCTTGTAACCAAAAGA	Eberhard et al. (2001)
tDNA ^{Glu} L (Lglu)	16739 ^b	GCCCTGAAARCCATCGTTG	Eberhard et al. (2001)
Control region H (CR522Rb)	576 ^c	TGGCCCTGACYTAGGAACCCAG	Eberhard et al. (2001)
Control region L (CRL380)	380 ^c	GCCCATAA YTGCCCGGGAACCTT	Present study
Control region L (CRL456)	456 ^c	CACGAGAGATCA YCAACCCGGTGT	Present study
Control region H (CRH1028)	1028 ^c	AGGTGTAACAACA AGTGATCAGGGT	Present study
tDNA ^{Phe} H (H1250)	1250 ^b	CTTGGCRTCTTCAGTGYCATGCT	Eberhard et al. (2001)

^a L and H stand for the light and heavy chains, respectively.

^b Location in the *Gallus gallus* map (Desjardins and Morais 1990).

^c Location in the *Amazona ochrocephala auripalliata* control region 2 sequence (Eberhard et al. 2001).

appropriate evolutionary model of DNA substitution prior to tree reconstruction using distance and maximum-likelihood (ML) approaches. Degree of saturation was evaluated by separately comparing the number of transitions and transversions as a function of the uncorrected sequence divergence (following Barker and Lanyon 2000). Each sequenced region was analyzed separately and two matrices were constructed using combined regions: (1) 12S rDNA, 16S rDNA, cytochrome *b*, and cytochrome oxidase I (total of 2,280 bp) from 17 taxa; and (2) 12S rDNA, 16S rDNA, cytochrome *b*, cytochrome oxidase I, and control region (total of 3,246 bp) from 13 taxa.

Phylogenetic analyses.—Phylogenetic tree reconstruction using maximum parsimony (MP), distance (minimum evolution, ME), and ML methods were performed in PAUP*. Parsimony analyses were conducted using a heuristic search algorithm with 100 random additional sequence replicates and tree bisection and reconnection (TBR) branch-swapping. Insertions and deletions (indels) were treated as missing data. Transitions in the third codon position of cytochrome *b* were down-weighted 0.01, 0.2, and 0.5 $\times$ using stepmatrices in PAUP*. Minimum evolution reconstructions were conducted using heuristic search with the same parameters as described for MP analyses. Maximum-likelihood analyses were performed by heuristic search with 10 random additional sequence replicates and TBR branch-swapping. Nonparametric bootstrapping (Felsenstein 1985) was used to assess support for branching topologies (1,000 bootstrap replicates for ME and MP, 100 replicates for ML).

Divergence times.—Two-cluster and the branch-length tests implemented in LINTREE (Takezaki et al. 1995) were used to examine the constancy of evolutionary rates among the 17 taxa from the matrix of 2,280 bp. Those sequences evolving excessively quickly or slowly at a high significance level (5%) were eliminated, and those tests were repeated until the hypothesis of constancy of evolutionary rates was accepted. The distance matrix obtained with the best-fit model for the 2,280 bp alignment matrix (general time-reversible [GTR] with gamma correction; Lanave et al. 1984, Rodríguez et al. 1990) was compared to the distance matrix obtained by the closest evolutionary model available in LINTREE (TrN93 with gamma correction; Tamura and Nei 1993) using a matrix correlation followed by a Mantel's test (Marroig and Cheverud 2001). Because those matrices are similar ($r = 0.988$; $P = 0.0001$), divergence times were estimated assuming TrN93 as the evolutionary model and using the branch lengths and standard errors of the linearized tree generated by the two-cluster test in LINTREE.

The fossil record does not provide a good calibration for divergence dates among Neotropical parrots. Thus, two alternative calibrations were used to estimate divergence times: (1) the split between

Galloanserae and Neoaves, estimated to be ~100 mya (Hedges et al. 1996, Cooper and Penny 1997, van Tuinen and Hedges 2001, Paton et al. 2002); and (2) mitochondrial DNA evolutionary rate of 2% pairwise divergence per millions of years, initially described for mammals (Brown et al. 1982), but also estimated (Shields and Wilson 1987, Randi 1996) and applied in birds (Klicka and Zink 1997, Lucchini et al. 2001).

RESULTS

DNA sequences.—The following supports a mitochondrial origin for our sequences: (1) amplified PCR products resulted in single bands and generated clean unambiguous sequences; (2) all obtained sequences aligned well with homologous ones from other parrots (Cooper and Penny 1997, Miyaki et al. 1998, Espinosa de los Monteros 2000, Eberhard et al. 2001); however, exceptions were the cytochrome-oxidase I sequences that were not previously available for parrots; (3) the inferred amino acid sequences of the coding genes (cytochrome *b* and cytochrome oxidase I) aligned to corresponding sequences from the outgroups *F. perigrinus* and *Ga. gallus*; (4) the lower proportion of guanine (Table 3) observed in cytochrome *b*, cytochrome oxidase I, and control region was in accordance with the results observed in other avian orders (Desjardins and Morais 1990, Baker and Marshall 1997, Moore and DeFilippis 1997, Hughes and Baker 1999, Espinosa de los Monteros 2000); and (5) as observed in other birds (Moore and DeFilippis 1997), the coding sequences accumulated more substitutions at third codon positions (Table 3).

The 16S rDNA sequence from *C. spixii* was already available (U707530; Miyaki et al. 1998), but its alignment with the other sequences revealed an unexpectedly high divergence. Thus, the same two individuals' samples were resequenced and a sample from a third bird was included (Table 1). Those three sequences are identical and are more easily aligned with the sequences from other taxa while differing from the previously published sequence. That suggests a 16S rDNA pseudogene may have been mistakenly amplified and sequenced by Miyaki et al. (1998). Thus, it is important to be cautious with sequences that are unexpectedly divergent.

Alignment.—Partial sequences obtained for 12S and 16S rDNAs, cytochrome *b*, and cytochrome oxidase I were aligned to homologues of *F. perigrinus* and *Ga. gallus*. Indels were present in

TABLE 3. Average base composition, percentage of variable sites, transition and transversion rates (Si/Sv) of mitochondrial regions from Neotropical parrots.

Mitochondrial regions	Base composition (%)				% variable sites	Si/ Sv
	A	C	G	T		
12S rDNA	33.1	27.9	21.3	17.7	19.51	6.2
16S rDNA	35.1	31.7	16.8	16.5	33.12	4.0
Cytochrome <i>b</i>	27.2	34.0	12.8	25.9	37.84	4.7
Cytochrome <i>b</i> (first position)	26.3	28.6	20.9	24.2	8.92	6.8
Cytochrome <i>b</i> (second position)	20.0	26.9	13.0	40.1	4.22	1.6
Cytochrome <i>b</i> (third position)	35.4	46.6	4.7	13.3	24.71	5.1
Cytochrome oxidase (COI) I	28.1	28.2	18.6	25.1	29.72	11.2
COI (first position)	27.1	18.8	31.9	22.1	3.07	27.9
COI (second position)	18.9	26.6	16.2	38.3	0.002	0
COI (third position)	38.4	39.2	7.6	14.7	26.46	11.0
Control region (966 bp) ^a	22.4	26.5	19.2	31.9	34.89	2.0

^aThose results exclude the sequences from *Amazona aestiva*.

the 12S and 16S rDNA alignment matrices but not in cytochrome-*b* and cytochrome oxidase I matrices. The parrot control region sequences show conserved blocks (C, D, and F) that are also observed in other orders of birds (Baker and Marshall 1997). Through PCR and sequencing analyses of a mitochondrial segment comprising the control region and its flanking genes from *An. hyacinthinus*, *Ara ararauna*, *D. nobilis*, *G. guarouba*, and *C. spixii* (Tavares et al. unpubl. data), we determined that those species have the same gene order as described for *Ga. gallus* (Desjardins and Morais 1990). Therefore, they do not present a duplication of the control region, as found in the parrots from the genera *Amazona* and *Pionus* (Eberhard et al. 2001).

Even though the control region sequences obtained for the species studied here could be aligned with the control region 2 sequence from *Amazona ochrocephala auropalliata* (Eberhard et al. 2001), homology could not be determined. In addition, the substantial variation and number of inferred indel events between the control region from parrot taxa, *F. peregrinus*, and *Ga. gallus* precluded confident alignment of sequences. Thus, the phylogenetic analyses including the control region sequences did not contain *Am. aestiva*, *F. peregrinus*, or *Ga. gallus*. The sequence alignment matrices for each region and for the two data sets with combined sequences (2,280 and 3,246 bp) are available from the authors on request.

The test for homogeneity of base frequencies suggested that base composition was unlikely to produce phylogenetic bias ($P = 1.0, 0.72, 0.71, 0.88, \text{ and } 0.70$ for 12S rDNA, 16S rDNA,

cytochrome *b*, cytochrome oxidase I, and control region, respectively). Values of average base composition, percentage of variable sites, and transition and transversion rates of each mitochondrial region were obtained for the Neotropical taxa studied (Table 3). Among the mitochondrial regions studied, the least variable one was the 12S rDNA. The fact that the control region was not the most variable (Table 3) could be due to the presence of *Am. aestiva* sequences in the comparisons of all other mitochondrial genes and its absence in the control region matrix. When that species is excluded from the other sequence matrices, the control region holds the highest variability among all mitochondrial segments analyzed. Within the parrot taxa studied, no saturation was detected for 12S rDNA, 16S rDNA, cytochrome oxidase I, and control region sequences; but we detected that transitions are saturated at the third codon position of cytochrome *b* when *Am. aestiva* is present in the data set. Also, when *F. peregrinus* and *Ga. gallus* are included, saturation occurs for transitions in 12S rDNA, 16S rDNA, cytochrome *b* (first and third positions) and cytochrome oxidase I (third position).

Phylogenetic analysis.—Because tree reconstructions for isolated mitochondrial regions always showed individuals of the same species clustered, analyses of the combined sequences included only one individual for each species. Ranges of noncorrected divergences between individuals from the same species were 0–1%, 0–0.7%, 0–1%, 0–0.6%, and 0–2.1% for 12S rDNA, 16S rDNA, cytochrome *b*, cytochrome oxidase I,

and control region, respectively. In general, MP analyses revealed that each separate mitochondrial region supports the following clades: (*An. hyacinthinus*, *An. leari*); (*Pr. maracana*, *Pr. auricollis*, *Pr. couloni*); (*D. nobilis*, *G. guarouba*); and (*Arat. aurea*, *Arat. cactorum*). Only topologies reconstructed using the cytochrome-*b* matrix did not support the group (*Arat. aurea*, *Arat. cactorum*). Relationships between those groups are not well resolved, and those deeper nodes show low support values (data not shown).

Analyses of combined sequences with 2,280 bp were initially performed including the *F. peregrinus* and *Ga. gallus* outgroups. The model selected for ME and ML analyses was GTR (Lanave et al. 1984, Rodríguez et al. 1990) with proportion of invariable sites ($P_i = 0.4771$), and rates for variable sites following a gamma distribution ($\gamma = 0.9160$). Estimated base frequencies were A = 0.3257, C = 0.3469, G = 0.1330, and T = 0.1944. All tree topologies based on MP, ME, and ML (bootstrap support values of 100, 85, and 80,

respectively) suggest that *Am. aestiva* is a basal taxon (Fig. 1). As *F. peregrinus* and *Ga. gallus* are too divergent from the ingroup, they were excluded from subsequent analyses, and *Am. aestiva* was considered an outgroup. The MP analyses resulted in four most parsimonious trees ([trees not shown] length = 1,491; CI = 0.440, excluding uninformative characters; RI = 0.454). Those trees present the same well-supported major clades in the ingroup that were obtained in the earlier analysis using *F. peregrinus* and *Ga. gallus* as outgroups. Because the cytochrome-*b* third-codon transitions were saturated, they were down-weighted using the transition and transversion ratio obtained from the data (0.2; Table 3) and two other arbitrary values: 0.5 and 0.01.

All weighted MP analyses resulted in one most parsimonious tree (tree not shown). The first two weighting values generated the same tree topology and recovered one of the four unweighted MP trees. The last weighting value

FIG. 1. Maximum-likelihood rooted tree ($-\ln$ likelihood = 12199.61986, model GTR + P_i + G) obtained with 2,280 bp of mitochondrial sequences. The ML (100 replications) and MP bootstrap values (1,000 replications) are presented above and under branches, respectively.

(0.01) generated one tree topology. All those four MP analyses (unweighted, three weighting values) supported the following clades with bootstrap support >96%: (*An. hyacinthinus*, *An. leari*); genus *Primolius*; (*G. guarouba*, *D. nobilis*); and (*Arat. aurea*, *Arat. cactorum*). The clade (*Ara ararauna*, *Ara severa*) was present in all the MP topologies with bootstrap values between 80 and 87. Hierarchical likelihood ratio test was performed for the remaining taxa, and the model selected for the ME and ML analyses was GTR with $P_i = 0.5279$, $\gamma = 1.1231$. Estimated base frequencies were A = 0.3025, C = 0.3318, G = 0.1563, and T = 0.2093. Similar topologies resulted from ME and ML analyses. Well-supported clades in the MP analyses were also recovered in the ME and ML analyses with high bootstrap values. An exception was the clade (*Ara ararauna*, *Ara severa*) with a low bootstrap of 58 in the ML analysis (Fig. 1). The deeper nodes obtained by

the ME and ML trees are different from the ones obtained in the MP topologies, but those nodes present bootstrap values <50%.

Analysis of longer sequences (3,246 bp) was performed with fewer taxa and no outgroup. The ME and ML trees were calculated according to the Tamura-Nei 93 model (TrN93; Tamura and Nei 1993), assuming that proportions of sites are invariable ($P_i = 0.4958$) and rates for variable sites follow a gamma distribution ($\gamma = 0.9372$). Base frequencies estimated from the data were A = 0.2801, C = 0.2983, G = 0.1688, and T = 0.2528. The MP analysis resulted in one most parsimonious tree (length = 1,859, CI = 0.497, RI = 0.526), with the same unrooted topology recovered by the ME and ML analyses (Fig. 2).

Divergence times.—The matrix with 2,280 bp was also used to estimate divergence times among the lineages studied. The hypothesis

FIG. 2. Maximum-likelihood unrooted tree ($-\ln$ likelihood = 13032.68957, model TrN93 + Pi + G) obtained with 3,246 bp of mitochondrial sequences. The ML (100 replications) and MP bootstrap values (1,000 replications) are presented, respectively, at the nodes. "N" represents bootstrap values <50.

FIG. 3. Distance (neighbor-joining) linearized tree obtained with 2,280 bp of mitochondrial sequences. Nodes are numbered according to Table 4.

of rate constancy was accepted when *F. peregrinus*, *Am. aestiva*, and *Py. picta* were excluded from the analysis. The best evolutionary model selected was GTR with $\gamma = 0.1843$. However, we used the similar model TrN93 with $\gamma = 0.1747$ available in LINTREE. Ages and standard errors for each node in Figure 3 are illustrated in Table 4. The split of the genera studied occurred within a relatively short period of time. Assuming the split of Galliformes and Neoaves at 100 mya, the average divergence times among the parrot genera lineages occurred between 7.5 and 18 mya (Miocene). In contrast, the separation of species lineages occurred over a broader range of time, from 1 to 12 mya (Pleistocene to middle Miocene). Assuming the calibration rate of 2% per millions of years, the split among genera lineages occurred between 4.2 and 13 mya (late Miocene) and the separation among species lineages occurred from around 0.7 to 7 mya (Pleistocene to late Miocene), with most of the splits occurring in the Pliocene and Pleistocene.

DISCUSSION

The results of the present study suggest that our understanding of the phylogenetic

relationships among Neotropical parrots can be improved by adding both taxa and sequences. Our results support that *Am. aestiva* is basal to the other 14 taxa studied. Thus, as expected, the eight new taxa included here are more closely related to the species belonging to one of the groups obtained in a previous study (Miyaki et al. 1998). Other independent studies also show

TABLE 4. Estimated dates of separation of parrot species with both calibration approaches (age [Ma] $\pm$ SE). Node numbers correspond to the numbers in Figure 3.

Nodes	Galloanserae-Neoaves	
	100 mya	2% Ma ⁻¹
1	1.32 $\pm$ 0.26	0.75 $\pm$ 0.15
2	2.31 $\pm$ 0.27	1.31 $\pm$ 0.15
3	3.82 $\pm$ 0.53	2.16 $\pm$ 0.30
4 ^a	7.50 $\pm$ 0.97	4.25 $\pm$ 0.55
5	7.58 $\pm$ 1.01	4.29 $\pm$ 0.57
6	12.01 $\pm$ 1.49	6.81 $\pm$ 0.85
7 ^a	12.27 $\pm$ 1.23	6.95 $\pm$ 0.70
8 ^a	13.90 $\pm$ 0.72	7.88 $\pm$ 0.41
9 ^a	15.99 $\pm$ 0.90	9.06 $\pm$ 0.51
10 ^a	16.00 $\pm$ 0.70	9.07 $\pm$ 0.40
11 ^a	17.89 $\pm$ 0.38	10.13 $\pm$ 0.21
12 ^a	18.29 $\pm$ 1.10	13.37 $\pm$ 0.62

^aSeparation between genera lineages.

that characteristics shared by some of those taxa are distinct from *Am. aestiva*: (1) a study of syrinx anatomy revealed *Am. aestiva* has a tendency for fusion of more Tympanum elements than taxa from the other group (Lima 2001); (2) chromosome morphology of five genera from our ingroup is distinct from that observed in *Am. aestiva* (see a revision in Francisco et al. 2001); (3) minisatellites linked to the W chromosome are absent in *Am. aestiva* and present in all the genera of our ingroup with the exception of *Py. picta* (Miyaki et al. 1992, 1993, 1995, 1997). Thus, the presence of two major groups within the tribe Arini is still supported, with some exceptions. However, additional taxa have yet to be studied. Ongoing analyses of more genera from the group that includes *Am. aestiva* are necessary to confirm that hypothesis.

As expected, the two species studied from the genus *Anodorhynchus* (*An. hyacinthinus* and *An. leari*) are closely related. Those species have been grouped in the same genus in all classifications (Salvadori 1891; Miranda Ribeiro 1920; Peters 1937; Pinto 1937, 1978; Forshaw 1989; Sick 1997; Collar 1997).

Some classification proposals (Salvadori 1891, Peters 1937, Forshaw 1989), mainly based on morphological characters, group 14 species in the genus *Ara*. Other authors (Sick 1990, 1997; Collar 1997) consider that five of those species should be separated into the genera *Primolius* (three species), *Diopsittaca* (one species), and *Orthopsittaca* (one species). The two *Ara* species studied here (*Ara ararauna* and *Ara severa*) form a group. Although the ML bootstrap value for that clade is not very high (58%), this group is well supported by the MP bootstrap (88%) with the same data set (Fig. 1). Higher bootstrap support values are obtained (MP = 97% and ML = 93%) in the analysis including longer sequences (Fig. 2). Thus, our data suggest that genus *Ara* is monophyletic. However, that result needs to be confirmed by a future study of all the species belonging to that genus.

The three species from the genus *Primolius* (*Pr. auricollis*, *Pr. couloui*, and *Pr. maracana*) also form a well-supported monophyletic group (Figs. 1 and 2). In some classifications, those species are included in the genus *Ara*; but according to Sick (1990), they present distinct vocalization traits and should be placed in another genus. The unrooted tree topology (Fig. 2) suggests that *Ara* and *Primolius* are sister groups,

with high bootstrap values (89% and 94% for ML and MP, respectively). However, that result must be taken with caution because that topology is unrooted.

High bootstrap values support the close relationship between *D. nobilis* and *Gu. guarouba* (Figs. 1 and 2). *Diopsittaca nobilis* has been placed in the genus *Ara* (Salvadori 1891, Peters 1937, Forshaw 1989). All species included in the genus *Ara* as described by those authors have blue-turquoise flight feathers; the exception is *D. nobilis*, which has green remiges, primaries, and secondaries (Collar 1997). Thus, our results support that *D. nobilis* is not closely related to the other species of the genus *Ara* and should be maintained in a separate genus.

Some authors include *Gu. guarouba* in the genus *Aratinga* (Peters 1937, Pinto 1978, Forshaw 1989). Others place that species in the genus *Conurus* with taxa from the genus *Aratinga* (Salvadori 1891, Miranda Ribeiro 1920). Our data do not support a close relationship between *Gu. guarouba* and any other taxa that we included from the genus *Aratinga*. However, our results clearly support a sister-taxon relationship between *Gu. guarouba* and *D. nobilis*.

As expected, the species *Arat. aurea* and *Arat. cactorum* are well-supported sister groups (Figs. 1 and 2). However, *Arat. leucophthalmus* does not seem to be closely related to those groups, which suggests that the genus *Aratinga*, as recognized today, is not monophyletic. A study including more species from the genus would help confirm that finding (Ribas and Miyaki 2004).

Relationships among the deeper lineages were not well resolved using mitochondrial DNA sequence data, as reflected in short branch lengths and low bootstrap support (Fig. 1). That result suggests that the resolution of our data was not sufficient to resolve those relationships. It is possible that the addition of more taxa, other gene sequences (such as the nuclear RAG-1; Groth and Barrowclough 1999), or both may result in higher resolution for that area of the tree topology. A possible alternative explanation is that the divergence of those taxa occurred during a very short interval of time and, thus a polytomy is recovered. Ongoing and future studies will enable us to get a clearer scenario of the phylogeny of Neotropical parrots.

Divergence times.—The short internodal branches of the linearized tree suggest that a relatively fast diversification occurred in the

Neotropical parrot lineages examined by our study. Both of the calibrations used to estimate divergence times (the split of Galloanserae and Neoaves ~100 mya and the rate of 2% sequence divergence per millions of years) suggest that the divergence of parrot genera lineages occurred during the Miocene, whereas the separation of most of the species lineages took place during the Pliocene and Pleistocene. The divergences of some genera of New World Thraupinae (Burns 1997) and Cracidae (Pereira et al. 2002) were also estimated to date from the Miocene. Therefore, major events during the Miocene seem to have contributed to avian diversification. One such event may have been the uplift of the Eastern Andean Cordillera in the late middle Miocene, resulting in the present Amazon River basin (Hoorn et al. 1995). Additionally, a combination of sea-level changes and tectonic loading of the foreland basin in the upper Amazon may have produced significant incursions of sea water into South America during the early Miocene (15–23 mya), also influencing the diversification of Neotropical communities (Lovejoy et al. 1998). That also coincided with a major global cooling event (Hoorn et al. 1995). The divergences between species of *Pteroglossus* (Rhamphastidae; Hackett 1995) and Thraupidae (Hackett and Lehn 1997) were also dated to the Pliocene and Pleistocene. Climatic oscillations occurring during those periods are related to fragmentation and re-expansion of tropical forests, ecological vicariance, river dynamics, marine transgressions, and other events (see revisions in Haffer 1993, Marroig and Cerqueira 1997). Those changes may be related to the diversification of Neotropical avian groups.

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Anexo III

Phylogenetic Relationships and Historical Biogeography of Neotropical Parrots (Psittaciformes: Psittacidae: Arini) Inferred from Mitochondrial and Nuclear DNA Sequences

ERIKA SENDRA TAVARES,¹ ALLAN J. BAKER,^{2,3} SÉRGIO LUIZ PEREIRA,² AND CRISTINA YUMI MIYAKI¹

¹Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, R. do Matão 277, 05508-090, São Paulo, SP, Brazil; E-mail: cymiyaki@ib.usp.br (C.Y.M.)

²Department of Natural History, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario, Canada M5S 2C6

³Department of Zoology, University of Toronto, Toronto, Ontario, Canada M5S 1A1

Abstract.—Previous hypotheses of phylogenetic relationships among Neotropical parrots were based on limited taxon sampling and lacked support for most internal nodes. In this study we increased the number of taxa (29 species belonging to 25 of the 30 genera) and gene sequences (6388 base pairs of RAG-1, cyt b, NADH2, ATPase 6, ATPase 8, COIII, 12S rDNA, and 16S rDNA) to obtain a stronger molecular phylogenetic hypothesis for this group of birds. Analyses of the combined gene sequences using maximum likelihood and Bayesian methods resulted in a well-supported phylogeny and indicated that amazons and allies are a sister clade to macaws, conures, and relatives, and these two clades are in turn a sister group to parrotlets. Key morphological and behavioral characters used in previous classifications were mapped on the molecular tree and were phylogenetically uninformative. We estimated divergence times of taxa using the molecular tree and Bayesian and penalized likelihood methods that allow for rate variation in DNA substitutions among sites and taxa. Our estimates suggest that the Neotropical parrots shared a common ancestor with Australian parrots 59 Mya (million of years ago; 95% credibility interval (CrI) 66, 51 Mya), well before Australia separated from Antarctica and South America, implying that ancestral parrots were widespread in Gondwanaland. Thus, the divergence of Australian and Neotropical parrots could be attributed to vicariance. The three major clades of Neotropical parrots originated about 50 Mya (95% CrI 57, 41 Mya), coinciding with periods of higher sea level when both Antarctica and South America were fragmented with transcontinental seaways, and likely isolated the ancestors of modern Neotropical parrots in different regions in these continents. The correspondence between major paleoenvironmental changes in South America and the diversification of genera in the clade of amazons and allies between 46 and 16 Mya suggests they diversified exclusively in South America. Conversely, ancestors of parrotlets and of macaws, conures, and allies may have been isolated in Antarctica and/or the southern cone of South America, and only dispersed out of these southern regions when climate cooled and Antarctica became ice-encrusted about 35 Mya. The subsequent radiation of macaws and their allies in South America beginning about 28 Mya (95% CrI 22, 35 Mya) coincides with the uplift of the Andes and the subsequent formation of dry, open grassland habitats that would have facilitated ecological speciation via niche expansion from forested habitats. [Biogeography; divergence times; mitochondrial DNA; molecular clock; molecular phylogeny; Neotropical parrots; nuclear DNA; tribe Arini.]

Neotropical parrots (Psittaciformes: Psittacidae: Arini) occur from Mexico to the extreme south of South America (Forshaw, 1989). They comprise the largest group within the order, numbering 30 genera and 149 of the total 330 recognized species of Psittaciformes (Collar, 1997; Rowley, 1997). They vary greatly in body mass, habitat preference, geographic distribution, and behavior (Forshaw, 1989). The systematics of Neotropical parrots has had a checkered history. Earlier workers, based on external morphology (Salvadori, 1891; Boetticher, 1943, 1959), anatomy (Verheyen, 1956), or behavior (Brereton, 1963), usually split them into two groups with similar composition, sometimes including African taxa (Salvadori, 1891; Boetticher, 1943, 1959). Verheyen (1956) suggested that the two groups Amazoninae and Arinae, which includes only Neotropical parrots, share a combination of anatomical characters that separates them from the other non-Neotropical parrots but did not suggest a category to group them. In a comprehensive review of the Psittaciformes, Smith (1975) rejected previous groupings of Neotropical genera because none of the characters were informative and placed all the Neotropical taxa in the tribe Arini (family Psittacidae) on the basis of two exclusive characters (e.g., chicks hatching with an imperforate ear canal, and copulatory stance on one leg). Smith's proposal has been accepted in all further classifications (e.g., Forshaw, 1989; Collar, 1997).

The monophyly of Neotropical parrots was supported by DNA-DNA hybridization studies (Sibley and Alquist, 1990), but with limited taxon sampling, and by DNA sequences from an intron of the spindlin gene located on the avian sex chromosomes (de Kloet and de Kloet, 2005).

Additionally, an analysis of 1771 base pairs (bp) of mitochondrial genes from nine genera of the Arini recovered two monophyletic groups (Miyaki et al., 1998), and referred to them as short-tailed and long-tailed groups, in accordance with an identification key (Sick, 1997). Branch support was generally low, except for the monophyly of Neotropical parrots (Miyaki et al., 1998). Later, this data set was increased to 3245 bp for 13 parrots, but the analyses still did not provide good overall support for the phylogenetic relationships among genera (Tavares et al., 2004). Despite efforts to understand the phylogenetic relationships among Neotropical parrot taxa using molecular data, the relationships among most genera remain unresolved or are not strongly supported in existing phylogenies. These studies have shown polytomies and lack of support at some nodes because of low phylogenetic signal in the DNA sequences analyzed. The recommended strategy to improve tree resolution is to increase the number of taxa and characters sampled for phylogenetic analysis (Poe and Swofford, 1999; Haddrath and Baker, 2001; Slowinski, 2001; Paton

et al., 2002; Pereira et al., 2002). Moreover, several studies indicated that *Aratinga*, *Amazona*, and *Pionopsitta* were not monophyletic genera (Tavares et al., 2004; Ribas and Miyaki, 2004; Russello and Amato, 2004; Ribas et al., 2005).

Estimates of divergence times assuming a time-constrained tree, and 100 million years ago (Mya) for the divergence of Galliformes and Psittaciformes, indicated the split between the Australian parakeet *Melopsittacus undulatus* and some Neotropical parrots occurred about 76 Mya, which suggests that continental drift could have been responsible for the isolation of these groups of parrots (Miyaki et al., 1998). The divergences between some Neotropical parrot genera were estimated to have occurred in the late Oligocene and the Miocene (between 27 and 16 Mya) and were likely associated with environmental changes such as sea level regression, orogenic movements in the Andes and expansion of forest/riparian habitat (Miyaki et al., 1998; Tavares et al., 2004). Divergence times between congeneric species of parrots were estimated between 0.5 and 1.3 Mya using the standard mitochondrial rate of 1.6–2.0%/Myr (Tavares et al., 2004; Eberhard and Bermingham, 2004, 2005; Ribas and Miyaki, 2004; Ribas et al., 2005).

In an attempt to better understand the evolution of this group, we obtained a large data set of mitochondrial and nuclear DNA sequences totaling 6388 bp for most genera of the tribe Arini, including representatives of putatively nonmonophyletic genera. We inferred their phylogenetic relationships based on several tree-building methods and checked the phylogenetic utility of morphological characters, some of which were used previously to define groups within Neotropical parrots. As the hypothesis of rate constancy was rejected for Neotropical parrot genera and the method of linearized tree does not account for rate variation of DNA substitution among lineages, divergence times were estimated using methods that accommodate rate variation as Bayesian technique (Thorne et al., 1998; Kishino et al., 2001) and penalized likelihood (Sanderson, 2002). Therefore, the dates estimated were used to help construct a biogeographic hypothesis for the diversification of the group.

MATERIALS AND METHODS

Taxon Sampling

We obtained samples from 29 species representing 25 of the 30 genera of the tribe Arini (Table 1). As previous studies suggested that the genera *Amazona*, *Aratinga*, and *Pionopsitta* are not monophyletic (Russello and Amato, 2004; Tavares et al., 2004; Ribas and Miyaki, 2004; Ribas et al., 2005), we sampled two or three species from each of these genera to represent the distinct lineages that have been reported. We chose the Australian *Melopsittacus undulatus* (tribe Platycercini), *Cacatua goffini* (family Cacatuidae), and the New Zealand *Strigops habroptilus* (tribe Strigopini, Genbank accession no. NC_005931, Harrison et al., 2004) as representatives of non-Neotropical par-

rots because each one belongs to a major group of parrots (de Kloet and de Kloet, 2005). *Falco peregrinus* (Genbank accession nos. NC_000878, Mindell et al., 1998; AY461399, Griffiths et al., 2004), and *Gallus gallus* (Genbank accession nos. X52392, Desjardins and Morais, 1990; AF143730, Groth and Barrowclough, 1999) were selected as more distant outgroups to root the topology. The classification of parrots adopted here follows Collar (1997), except for *Propyrrhura*, which was referred to as *Primolius* following Penhallurick (2001) (Table 1).

DNA Extraction and Sequencing

DNA was extracted from blood (except *Brotogetis chiriri*, *Nannopsittaca dachilleneae*, and *Pionopsitta barra-bandi*, which were from muscle) in a solution containing 0.1% SDS, 100 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA, and 10 mg/ml proteinase K kept overnight at 55°C. DNA purification was performed by standard phenol-chloroform-isoamyl alcohol method (Bruford et al., 1992). Polymerase chain reaction (PCR) amplifications were performed according to Hagelberg (1994) using 25- μ l reactions, with a buffer solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin, and 160 μ g/ml bovine serum albumin (BSA), 0.4 mM dNTPs, 0.2 μ M of each primer, 1 U *Taq* polymerase Platinum (Gibco), and 25 to 50 ng DNA. PCR cycle conditions for RAG-1 amplifications follow Groth and Barrowclough (1999). Mitochondrial genes were amplified in 36 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 1 min, with an initial denaturation of 94°C for 5 min and a final extension at 72°C for 7 min. Amplified segments were purified by excising bands from agarose gels and centrifuging each through a filter tip. Sequences were obtained on an Li-Cor 4200 bidirectional automated DNA sequencer (Li-Cor Biotechnology) or ABI3100 (Applied Biosystems) according to the manufacturers' suggested protocols. Sequences were obtained from the nuclear gene RAG-1, and the mitochondrial genes *cyt b*, *NADH2*, *ATPase 6*, *ATPase 8*, *COIII*, *12S rDNA*, and *16S rDNA*.

The primers used to amplify RAG-1 were R13, R17, R18, R21, R24, and R2B (Groth and Barrowclough, 1999). Additional primers used only in sequencing reactions were R1, R11, R14, R19, R20, and R22 (Groth and Barrowclough, 1999). The primers used to amplify cytochrome *b* were b1, b52, and b6 (Kocher et al., 1989). The following mitochondrial primers were developed by Oliver Haddrath (personal communication): for *NADH2* amplifications the external primers used were MetL (AAG CTA TCG GGC CCA TAC CCG) and ASNH (GAT CRA GGC CCA TCT GTC TAG), and sequencing primers were ND2H (CCT TGA AGC ACT TCT GGG AAT CAG A) and ND2-54H (GGG GTG GTG AGA TTT TGC GA). For a segment including *ATPase8*, *ATPase6*, and *COIII* the primers used in amplifications were LysL (CAG CAC TAG CCT TTT AAG CT), and COIIRH (ATT ATT CCG TAT CGN AGN CCY TTT TG), and sequencing primers were A5ATP6 (TAG GAG TGT

TABLE 1. Taxa sampled for this study. Ratio of wing length:tail length and museum skins used to verify the states of the shape of the tail, nostrils and periophthalmic ring are also given.

Species	Sample number ^a	Wing/tail ^b	Skin examined MZUSP ^c	Shape of the tail ^d	Nostrils ^e	Periophthalmic ring ^f
<i>Amazona farinosa</i>	3683	1.9	6731	R	E	N
<i>Amazona xanthops</i>	1876	2.6	4330	R	E	N
<i>Anodorhynchus hyacinthinus</i>	5281*	0.8	32290	C	H	N
<i>Ara ararauna</i>	13	0.8	13992	C	E	N
<i>Aratinga aurea</i>	346	1.2	14893	C	E	N
<i>Aratinga leucophthalmus</i>	2090*	1.2	10653	C	E	N
<i>Aratinga solstitialis</i>	2042	1.1	6490	C	E	N
<i>Bolborhynchus lineola</i>	4393	1.9	13080	C	E	F
<i>Brotozeris chiriri</i>	5486*	1.3	74601	C	E	N
<i>Cacatua goffini</i>	4582	—	—	—	—	—
<i>Cyanoliseus patagonus</i>	4967	1.0	2272	C	E	F
<i>Cyanopsitta spixii</i>	409	0.8	76154	C	E	N
<i>Derophtus accipitrinus</i>	395	1.4	44059	R	E	N
<i>Diopsittaca nobilis</i>	973	1.2	15897	C	E	N
<i>Enicognathus leptorhynchus</i>	5173	1.2	21754	C	H	F
<i>Forpus crassirostris</i>	501	2.1	39569	R	E	F
<i>Graydidascalus brachyurus</i>	1624*	2.9	22573	R	E	F
<i>Guarouba guarouba</i>	69	1.4	43982	C	E	N
<i>Melopsittacus undulatus</i>	4999	—	—	—	—	—
<i>Myiopsitta monachus</i>	2821	1.1	2277	C	H	F
<i>Nandayus nenday</i>	143	1.2	13083	C	E	N
<i>Nanopsittaca dachilleae</i>	5495*	2.3	—	R ^e	E ^e	F ^e
<i>Orthopsittaca manilata</i>	1852	1.1	38318	C	E	N
<i>Pionites leucogaster</i>	2377	2.1	12226	C	E	N
<i>Pionopsitta pileata</i>	3467	2.1	69441	R	E	N
<i>Pionopsitta barrabandi</i>	389693*	2.4	3501	R	E	N
<i>Pionus maximiliani</i>	1219	2.2	14015	R	E	N
<i>Primolius auricollis</i>	1742	1.1	44077	C	E	N
<i>Pyrrhura leucotis</i>	3921	1.0	34495	C	E	N
<i>Rynchopsitta pachyrhyncha</i>	5237	1.6	—	C ^e	H ^e	N ^e
<i>Triclaria malachitacea</i>	415	1.4	28102	R	E	N

^aCatalogue number at the genetic collection, LGEMA, Universidade de São Paulo, except 389693 from Field Museum of Natural History, 5495 from Museu Paraense Emílio Goeldi (voucher MPEG52710), 5486 at Museu de Zoologia da Universidade de São Paulo; ^bratio obtained from males, measures from Forshaw (1989); ^cvoucher number at the skin collection of the Museu de Zoologia da Universidade de São Paulo; ^dR, rounded; C, cunneated; ^edata from the literature (Salvadori, 1891; Forshaw, 1989; Collar, 1997); ^fE, exposed; H, hidden; ^gN, naked and evident; F, feathered; *wild-caught birds, otherwise captive.

GCT TGG TGT GCC ATT) and ATP6L (AAA YAT YTA ATG GCA CAC CAA GC). To amplify the rDNAs 12S and 16S the primers used were L1537 (AAT CTT GTG CCA GCC ACC GCG G) and 12Send (GTG CAC CTT CCG GTA CAC TTA CC), and 16Sa (AAG CCW ANC GAG CYG GGT GAT AGC TGG) and 16Se (GCA CGG TTA GGA TAC CGC GGC CG). Sequencing primers for the 16S segment were 16Sb (CAT AGA TAG AAA CCG ACC TGG) and 16Sc (TTC TTC AAG GTC GCC CCA ACC). The DNA sequences are deposited in GenBank under accession numbers: DQ143208 to DQ143324 and DQ150989 to DQ150996. NADH2 sequences were obtained from GenBank for *Amazona farinosa* (AY194461, Eberhard and Bermingham, 2004), *Pionopsitta barrabandi*, *Pionopsitta pileata*, and *Triclaria malachitacea* (AY669468, AY669481, and AY669486; Ribas et al., 2005), as well as cyt b for *Diopsittaca nobilis* (AF370769; Tavares et al., 2004).

Sequence Analysis

Sequences generated by the automated sequencers were checked for ambiguities using Sequencher 4.1.2 (GeneCodes Corp., Ann Arbor, Michigan). Alignments

of coding genes were verified visually in MacClade 4.0 (Maddison and Maddison, 2000). Indels, ambiguously aligned sites, and overlapping bases between ATPase 8 and ATPase 6 and ATPase 6 and COIII were removed from phylogenetic analyses. Base composition, transition and transversion rates, and genetic distances among nucleotide sequences were calculated in PAUP* 4.0b10 (Swofford, 2002). The degree of sequence saturation was evaluated for each gene by plotting the number of transitions and transversions against the corrected pairwise distances. The variable sites in each gene alignment were tested for stationarity in base composition using TREEPUZ-ZLE 5.0 (Schmidt et al., 2002) because nonstationarity in base composition can bias methods of tree inference and result in erroneous tree topologies (Penny et al., 1990; Lockhart et al., 1994; Foster and Hickey, 1999; Chang and Campbell, 2000; Haddrath and Baker, 2001; Paton et al., 2002). The invariable sites were excluded since they can hinder detection of compositional bias among taxa (Foster and Hickey, 1999). Symmetrical directional mutation pressure acting on all coding gene sequences was estimated with DMP 2.0 (Jermini et al., 1996) and was

compared with the G+C content in the third codon positions where bias is expected to be greatest.

Phylogenetic Analysis

The best-fit models of nucleotide evolution for each partition and for the combined data set were selected with a hierarchical likelihood-ratio test in ModelTest 3.7 (Posada and Crandall, 1998). Initial Bayesian analyses with Markov chain Monte Carlo (MCMC) sampling was performed with MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) for each gene separately, for the mitochondrial and nuclear partitions independently, and for the concatenated data set to evaluate whether mitochondrial and nuclear partitions were congruent and whether partitioning of genes with different models improved the fit to the data. The final Bayesian analysis was conducted for the combined data set using a partitioned likelihood approach (one partition for each gene), in which parameters were estimated separately for each data partition. Four different runs were executed, each for two million generations, with one cold and four heated chains, sampling once every 1000 trees and with a burn-in time determined by the time to convergence of the likelihood scores. The posterior probabilities of each node were computed for all Bayesian analyses across the sampled trees after burn-in.

The parameters of the models of DNA substitution were estimated in ModelTest 3.7 (Posada and Crandall, 1998) and used as initial estimates for a maximum likelihood (ML) search in PAUP* 4.0b10 (Swofford, 2002), performed with a heuristic search, stepwise addition (five random taxa additions), and tree bisection reconnection (TBR) algorithm. Support at the nodes was estimated by the nonparametric bootstrap (Effron, 1979; Felsenstein, 1985) with 1000 replicates and random addition of taxa in PHYML (Guindon and Gascuel, 2003), using the same parameter values as in the ML search. We compared the resulting tree with that obtained with an ML analysis under a nonhomogeneous model of substitution using the program NHML 3.0 (Galtier and Gouy, 1998), with a correction for rate variation among sites (HKY+ Γ). The high number of terminal taxa ($n = 32$) precluded a tree search in NHML; thus, a topological constraint was used on the nodes supported by at least 90% bootstrap support in the homogeneous ML analysis.

Comparison of the Tree Topologies

An approximately unbiased (AU) test (Shimodaira, 2002) implemented in CONSEL 0.1f (Shimodaira and Hasegawa, 2001) was used to compare distinct tree topologies. The AU test uses a multiscale bootstrap technique, where several sets of bootstrap replicates are generated by changing the sequence length, which may differ from that of the original data. The number of times the hypothesis is supported by the replicates is counted for each set to obtain bootstrap probabilities (BP) values along the matrices of different sequence lengths (Shimodaira, 2002).

Mapping and Comparing Morphological, Behavioral, and Sex-Linked Minisatellite Characters

Eight characters that were used to group or diagnose Neotropical parrot genera in previous classifications (Salvadori, 1891; Brereton and Immelmann, 1962; Forshaw, 1989), that were considered in systematic studies (Smith, 1975), or that were suggested to be exclusive to a group (Miyaki et al., 1997) were mapped onto the Bayesian consensus topology using Mesquite 1.05 (Maddison and Maddison, 2004), under likelihood method and Markov k-state one-parameter model to evaluate whether they were phylogenetically informative (assuming that the DNA sequence tree is correct). The characters selected and their states were (1) the length of the tail (Salvadori, 1891); as the definition of the states of this character is not clear, we assumed two states: long (ratio of wing length: tail length lower than 1.7) or short (ratio of wing length: tail length higher than 1.8). The definition of these cutoffs was based on the fact that in the distribution of ratios, no values between 1.6 and 1.9 were observed (Table 1); (2) the shape of the tail: cuneated (feathers are gradually longer from the edges towards the center) or rounded (all the feathers have approximately the same size) (Salvadori, 1891); (3) morphological sexual dimorphism: present or absent (Smith, 1975); (4) bicolored 'pericyclic' iris: present or absent (Smith, 1975); (5) scratching the head with a foot: by pulling the leg 'directly' forward under the wing, or lifting the foot 'indirectly' over the wing (Brereton and Immelmann, 1962); (6) nostrils: exposed or hidden (Salvadori, 1891); (7) periophthalmic ring: naked and evident or feathered (Forshaw, 1989); (8) minisatellites linked to the W chromosome: present or absent (Miyaki et al., 1997). The latter character was used in the comparison but its ancestral states were not reconstructed because not all the taxa sampled here were included in Miyaki et al. (1997); therefore, the presence or absence of the W-linked minisatellite in these taxa is unknown. The lengths of the tail and the wing followed Forshaw (1989; obtained from museum skins). The states of shape of the tail, nostrils, and periophthalmic ring were also determined from an examination of museum skins (Table 1).

Divergence Times

The assumption of rate constancy of DNA substitution through time within the Neotropical parrots was tested using a likelihood ratio test comparing the ML topology obtained with and without a clock constraint in PAUP* 4.0b10 (Swofford, 2002). The difference in likelihood values was compared with a chi-square distribution, with the number of taxa minus 2 as the number of degrees of freedom. Estimates of divergence times were obtained using two methods that do not assume constant rates of evolution along the lineages: a penalized likelihood (PL) method and Bayesian inference (Thorne et al., 1998; Kishino et al., 2001).

PL was performed in the program r8s 1.5 (Sanderson, 2003), which assumes a parametric model with a

different substitution rate along each branch and a non-parametric roughness penalty that costs the model more if rates change too quickly between branches (Sanderson, 2002). Prior to the estimation of divergence times, a cross-validation analysis (Sanderson, 2002) was performed in r8s to determine the best smoothing parameter for the data. The branch-lengths used were from the partitioned likelihood Bayesian tree topology including the same taxa used in phylogeny reconstruction, but only with the mitochondrial genes as RAG-1 sequences were not available for *Strigops*.

The Bayesian inference for divergence time estimation was performed in MULTIDIVTIME (MULTIDISTRIBUTE package; <http://statgen.ncsu.edu/thorne/multidivtime.html>), which uses a probabilistic model to describe the change in evolutionary rate over time. It also uses the Markov chain Monte Carlo (MCMC) procedure to derive the posterior distribution of rates (Thorne et al., 1998; Kishino et al., 2001) and times, and in a multigene data set allows each gene to be analyzed with an independent model (Thorne and Kishino, 2002). Initially, the baseml program in PAML 3.14 (Yang, 1997) was used to obtain estimations of transition/transversion rates, values of substitution parameters under the model HKY85 plus Γ , and a value of the α parameter for Γ distribution for each gene. These data were converted in paml2modelinf of the MULTIDISTRIBUTE package to a file recognized by estbranches, which estimates branch lengths and their variance-covariance matrix. MCMC analyses are conducted in MULTIDIVTIME to approximate the posterior distribution of substitution rates and divergence times. The gamma prior distribution for the expected time between the tip and the ingroup (rtrate) and its standard deviation (SD) were set to 90 and 20 Myr, respectively; rate of root node (rtrate) and its SD were both set 0.094 substitutions/site/unit time as estimated from the median of all tip-to-root of the ingroup branch lengths divided by rtime; rate of change between the ancestral and descendent node (brownmean) and its SD were set such that rtrate times brownmean = 1. A large SD was chosen because it allows a gene to have a large variation in rate change over time and because a priori information for rate change is unknown (Thorne and Kishino, 2002). Multiple runs with different initial states were performed to check for the convergence of the MCMC algorithm by comparing each run's posterior distribution of divergence times, branch lengths, and the proportion of successful changes of those parameters along the Markov chain. The data set used in these estimates was the same used to infer phylogenetic relationships among taxa. As this method allows incomplete taxon sampling across genes, RAG-1 was included in the analysis even though we did not have a sequence for *Strigops*.

Several independent studies of molecular dating of divergence times in birds support the origin of the Orders of the Neoaves around 90 Mya, in the early-mid Cretaceous (Cooper and Penny, 1997; van Tuinen and Hedges, 2001; Paton et al., 2002). This evidence is in agreement with a hypothesis of a vicariant origin for

the parrots and other bird lineages mediated by the breakup of Gondwanaland (Cracraft, 1973, 2001), and it is also corroborated by high-level phylogenetic inferences among parrot lineages (Barrowclough et al., 2004; de Kloet and de Kloet, 2005). These analyses suggest that the large New Zealand parrots are sister to all the other parrots (Barrowclough et al., 2004; de Kloet and de Kloet, 2005), and the next most basal monophyletic clade is the traditional Cacatuidae, including *Nymphicus* (Barrowclough et al., 2004). To test if the time of isolation of New Zealand (82 to 85 Mya; Cooper and Millener, 1993) is a reasonable constraint to estimate divergence times in parrots, a data set including the same genes and taxa used in phylogenetic estimates and additional taxa of other bird orders were run in MULTIDIVTIME to independently estimate the age of the node that separates *Strigops* from the other parrots. The taxa added in this analysis were *Rhea americana* (Genbank accession nos. NC_000846; Harlid et al., 1998), *Anas platyrhynchos* (Genbank accession nos L16770, Liu et al., 1996; L22476, Ramirez et al., 1993; AF059082 and AF059142, Johnson and Sorenson, 1998), *Aythya americana* (Genbank accession no. NC_000877; Mindell et al., 1999), and *Anseranas semipalmata* (Genbank accession no NC_005933; Harrison et al., 2004). The topology was constrained to match well-supported phylogenetic relationships in other studies (Groth and Barrowclough, 1999; Livezey and Zusi, 2001; Paton et al., 2002; García-Moreno et al., 2003). Prior information assumed was (1) minimum age of 65 Myr for the common ancestor of screamers and true ducks (Clarke et al., 2005; Pereira and Baker, 2006); (2) the separation of Galloanseres and Neoaves at a lower limit of 90 Mya (Haddrath and Baker, 2001) and an upper limit of 130 Mya (van Tuinen and Hedges, 2001); and (3) a lower limit of 65 Mya for the split of the Psittaciformes and other Neoaves (Cooper and Penny, 1997). The estimated divergence time for the node that separates *Strigops* from the other parrots was 116 (95% CrI 98,127 Mya), which is older than the separation of New Zealand from Antarctica. We therefore calibrated the estimates of divergence times among the genera within the tribe Arini by fixing the age of the node that separates the New Zealand taxon *Strigops* from the other parrots at 85 to 82 Mya, because this split could have predated the vicariant event. As the fossil record for parrots and allies is scanty, and the phylogenetic placement of fossils has not been determined in a cladistic framework, we decided not to use them as minimum age constraints in our analyses.

RESULTS

Sequence Statistics

The PCR amplifications resulted in single products of about 800 to 1300 bp, which are larger than the size of most putative mitochondrial genes translocated to the nuclear genome of chicken (Pereira and Baker, 2004). Additionally, the translation of the protein-coding genes sequenced did not indicate unexpected stop codons or frameshift mutations, and codon positions showed the

expected levels of variation. Given the paucity of nuclear pseudogenes of mitochondrial origin in birds, it is extremely unlikely that any were amplified in our study.

The sequences obtained for each sample totaled 6388 base pairs (bp) long, composed of 2703 bp of nuclear DNA and 3685 bp of mitochondrial DNA (mtDNA). Sequence alignment and phylogenetic trees obtained were deposited in TreeBase (Author: Tavares, PIN number: 1772). Substitutions accumulated linearly against corrected distances for RAG-1, 12S, and 16S rDNAs. Transitions at third codon positions were saturated for the remaining genes in comparisons between Neotropical parrots and outgroups, as were transitions at third codon position of ATPase6, ATPase8, and COIII among Neotropical parrots. The average uncorrected pairwise distances calculated separately for each gene show that RAG-1 was the slowest evolving gene (p-distances among Neotropical parrots in the range 0.04% to 4%), as expected for an exon of a nuclear coding gene. Among the mitochondrial genes, the most slowly evolving genes were 12S rDNA (467 bp) and 16S rDNA (483 bp), with p-distances in the range 1.1% to 9.2%, followed by the protein-coding genes COIII (191 bp) and cyt b (888 bp) with p-distances 3.1% to 16.3%, and NADH2 (894 bp)

with p-distances 5% to 20%. The most rapidly evolving genes were ATPase 6 (673 bp) and ATPase 8 (89 bp), with p-distances ranging from 4.3% to 26.1%. The transition/transversion ratio for the Neotropical parrots was 3.15, 4.33, and 4.26 for RAG-1, the combined mitochondrial genes, and the concatenated sequences from both genomes, respectively.

The percent base composition for the Neotropical parrot sequences was A = 31.6–31.9, C = 19.7–20.2, T = 24.4–24.9, G = 23.5–23.8 for RAG-1, and A = 29.8–32.5, C = 31.2–34.6, T = 21.2–24.5, G = 12.3–14.2 for the mitochondrial genes combined. These values are in agreement with the expected ranges for nuclear and mitochondrial genes observed in other birds (e.g., Pereira et al., 2002). Significant variation in nucleotide composition ($P < 0.05$) was detected in third codon positions of mitochondrial coding genes of 14 taxa (Table 2), whereas RAG-1 sequences did not have a bias in base composition among taxa. Significant symmetrical directional mutation pressure ($P < 0.05$) towards G+C content was detected for six taxa and towards A+T content for one taxon. Variation in the G+C content was expressed mostly at synonymous sites (Table 2). The concatenated nuclear and mtDNA sequences also were not stationary, but heterogeneity in base composition was reduced from

TABLE 2. Symmetrical directional mutation pressure (μ_D) for the mitochondrial protein coding genes. $\mu_D < 0.5$ and $\mu_D > 0.5$ represent greater A+T and greater G+C contents, respectively. The values represent the GC% observed in the total sequence (Pobs) at synonymous (Psyn) sites, nonsynonymous (Pnon) sites, and third codon positions (P₃). Significant variation in base composition is indicated by * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Taxon	μ_D	Pobs	Pnon	Psyn	P ₃
<i>Ara</i> ^a	0.519	0.460	0.412	0.534	0.474
<i>Primolius</i>	0.523	0.460	0.410	0.537	0.479
<i>Orthopsittaca</i>	0.540	0.472	0.418	0.554	0.489
<i>Cyanopsitta</i>	0.539	0.466	0.409	0.553	0.491
<i>Nandayus</i>	0.508	0.452	0.406	0.524	0.463
<i>Aratinga solstitialis</i> ^b	0.502	0.447	0.402	0.517	0.459
<i>Aratinga leucophthalmus</i>	0.533	0.467	0.414	0.548	0.493
<i>Aratinga aurea</i>	0.525	0.460	0.408	0.540	0.475
<i>Guarouba</i>	0.519	0.454	0.400	0.535	0.467
<i>Diopsittaca</i>	0.522	0.458	0.406	0.537	0.470
<i>Cyanoliseus</i> ^b	0.502	0.448	0.403	0.517	0.462
<i>Anodorhynchus</i> ^c	0.529	0.462	0.408	0.543	0.479
<i>Enicognathus</i>	0.541	0.468	0.410	0.559	0.489
<i>Rhynchopsitta</i> ^{a,b}	0.485	0.444	0.406	0.500	0.435
<i>Pyrrhura</i> ^a	0.572**	0.481	0.413	0.585	0.523
<i>Derophtus</i>	0.556**	0.473	0.410	0.570	0.501
<i>Pionites</i>	0.563***	0.475	0.409	0.577	0.513
<i>Forpus</i> ^d	0.514	0.455	0.406	0.529	0.458
<i>Graydidascalus</i> ^d	0.540	0.465	0.407	0.555	0.487
<i>Amazona xanthops</i>	0.535	0.467	0.412	0.549	0.480
<i>Amazona</i> ^b	0.563***	0.474	0.406	0.576	0.512
<i>Pionus</i>	0.551**	0.473	0.413	0.565	0.491
<i>Pionopsitta barrabandi</i>	0.519	0.457	0.407	0.534	0.478
<i>Triclaria</i> ^b	0.520	0.460	0.412	0.535	0.466
<i>Pionopsitta pileata</i>	0.528	0.463	0.411	0.543	0.479
<i>Myiopsitta</i> ^a	0.470**	0.434	0.402	0.483	0.417
<i>Brotogeris</i> ^b	0.565***	0.475	0.408	0.578	0.513
<i>Bolborhynchus</i>	0.541	0.469	0.412	0.555	0.486
<i>Nannopsittaca</i>	0.537	0.466	0.411	0.551	0.477
<i>Cacatua</i> ^e	0.540	0.470	0.414	0.555	0.486
<i>Melopsittacus</i> ^d	0.511	0.455	0.409	0.526	0.457
<i>Falco</i>	0.500	0.454	0.415	0.515	0.455

^aATPase 6; ^bcyt b; ^cATPase 8; ^dNADH2; ^eCOIII.

14 to 10 taxa in which the mtDNA bias was evident. The four taxa that have base compositional heterogeneity in mitochondrial sequences, but reached stationarity in the combined data set, were *Anodorhynchus*, *Ara*, *Melopsittacus*, and *Triclaria*.

Phylogenetic Analyses

Bayesian analysis using only the RAG-1 gene or only the combined mitochondrial genes resulted in similar tree topologies (Fig. 1). The RAG-1 tree has higher posterior probabilities at most of the deeper nodes, but lacks resolution in shallower nodes that were better resolved with the mitochondrial data set. The taxa with significantly biased mtDNA base compositions (Table 2) are in the same positions in both trees, suggesting that the bias in base composition is not negatively affecting tree inference (Fig. 1). This conclusion is also reinforced by the fact that the ML analyses with a non-homogeneous evolution model recovered basically the same topology (not shown), differing only in the position of nodes that form a polytomy in the mitochondrial Bayesian consensus tree. Therefore, we opted to use concatenated sequences from both genomes and discuss our results based on this combined data set. Evolutionary models used were NST = 2 plus Γ for Rag1 and NST = 6 plus Γ and proportion of invariable sites (I) for each one of the mitochondrial genes.

The partitioned Bayesian analysis of the combined nuclear and mtDNA data sets resulted in a consensus topology very similar to the one obtained with the mitochondrial genes. In all trees three major clades were recovered (Fig. 2): clade A, parrotlets (*Bolborhynchus* and *Nannopsittaca*); clade B, amazons and allies (*Amazona*, *Pionus*, *Graydidascalus*, *Pionopsitta*, *Triclaria*, *Myiopsitta*, and *Brotogeris*); and clade C, macaws, conures, and allies (*Ara*, *Primolius*, *Orthopsittaca*, *Cyanopsitta*, *Diopsittaca*, *Guarouba*, *Nandayus*, *Aratinga*, *Anodorhynchus*, *Cyanoliseus*, *Enicognathus*, *Rhynchopsitta*, *Pyrhura*, *Derophtus*, *Pionites*, and *Forpus*). The node between clade B and clade C was not highly supported (52% and 0.87, ML bootstrap and posterior probabilities of Bayesian analysis, respectively). However, when only *Falco* is used as an outgroup the support of this node is increased to a posterior probability of 0.96.

A nonpartitioned ML search using starting parameters from ModelTest 3.6 recovered a similar topology to the partitioned Bayesian tree (Fig. 2), except that *Pyrhura* was a sister genus to all other genera in clade C (excluding a clade containing *Derophtus* and *Pionites*). Both bootstrap and Bayesian posterior probabilities provided good support for most of the clades. However, some nodes with low bootstrap support values in ML analysis have high posterior probabilities. For example, the sister-group relationship between *Forpus* and the rest of the macaws and conures clade had 78% bootstrap support and a posterior probability of 1.0. The homogeneous ML and nonhomogeneous ML searches recovered the same topology, differing only in branches with low posterior probabilities or bootstrap support. These relatively

minor topological differences between the trees from the three methods were not significant at the 5% level in the AU test.

Mapping Morphological, Behavioral, and Sex-Linked Minisatellite Characters on the Molecular Tree

Morphological and behavioral characters used in previous classifications were inferred to be homoplasious when mapped onto our phylogenetic hypothesis (Table 1, Fig. 3). Additionally, the minisatellites linked to chromosome W are present in taxa from macaws, conures, and allies, excluding the basal genera *Pionites*, *Derophtus*, and *Pyrhura*, and are absent in other genera surveyed within amazons and allies (Fig. 3h). However, the state is not known in the parrotlet clade, in two taxa from amazons and allies, and in four taxa from macaws, conures, and allies. Thus, a wider survey of taxa to investigate the presence of such minisatellites is needed to better evaluate phylogenetic utility of these markers.

Divergence Times

Rate constancy among the Neotropical parrot sequences was rejected by the likelihood ratio test between trees with and without an enforced molecular clock, including the outgroups *Falco* and *Gallus* (log-likelihood of clock tree = -39,016.92; log-likelihood of nonclock tree = -38,962.05; $2\Delta = 109.73$, $df = 33$; $P < 0.001$), and excluding them (log-likelihood of clock tree = -33,714.15; log-likelihood of non-clock tree = -33,664.22; $2\Delta = 99.88$; $df = 30$; $P < 0.001$). Cross-validation indicated a nonparametric rate-smoothing value of 3.16 to estimate divergence times under the PL method. Divergence times estimated with the PL and Bayesian approach were similar, and thus we discuss the events based on the results of the Bayesian approach as it accounts for uncertainty of branch lengths and time estimates (Table 3, Fig. 4).

Based on 95% credibility intervals (95% CrI), the divergence between the Neotropical and Australian parrot lineages was estimated to have occurred around 66 to 51 Mya, in the Early Eocene. The three extant major lineages of the Arini started their diversification with the separation of the lineage leading to the small parrotlets (clade A) around 57 to 42 Mya (Fig. 4, Table 3), followed by the separation of amazons and allies (clade B) and the macaws, conures, and allies (clade C) at 56 to 41 Mya. The pattern of cladogenesis was distinctively different in clades B and C. Genera of amazons and allies (clade B) radiated in a continuous fashion from as early as 54 Mya to as late as 12 Mya. In contrast, in clade C the separation of the *Forpus* lineage occurred around 54 to 39 Mya, but further cladogenesis of genera followed later from 35 to 4 Mya, after a 20-Myr gap.

DISCUSSION

Phylogenetic Relationships and Implications for Parrot Systematics

In the present study, we sampled most genera of Neotropical parrots and gathered more mitochondrial and nuclear sequence data than previous studies of

FIGURE 1. Bayesian analysis of 29 Neotropical parrots taxa based on: (a) 2703 base pairs of nuclear RAG-1 sequences; and (b) 3685 base pairs of mitochondrial sequence. Taxa with significant bias in base composition in the mitochondrial genes are underlined. Numbers at the nodes are Bayesian posterior probabilities. Scale bar corresponds to number of expected substitutions per site.

FIGURE 2. Bayesian analysis of 29 Neotropical parrots taxa (representing 25 of the 30 recognized genera) based on combined analyses of 6388 base pairs of nuclear and mitochondrial sequences. Numbers on the nodes are ML bootstrap percentages/Bayesian posterior probabilities, which are represented as asterisks when 100/100. Three major clades are indicated: A, parrotlets; B, amazons and allies; and C, macaws, conures, and allies. The scale bar depicts expected number of substitutions per site.

phylogenetic relationships of Psittaciformes at the genus level (Miyaki et al., 1998; Tavares et al., 2004; de Kloet and de Kloet, 2005). Branch support and resolution were improved by this study as a result of a more complete taxon sampling and the combined analysis of mitochondrial and nuclear sequences (Poe and Swofford, 1999; Haddrath and Baker, 2001; Slowinski, 2001; Paton et al., 2002). Also, it has been shown previously that the combined analysis of the mitochondrial and nuclear sequences provides resolution in different parts of the tree, complementing each other and resulting in a more robust topology (e.g., Pereira et al., 2002).

We detected three major clades within Neotropical parrots: parrotlets of the genera *Bolborhynchus* and *Nannopsittacus* (clade A), amazons and allies of the genera *Amazona*, *Pionus*, *Graydidascalus*, *Pionopsittacus*, *Triclaria*, *Myiopsittacus*, and *Brotogeris* (clade B), and macaws, conures, and relatives in the genera *Ara*, *Primolius*, *Orthopsittacus*, *Cyanopsittacus*, *Nandayus*, *Aratinga*, *Guarouba*, *Diopsittacus*, *Anodorhynchus*, *Cyanoliseus*, *Rhynchopsittacus*, *Enicognathus*, *Pyrrhura*, *Pionites*, *Derophtus*, and *Forpus* (clade C). The

inclusion of *Forpus* in clade C is well supported in both Bayesian and ML trees (1.00 and 78%, respectively), but it is on a long branch and its placement may be phylogenetically problematical. Although clades B and C have been recovered in other studies using less dense taxon sampling (Miyaki et al., 1998; Tavares et al., 2004; de Kloet and de Kloet, 2005), a novel proposal in our results is the suggestion that the parrotlets (clade A) are a sister group of all the other taxa. The nonmonophyly of *Aratinga*, *Amazona*, and *Pionopsittacus* was also confirmed as previously suggested (Ribas and Miyaki, 2004; Russello and Amato, 2004; Ribas et al., 2005).

Morphological, Behavioral, and Sex-Linked Minisatellite Characters Mapped on the Molecular Tree

None of the morphological or behavioral characters analyzed were congruent with our molecular phylogenetic hypothesis. The size and shape of the tail were the main characters adopted in a previous classification to

define two groups that includes the Neotropical parrots (Salvadori, 1891). Furthermore, the importance of the size and shape of the tail was raised again in a more recent molecular study, where character state variation agreed with the inferred phylogenetic relationships among nine taxa sampled (Miyaki et al., 1998). However, meager taxon sampling may have led to the erroneous conclusion that size or shape of the tail could be phylogenetically informative. The use of these characters as diagnostic descriptors in systematic studies of Neotropical parrots (e.g., Ribas and Miyaki, 2004; Russello and Amato, 2004; Ribas et al., 2005; de Kloet and de Kloet, 2005) should be discontinued.

Divergence Times and Reconstruction of Historical Biogeography

Previous estimates of divergence times among parrots have been proposed based on methods that do not account for rate variation in DNA substitution among lineages. For example, Miyaki et al. (1998) and Tavares et al. (2004) used the linearized tree method (Takezaki et al., 1995), which detects taxa that are evolving at rates that

are significantly different from the average and excludes them from the analysis until a set of taxa evolving at a similar rate remains. They then assumed that Galliformes and Anseriformes separated from other Neognath birds around 100 Mya to calibrate their clock. Tavares et al. (2004) also applied the 'standard' mitochondrial rate of DNA substitution of 2.0%/Myr to a set of clock-like sequences. This procedure has also been applied to closely related species of parrots, based on rates of 1.6–2.0%/Myr (Ribas and Miyaki, 2004; Eberhard and Bermingham, 2004, 2005; Ribas et al., 2005). In general, these studies resulted in dates that are younger than the estimates obtained in this study. We believe that our approach to estimate divergence times is more appropriate because we relaxed the assumption of a strict molecular clock by using methods that accounted for rate variation among lineages within Neotropical parrots, accounted for uncertainty in branch lengths and time estimates by a Bayesian approach, and used a hypothesis for the phylogenetic relationships among genera that was better supported at the genus level than previous hypotheses (Miyaki et al., 1998; Tavares et al., 2004). Moreover, the use of an independent geological calibration point

FIGURE 3. Mapping of characters on the molecular phylogeny. (a) Length of the tail; (b) shape of the tail; (c) morphological sexual dimorphism; (d) bicolored pericyclic iris; (e) scratching the head with a foot; (f) nostrils; (g) periphthalmic ring; and (h) minisatellites linked to the W chromosome. The circles at terminal nodes represent the observed character state for the corresponding species to the right. Pie charts at internal nodes represent proportions of the marginal probabilities for the reconstructed character states at that node. Numbers above the charts (a–g) are corresponding proportions of the states represented in white along the characters, when these values are not 0 or 1. The circles on the terminals are states in extant taxa. (Continued)

FIGURE 3. (Continued)

FIGURE 3. (Continued)

(i.e., the separation of New Zealand from the rest of the Gondwanaland) allowed rates to be estimated from the data set, instead of assuming rates estimated for other avian groups (Lovette, 2004).

Based on 95% credibility intervals, the estimated time of divergence of the ancestral Neotropical lineage from the Australian parrots between 66 and 51 Mya implies that at this time the Psittaciformes were widely distributed across the southern continents including Antarctica and South America. This transantarctic pattern of relationship between the fauna of South America and that of Australia and New Zealand has been suggested before for many avian groups, including Psittaciformes (Cracraft, 2001). The separation of Australia from the Antarctica/South America landmass around 55 to 45 Mya, as well as a rise in sea level due to global increase in temperatures (Woodbourne and Case, 1996), is a candidate vicariant event for the split of Neotropical and Australian parrots. Between 60 and 50 Mya, Antarctica was covered with lush, green cool-temperate forests similar to those found today in New Zealand and Tasmania (Poole et al., 2001, 2005) and could have supported a limited diversity of parrots.

The diversification of Neotropical parrots into three major lineages occurred between 57 and 41 Mya, which corresponds to the Late Paleocene and most of the

Eocene. At this time, Antarctica was ice-free, wetter, and warmer than today and still connected to South America (Lawver and Gahagan, 2003). Sea level was on average 100 to 200 m above current level (Haq et al., 1987), fragmenting the continent into East Antarctica and West Antarctica by a seaway flowing from the Ross Sea towards the Weddell Sea (Lawver and Gahagan, 2003). Similarly, the southern cone of South America was isolated from the rest of South America by a transcontinental seaway throughout the Eocene and Early Oligocene until at least 30 Mya (Smith et al., 1994). We hypothesize that these isolated areas could have harbored the common ancestors of the three main lineages of extant Neotropical parrots (clades A, B, and C in Fig. 4).

Although it is not clear from our data or from the fossil record which clades were present in those areas, the two contrasting temporal patterns of genus diversification found among clades of Neotropical parrots may give us some clues (Fig. 4). The pattern observed within clade B (amazons and allies) is one of continuous increase in genus diversity throughout the Eocene, Oligocene, and Miocene after the clade originated in the Eocene between 54 and 39 Mya. Therefore, we hypothesize that the ancestor of this clade may already have been present in the southern region of South America or was able to cross the Patagonian transcontinental sea. Because the

TABLE 3. Estimated divergence times of parrot lineages (million of years ago, Mya) using the PL method fixing the node that splits *Strigops* from the other parrots at 85 and 82 Mya and associated 95% confidence intervals (95% CI); and with a Bayesian method and the 95% credibility intervals (95% CrI). Nodes are numbered as in Figure 4.

Node	PL method				Bayesian Method	
	Date	95% CI	Date	95% CI	Date	95% CrI
1	85	Fix	82	Fix	82.9	(82.0; 84.6)
2	72.3	(69.3; 75.0)	69.8	(66.9; 72.3)	67.6	(60.4; 74.8)
3	64.3	(60.8; 68.5)	62.1	(59.0; 65.1)	59.0	(51.5; 66.8)
4	54.5	(51.0; 57.8)	52.6	(49.3; 59.0)	49.7	(42.5; 57.3)
5	21.0	(18.1; 24.0)	20.3	(17.7; 23.2)	18.4	(13.4; 24.2)
6	52.7	(49.3; 56.0)	50.9	(47.7; 54.1)	48.3	(41.2; 55.9)
7	51.2	(47.7; 54.6)	49.4	(16.2; 52.7)	46.1	(39.0; 53.8)
8	37.9	(34.5; 41.4)	36.7	(33.3; 40.0)	32.5	(25.5; 40.2)
9	41.5	(36.8; 45.1)	40.1	(36.7; 43.6)	39.2	(32.2; 46.6)
10	38.7	(35.2; 42.3)	37.4	(34.0; 40.9)	36.2	(29.7; 43.4)
11	37.0	(33.4; 40.6)	35.7	(32.4; 39.2)	33.2	(26.8; 40.2)
12	29.9	(26.3; 33.6)	28.9	(25.4; 32.4)	24.9	(19.4; 31.1)
13	25.8	(22.4; 29.4)	24.9	(21.6; 28.4)	22.4	(17.0; 28.7)
14	20.4	(17.4; 24.2)	19.7	(16.8; 22.9)	16.6	(11.7; 22.1)
15	50.0	(46.6; 53.4)	48.3	(45.1; 51.6)	46.2	(39.0; 53.8)
16	31.4	(28.6; 34.5)	30.4	(27.6; 33.3)	28.2	(22.5; 34.8)
17	23.9	(20.0; 27.0)	23.1	(20.1; 26.1)	22.3	(16.6; 28.7)
18	26.3	(23.5; 29.3)	25.4	(22.7; 28.3)	25.2	(20.0; 31.2)
19	26.0	(23.1; 29.1)	25.1	(22.4; 28.1)	23.4	(18.1; 29.6)
20	24.1	(21.7; 26.9)	23.3	(20.9; 26.7)	23.4	(19.0; 29.2)
21	22.8	(21.0; 25.6)	22.1	(19.8; 24.6)	22.1	(17.3; 27.5)
22	20.9	(18.7; 23.3)	20.2	(17.9; 22.6)	20.6	(16.1; 25.7)
23	9.7	(7.9; 11.8)	9.4	(7.7; 11.3)	8.9	(6.1; 12.3)
24	19.4	(17.3; 21.8)	18.7	(16.7; 21.1)	19.9	(15.5; 24.9)
25	16.7	(14.7; 19.0)	16.2	(14.3; 18.4)	18.3	(13.9; 23.2)
26	18.4	(16.3; 20.7)	17.8	(15.9; 20.1)	19.0	(14.8; 24.0)
27	5.1	(4.3; 6.0)	4.9	(4.2; 5.8)	5.5	(3.6; 7.7)
28	16.7	(14.9; 19.0)	16.3	(14.4; 18.4)	17.2	(13.0; 22.0)
29	14.4	(12.5; 18.6)	13.9	(12.1; 15.9)	15.0	(11.2; 19.5)
30	13.3	(11.4; 15.3)	12.8	(11.1; 14.8)	13.6	(9.9; 18.0)

pattern of cladogenesis corresponds to times of major paleoenvironmental changes in South America, we infer that this diversification could well have occurred exclusively in South America. Conversely, ancestors of parrotlets and of macaws, conures, and allies may have been isolated in Antarctica and/or the southern cone of South America, and only dispersed out of these southern regions when climate cooled and Antarctica became ice-encrusted about 35 Mya (Dingle and Lavelle, 1998; Shevenall et al., 2004). This 'ice house' climate caused a significant drop in sea level during the Early Oligocene (Haq et al., 1987) and caused considerable changes in oceanic and atmospheric circulation (Flynn and Wyss, 1998). A similar pattern of cladogenesis showing a gap between the origin of the group and the radiation of genera has been detected in other Neotropical birds, such as cracids (Pereira et al., 2002) and toucans (Nahum et al., 2003).

The upheaval of the Andes, especially in the last 30 to 8 Mya, changed river systems, atmospheric circulation and rainfall throughout the continent and altered the geographic distribution of the Neotropical biota to a large extent (Lundberg et al., 1998; Nore, 2004). The Andes blocked humid Pacific winds from reaching the interior of the continent (Hooghiemstra and van der Hammer, 1998), leading to the formation of open habitats and

grasslands, as inferred by the estimates for the time of diversification of plants of dry environments (Pennington et al., 2004) and by the observation that the mammalian fauna was dominated by hypsodont herbivores, which are usually inferred to be grazers (Flynn and Wyss, 1998). The origin of Neotropical parrots that are today found in open areas, grasslands and savannah, especially in clade C, coincides with the formation of these habitats in South America. The rapid radiation of genera in clade C (macaws, conures, and allies) points to an important role for ecological speciation (Schluter, 2000) via niche diversification as these open-dry areas were colonized from forests.

Although we did not include African parrots in our study, we do not expect their phylogenetic placement to change our biogeographic interpretation of the evolution of Neotropical parrots. Previous work has suggested that African parrots are not monophyletic and may have invaded Africa twice, via Southeastern Asia, and separately via South America (Barrowclough et al., 2004; de Kloet and de Kloet, 2005). The putative colonists from South America are the sister group to the monophyletic Neotropical parrots and therefore their divergence time cannot be younger than 57 to 41 Mya, which is the estimated age for the beginning of the diversification of Neotropical parrots based on our nuclear and

FIGURE 4. Chronogram showing divergence times among the parrot genera and paleoevents possibly related to the Neotropical diversification. Numbers on nodes correspond to the estimated divergence times of lineages as given in Table 3. Horizontal bars at nodes are 95% credibility intervals of divergence times. Clades are defined as A, parrotlets; B, amazons and allies; and C, macaws, conures, and allies. Eustatic curves of sea level are from Haq et al. (1987), and Antarctic ice sheet scale is from Zachos et al. (2001).

mitochondrial DNA sequences. This implies that the invasion of Africa from South America occurred at a time when Africa was already isolated from a supercontinent that would later be fragmented into Antarctica, Australia, and South America and reinforces our hypothesis that the ancestor of parrots and allies were widespread in Gondwanaland.

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Cyanopsitta spixii—monotypic species of its genus, extinct in the wild and last seen in Curaçá (Bahia, Brazil).

Anexo IV

Multiple independent origins of mitochondrial control region duplications in the order Psittaciformes

Erin E. Schirtzinger^{a,*}, Erika S. Tavares^{b,d}, Lauren A. Gonzales^a, Jessica R. Eberhard^c, Cristina Y. Miyaki^d, Juan J. Sanchez^e, Alexis Hernandez^e, Heinrich Müller^f, Gary R. Graves^{g,h}, Robert C. Fleischerⁱ, Timothy F. Wright^a

^a Department of Biology, New Mexico State University, Las Cruces, NM 88003, USA

^b Department of Natural History, Royal Ontario Museum, 100 Queen's Park, Toronto, ON, Canada M5S 2C6

^c Department of Biology and Museum of Natural Science, Louisiana State University, Baton Rouge, LA 70803, USA

^d Departamento de Genética e Biologia Evolutiva, Universidade de São Paulo, São Paulo, SP, Brazil

^e Instituto Nacional de Toxicología y Ciencias Forenses, 38320 Tenerife, Canary Islands, Spain

^f Department of Veterinary Medicine, Laro Parque Fundación, 3840 Puerto de la Cruz, Tenerife, Spain

^g Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, PO Box 37012, MRC-116, Washington, DC 20013-7012, USA

^h Center for Macroecology, Evolution and Climate, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark

ⁱ Center for Conservation and Evolutionary Genetics, Smithsonian Conservation Biology Institute, National Zoological Park, PO Box 37012, MRC 5503, Washington, DC 20013-7012, USA

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ABSTRACT

Mitochondrial genomes are generally thought to be under selection for compactness, due to their small size, consistent gene content, and a lack of introns or intergenic spacers. As more animal mitochondrial genomes are fully sequenced, rearrangements and partial duplications are being identified with increasing frequency, particularly in birds (Class Aves). In this study, we investigate the evolutionary history of mitochondrial control region states within the avian order Psittaciformes (parrots and cockatoos). To this aim, we reconstructed a comprehensive multi-locus phylogeny of parrots, used PCR of three diagnostic fragments to classify the mitochondrial control region state as single or duplicated, and mapped these states onto the phylogeny. We further sequenced 44 selected species to validate these inferences of control region state. Ancestral state reconstruction using a range of weighting schemes identified six independent origins of mitochondrial control region duplications within Psittaciformes. Analysis of sequence data showed that varying levels of mitochondrial gene and tRNA homology and degradation were present within a given clade exhibiting duplications. Levels of divergence between control regions within an individual varied from 0–10.9% with the differences occurring mainly between 51 and 225 nucleotides 3' of the goose hairpin in domain I. Further investigations into the fates of duplicated mitochondrial genes, the potential costs and benefits of having a second control region, and the complex relationship between evolutionary rates, selection, and time since duplication are needed to fully explain these patterns in the mitochondrial genome.

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1. Introduction

Conservation of genome size, consistent gene content, and a lack of introns or intergenic spacers in animal mitochondria are generally interpreted as evidence that mitochondrial genomes are under selection for small size (Brown et al., 1979; Quinn and Wilson, 1993; Rand, 1993). This selection regime suggests that gene duplications in the mitochondria should be very rare or quickly eliminated because smaller genomes can replicate more quickly (Attardi, 1985; Diaz et al., 2002; Gray, 1989; Rand, 2001;

Selosse et al., 2001; Sogin, 1997). As more mitochondrial genomes are sequenced, however, duplications of mitochondrial genes have been identified with increasing frequency in diverse species such as birds, lizards, ostracods, fish, arthropods, and snakes (Abbott et al., 2005; Arndt and Smith, 1998; Bensch and Härlid, 2000; Black and Roehrdanz, 1998; Campbell and Barker, 1999; Desjardins and Morais, 1990; Eberhard et al., 2001; Gibb et al., 2007; Kumazawa et al., 1996, 1998; Lee and Kocher, 1995; Lee et al., 2001; Macey et al., 1997; Mindell et al., 1998; Moritz and Brown, 1987; Ogoh and Ohmiya, 2004, 2007; Quinn and Mindell, 1996; Shao and Barker, 2003; Shao et al., 2005). It is now clear that duplications do occur in the mitochondrial genome and are much more common than previously thought. However, understanding the underlying mechanisms, evolutionary dynamics, and fitness consequences of

* Corresponding author. Address: Department of Biology, New Mexico State University, PO Box 30001, MSC 3AF, Las Cruces, NM, USA. Fax: +1 575 646 4791.

E-mail address: eesem@nmsu.edu (E.E. Schirtzinger).

Fig. 1. (a) The typical avian mitochondrial gene order identified by Desjardins and Morais (1990). A tandem duplication followed by random loss of the control region and tRNAs can explain the rearrangement from the typical vertebrate gene order. (b) An alternative avian mitochondrial gene order identified by Mindell et al. (1998). A single tandem duplication followed by incomplete loss of the duplicated region may explain this rearrangement from the typical avian mitochondrial gene order. (c) The mitochondrial gene order identified by Eberhard et al. (2001) in *Amazona* parrots, in which two complete and putatively functional mitochondrial control regions appear to be maintained. (d) The mitochondrial gene order discovered by Abbott et al. (2005) in *Thalassarche* albatrosses in which cytochrome *b* to the control region is tandemly duplicated. Only the second copy of cytochrome *b* shows any degradation as depicted by the labels pcyt**b** and dcyt**b**, which are regions with high similarity to the full-length cytochrome *b* gene.

these duplications remains an ongoing challenge for the field of molecular evolution.

Mitochondrial duplications often occur as tandem arrays, with a gene or group of genes repeated one after the other (Campbell and Barker, 1999; Eberhard et al., 2001; Abbott et al., 2005). Several mechanisms have been proposed that would result in this type of structure. Slipped strand mispairing can frequently result in tandem duplications in the presence of repeat units or sequences that form secondary structures (Levinson and Gutman, 1987; Madsen et al., 1993; Mueller and Boore, 2005). During DNA replication, a portion of the DNA strand dissociates between two repeats forming a loop. The polymerase then reassociates at the first repeat and duplicates the looped section (Levinson and Gutman, 1987; Madsen et al., 1993; Boore, 2000). The consistent presence of repeats at either end of the junctions of mitochondrial tandem duplications in parthenogenetic lizards led Fujita et al. (2007) to conclude that slipped strand mispairing was likely the cause of duplications. Over-running the termination signal during DNA replication has also been suggested as a way to form tandem duplications (Boore, 2000; Mueller and Boore, 2005). In this case, the duplications would include the genes flanking the origin of replication, which is often seen in mitochondrial duplications (San Mauro et al., 2006). Initiation of replication at sites of secondary structure other than the origin has also been suggested to result in tandem duplications (Levinson and Gutman, 1987; Madsen et al., 1993; Stanton et al., 1994; Lunt and Hyman, 1997; Macey et al., 1997; Boore, 2000). tRNAs or other sequences that are capable of forming secondary structures often are found at the ends of duplicated regions, suggesting that these structures may cause illicit priming of mitochondrial replication (Stanton et al., 1994; San Mauro et al., 2006). Finally, unequal crossing over could potentially result in tandem duplications when two mitochondrial genomes within a single mitochondrion recombine with one genome donating its copies of a group of genes to the other genome (see Ohno, 1970; Zhang, 2003 for this mechanism in the nucleus).

Consideration of the fates of duplicated nuclear genes suggests four potential fates for duplicated mitochondrial genes: (1) non-functionalization in which one copy becomes a pseudogene and is eventually eliminated from the genome, (2) subfunctionalization, in which copies of a multifunctional gene can each become specialized for one of the different original functions, and will each be stably maintained within the genome because they are under

selection to carry out different functions, (3) neofunctionalization, in which a duplicated gene acquires a novel function due to mutations in the regulatory region or within the gene copy, and (4) redundant maintenance, in which multiple copies of a gene are maintained through gene conversion or purifying selection because the extra copies help meet high expression demands (Force et al., 1999; Lynch et al., 2001; Rastogi and Liberles, 2005; Roth et al., 2007; Zhang, 2003). However as mitochondria are believed to be under selection for compactness, it would seem that nonfunctionalization and elimination of extra gene copies would be the most likely fate of mitochondrial duplications (Rand and Harrison, 1986). Depending upon which copy of a gene is eliminated, a new gene arrangement may arise or the original gene order may be restored (Boore, 2000). This hypothesis has come to be known as the tandem duplication/random loss model of mitochondrial genome rearrangement (Boore, 2000; Macey et al., 1997; Mindell et al., 1998; Mortiz, 1991). In this model, loss of duplicated genes is thought to occur rapidly relative to evolutionary time (Mortiz, 1991; Quinn, 1997). Therefore, residual evidence of a previous duplication such as the presence of pseudogenes may be suggestive of a relatively recent event (Mortiz, 1991; Quinn, 1997).

Four different mitochondrial genome arrangements have been identified within birds (Class Aves) that differ from that of the typical vertebrate (Fig. 1). The common avian arrangement, first identified in the chicken by Desjardins and Morais (1990), can be derived from the common vertebrate arrangement (ND6/tRNA^{Glu}/cyt *b*/tRNA^{Thr}/tRNA^{Pro}/control region) by one tandem duplication/random loss event (involving cyt *b*/tRNA^{Thr}/tRNA^{Pro}/ND6/tRNA^{Glu}/control region). Later, Mindell et al. (1998) described a second arrangement in which a non-coding region of variable length and with some similarity to the control region was found in the typical location of the control region, while the full-length control region was located after tRNA^{Thr}. A single tandem duplication/random loss event is necessary to derive this arrangement from the common avian mitochondrial arrangement (Mindell et al., 1998). This second gene order has been found in several diverse orders of birds, such as Piciformes (woodpeckers), Cuculiformes (cuckoos), Falconiformes (falcons), Passeriformes (oscines and suboscines), and Tinamiformes (tinamous) (Bensch and Härlid, 2000; Haddrath and Baker, 2001; Mindell et al., 1998). A third arrangement of mitochondrial genes was found in several species of *Amazona* parrots (Eberhard et al., 2001). In this case, one degenerate copy of the

duplicated ND6 and tRNA^{Glu} was still present making the extent of the duplication more easily defined. Additionally, the second non-coding region showed high similarity with the control region and appeared to be functional. This arrangement has also been found in the osprey (*Pandion haliaetus*), ivory-billed aracari (*Pteroglossus azara*) and Philippine hornbills (*Aceros waldeni* and *Penelopides panini*) (Gibb et al., 2007; Sammler et al., 2011). The fourth arrangement was identified in *Thalassarche* albatrosses (Abbott et al., 2005). Here, the genes from cytochrome *b* to the control region were tandemly duplicated and most appeared to still be functional. However, the second copy of cytochrome *b* appeared greatly reduced in size with only portions of the 5' and 3' ends (designated as d-cyt *b* and p-cyt *b*) being alignable with the full-length copy. The two control regions were also easily alignable and appeared to be functional, but differed in sequence and length of domain III, with control region 1 lacking repeats at the 3' end (Abbott et al., 2005). A similar rearrangement has also been found in the black-faced spoonbill (*Platalea minor*) (Cho et al., 2009), the ruff (*Philomachus pugnax*) (Verkuil et al., 2010), three species of booby in the genus *Sula* (Morris-Pocock et al., 2010) and two species of Philippine hornbills (Sammler et al., 2011).

Despite the many descriptions of avian mitochondrial gene arrangements that have been published, we still lack a clear understanding of when or how often mitochondrial duplications have occurred in birds. Few orders have been systematically surveyed for gene arrangements or have been paired with a well-sampled phylogeny to allow robust conclusions about the evolutionary history of mitochondrial duplications and genome rearrangements. The order Psittaciformes (parrots and cockatoos, hereafter 'parrots'), presents an excellent opportunity to identify the frequency with which mitochondrial control region duplications occur within a clade. Eberhard et al. (2001) established that several species of Neotropical parrots contained a duplicated control region, while preliminary data from other parrots suggested that these duplications were not shared by the entire order (T.F. Wright, J.R. Eberhard, unpublished data; E.S. Tavares, C.Y. Miyaki, unpublished data).

The current study seeks to address the following two questions: (1) Does the mitochondrial control region duplication, first identified in *Amazona* parrots, exist in other parrot genera? (2) If so, was there a single origin or were there multiple independent origins of these duplications? To answer these questions, we surveyed 117 parrot species by PCR for the presence of mitochondrial control region duplications and mapped these results onto a phylogeny reconstructed from mitochondrial and nuclear intron DNA sequences.

2. Materials and methods

2.1. Taxon and character sampling

For the phylogeny and survey of mitochondrial control region duplications, we added 51 new taxa to the dataset of Wright et al. (2008) for a total of 117 parrot species representing 79 of the 82 extant genera (Tables 1S and 2S). We used a stratified sampling method to determine the number of species sampled per genus such that genera with one to four species were represented by a single species or 25–100% coverage, genera with 5–11 species had two representatives (18–40% coverage), genera with 12–16 species were represented by three species (19–25% coverage) and genera with more than 17 species had four representatives (13–24% coverage). The new species included in this study were chosen based upon the accessibility of tissue or blood samples in museum or zoo collections. Samples for three genera (*Geopsittacus*, *Ognorhynchus* and *Oreopsittacus*) were unobtainable. Taxonomic nomenclature follows Forshaw (2006) for Old World species and the 2010 AOU North American and South American checklists for

New World species (Chesser et al., 2010; Remsen et al., 2010). *Coccyzus americanus* (Cuculiformes), *Colinus colinus* (Coliiformes), *Columbina passerina* (Columbiformes), *Falco peregrinus* (Falconiformes), *Otis sunia* (Strigiformes), *Picus canus* (Piciformes), *Serinus canarius* (Passeriformes) and *Tockus flavirostris* (Coraciiformes) were included as outgroups as each has been identified as an ally or a sister group of the parrots in previous studies (Ericson et al., 2006; Fain and Houde, 2004; Hackett et al., 2008; Sibley and Ahlquist, 1990; Sorenson et al., 1999).

For the phylogenetic analyses, we sampled two mitochondrial protein-coding loci (cytochrome *c* oxidase I (COI) and nicotinamide adenosine dehydrogenase subunit 2 (ND2)) and two nuclear introns (tropomyosin intron five (TROP) and transforming growth factor beta 2 intron one (TGFβ2)). These genes have proven to be informative in other phylogenetic studies of parrots (Wright et al., 2008; Joseph et al., 2012).

2.2. DNA extraction, PCR and sequencing

We extracted DNA from tissue or blood samples, performed polymerase chain reaction amplification (PCR), and sequenced the PCR products at laboratories in three locations due to legal restrictions on transporting specimens from endangered species. The laboratories were New Mexico State University (NMSU) in Las Cruces, New Mexico; the Instituto Nacional de Toxicología y Ciencias Forenses (Spain) in Tenerife, Canary Islands, Spain; and the University of São Paulo (Brazil) in São Paulo, Brazil.

At NMSU and in Spain we extracted DNA using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol for each tissue type. In Brazil, we extracted DNA from blood samples using a phenol/chloroform protocol (Bruford et al., 1992). At all locations we amplified the four gene regions by PCR using primers, reactions, and cycling conditions as described in Wright et al. (2008). PCR products were checked for correct size and the presence of multiple bands by electrophoresis on a 0.5–2% agarose gel and stained with ethidium bromide.

We cleaned PCR products using a QiaQuick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions at NMSU and in Spain, while in Brazil we used 1 µL exonuclease I and 0.5 µL of shrimp alkaline phosphatase per 10 µL PCR reaction incubated at 37 °C for 30 min, then 80 °C for 15 min to clean PCR products. We sequenced each PCR product in both directions using the PCR primers and Big Dye v3.1 Terminator Cycle Sequencing chemistry (Applied Biosystems Inc, Foster City, CA). Each sequencing reaction at NMSU and in Spain consisted of 2 µL of BigDye, 1 µL of 5X sequencing buffer, 3.2 µL of 1µM primer, 2 µL of clean PCR product, and 11.8 µL of water. In Brazil each sequencing reaction consisted of 2 µL of BigDye, 2–4 µL of clean PCR product, and 1 µL of primer. Sequencing conditions at all locations were 25 cycles of 95 °C for 25 s, 50 °C for 5 s, and 60 °C for 4 min. We cleaned sequencing reactions at NSMU by centrifugation through Sephadex columns. Clean reactions were dried and resuspended in 20 µL of HiDi Formamide (Applied Biosystems Inc., Foster City, CA) before sequencing on an ABI 3100 Avant automated sequencer. In Brazil sequencing reactions were cleaned by isopropanol/ethanol precipitation, dried, resuspended in 1.8 µL of formamide, heated to 95 °C for 2 min, placed on ice until loaded on an ABI 377 automated sequencer. In Spain, we cleaned sequencing reactions by centrifugation through Centri-Sep columns in a 96 well format (Applied Biosystems Inc., Foster City, CA). The cleaned reaction was dried, resuspended in 20 µL of formamide, and sequenced on an ABI 310 automated sequencer.

2.3. Phylogenetic analysis

Raw sequences were checked for ambiguous base calls in Sequencher 4.7 (Gene Codes, Ann Arbor, MI) and combined into

Fig. 2. Location of primer pairs and relative fragment lengths used to classify control region duplication state. (a) Schematic of fragment sizes based upon the presence of a single or duplicated mitochondrial control region. (b) A representative agarose gel of fragment sizes for a duplicated and single control region.

contigs by locus and taxon. Sequences were aligned using Clustal W with default parameters as implemented at <http://www.ebi.ac.uk/Tools/clustalw> and adjusted by eye. Gaps within introns were coded by the simple indel coding method (Simmons and Ochoteren, 2000) as implemented in IndelCoder 0.5 in the SeqState 1.40 program (Müller, 2005, 2006).

Maximum likelihood methods of phylogenetic reconstruction were implemented in GARLI v0.951 using the default settings (Zwickl, 2006). The General Time Reversible model with a gamma distribution of among site heterogeneity and a proportion of invariant sites was chosen, with parameter values estimated by the software and SPR branch swapping (Zwickl, 2006). To ensure that the tree was not located in a local optimum, 20 independent runs were conducted in GARLI and the tree with the highest likelihood was chosen for subsequent analyses (Zwickl, 2006). Nodal support was evaluated by 100 maximum likelihood bootstraps calculated in GARLI.

Bayesian methods of phylogeny reconstruction were implemented using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). A separate evolutionary model was determined for each gene region in MrModelTest 2.3 under the Akaike Information Criterion (Nylander, 2004). Gaps were coded as restriction sites using the default settings. The analysis consisted of two parallel runs, each with one cold chain and three heated chains with default parameters. The mixed model analysis of the combined dataset was run for 15,000,000 generations with trees sampled every 1000 generations and a burn-in of 25%. Convergence was assumed when the average standard deviation of split frequencies was less than or equal to 0.01 and when the effective sample size (ESS) value for parameter values was greater than 200 when viewed in Tracer v1.4 (Rambaut and Drummond, 2007).

2.4. Mitochondrial control region survey

Of the 117 species included in the phylogeny 112 were surveyed at NMSU for the presence of a mitochondrial control region duplication using PCR to amplify diagnostic fragments from the regions predicted to differ in length depending on the presence or absence of a duplicated control region. We could not survey five taxa using the PCR approach (*Strigops habroptilus*, *Cyanoramphus auriceps*, *Cyanopsitta spixii*, *Enicognathus leptorhynchus* and *Tricharia malachitacea*) because tissue samples were not available at NMSU. We used the primer pairs L15725p – AAACCAGARTGATAYTTTC TMTTYGCAT (modified from Sorenson et al., 1999) and H520p –

TGKSCCTGACCKAGGAACCCAG (modified from Sorenson et al., 1999), L16087p – TGGYCTTGTAARCCAAARRAYGAAG (modified from Sorenson et al., 1999) and H520p and L16087p with H16191 – TCTCGDGGGGCDATTCGGGC (Sorenson et al., 1999) to amplify diagnostic Segments 15, 16 and ND6 respectively. An additional primer pair, LGlu – GCCCTGAAAARCCATCGTTG (Eberhard et al., 2001) in conjunction with H520p was used to amplify Segment Glu to verify the presence of a second control region that is usually flanked by the intact tRNA^{Glu}. The expected location of each primer and the genes contained within each segment are depicted in Fig. 2. PCR reaction conditions were the same as for the phylogeny except for the cycling conditions which were 94 °C for 4 min followed by 29 cycles of 94 °C for 25 s, 60 °C for 30 s on the initial cycle with a decrease of 0.4 °C each cycle, and 72 °C for 2 min. This was followed by 6 cycles of 94 °C for 25 s, 45 °C for 30, 72 °C for 2 min, with a final extension of 72 °C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Gels were imaged on a BioRad Gel Doc XR and band sizes for each segment were estimated using the BioRad Amplisizer 50–2000 bp Molecular Ruler and the Band Analysis protocol in the Quantity One software (BioRad Life Sciences, Hercules, CA).

Expected sizes of the diagnostic segments used to score each species as a single or duplicated control region were as follows: Single Control Region, Segment 15 = 1579–1896 base pair (bp), Segment 16 = 1225–1542 bp, Segment ND6 = 132 bp; Duplicated Control Region, Segment 15 = 800–1250 bp, Segment 16 = 600–1000 bp, Segment ND6 ≥ 1200 bp. These segment sizes were derived from preliminary studies of parrot control region duplications and then further refined by analysis of the GenBank parrot mitochondrial sequences (Table 3S and T.F. Wright, J.R. Eberhard, E.E. Schirtzinger, unpublished data). Band sizes were expected to show some variation due to the variation in size of domains I and III of the control region (Baker and Marshall, 1997) as determined by alignment of primer H520p to parrot control region sequences available on GenBank. Another source of variation is the presence and size of intergenic spacers. This variation was evaluated by counting the base pairs between the 3' end of one annotated gene and the 5' end of the next annotated gene in the region of cytochrome *b* to tRNA^{Phe} from three parrot mitochondrial genomes on GenBank (*Strigops habroptilus*, *Agapornis roseicollis* and *Melopsittacus undulatus*). These ranges were added to the sizes of the genes included in each segment to get the total estimated range of variation. A species was scored as having a single or duplicated control region based on the correspondence of its measured

segment sizes to the expected sizes for each segment (Fig. 1S). Taxa that did not amplify at least two diagnostic segments were classified as unscorable.

2.5. Sequencing of selected taxa

Because the expected band sizes encompass a large range, diagnostic Segment 16 and Segment Glu, from selected species were sequenced at NMSU or Brazil to confirm the status of the mitochondrial control region as classified by our PCR survey. At least one representative of each clade that contained an inferred duplicated control region was sequenced. In addition, species that were ambiguous in their classification were also sequenced. Finally, the GenBank mitochondrial sequences for *Agapornis roseicollis*, *Strigops habroptilus* (single control regions) and *Melopsittacus undulatus*, *Amazona farinosa*, *Amazona ochrocephala* and *Psittacus erithacus* (duplicated control regions) were also used as confirmation of the PCR survey results.

At NMSU, PCR products were amplified, cleaned as described above, and sent to the University of Chicago Cancer Sequencing Facility for sequencing on an ABI 3730 automated sequencer using Big Dye chemistry. In Brazil, amplifications for sequencing were performed in 10 μ L reactions with 1X buffer (GE Healthcare or Biotools), 2 μ M of dNTP, 1 μ M of each primer, 0.5 U of *Taq* polymerase, and 20–50 nanograms (ng) of template DNA, or in 25 μ L reactions with 1X buffer (Biotools), 2 μ M of dNTP, 1 μ M of each primer, 1 U of *Taq* polymerase, and 25–50 ng of DNA. PCR conditions were: initial denaturation 96 °C for 5 min, 30 cycles of 95 °C for 60 s, 50–54 °C for 25 s, and 65 °C for 40–80 s, with a final extension of 65 °C for 5 min. The size and quality of PCR products were verified, purified as described above or bands were excised from agarose gels and the product was isolated by centrifugation through filter tips (Axigen). Sequencing reactions were prepared, cleaned, and run as described above.

Raw sequences were proofread as previously described and combined into a consensus sequence by taxon and segment using Sequencher 4.7 (Gene Codes, Ann Arbor, MI). The location of tRNA^{Thr}, tRNA^{Pro}, ND6, and tRNA^{Glu} were identified by comparison with the homologous genes from the mitochondrial genome of *Melopsittacus undulatus* (NC_009134), while the control region was identified by the presence of the goose hairpin (C₇TAC₇) near the 5' end. The identity of pseudogenes was based on similarity with known sequences from the *Melopsittacus undulatus* mitochondrial genome or comparison with the pseudogenes defined by Eberhard et al. (2001). Functionality of tRNAs was assessed by simulation in tRNA scan-SE (Lowe and Eddy, 1997).

For each species sequenced, the gene order from tRNA^{Thr} through domain I of the control region was identified by similarity with previously described avian gene orders (Abbott et al., 2005; Desjardins and Morais, 1990; Eberhard et al., 2001; Mindell et al., 1998). For those species with a duplication, we measured the length of the non-coding region (calculated as the number of nucleotides from the end of tRNA^{Thr} to the goose hairpin), and the number of nucleotide differences between the two control regions (calculated as the number of differences when the two control region fragments were aligned divided by the total length of the aligned control region segment). Because the 5' end of the control region does not have a definitive starting motif and the 3' end of the tRNA^{Glu} could not be identified for all species, the goose hairpin was used as a proxy for the beginning of the control region.

2.6. Ancestral state reconstruction

Using the classifications from the PCR survey, sequences from selected taxa and GenBank sequences, the presence of a single (0) or duplicated (1) control region was coded into a matrix and

mapped onto the phylogeny. Three species (*Aprosmictus erythropterus*, *Eos reticulata*, *Pionites melanocephala*) were coded as unscorable because they either failed to amplify the diagnostic fragments by PCR or they produced ambiguous results and no confirmatory sequence was available. Ancestral state reconstructions were undertaken in Mesquite 2.01 (Maddison and Maddison, 2007) under parsimony (Fitch) and the maximum likelihood criterion. Likelihood reconstructions were undertaken using the symmetric MK1 model, which is a generalization of the Jukes–Cantor model with equal probability of changing states and the AsymmMK model, which uses different rates of change between states (Maddison and Maddison, 2007). To test the robustness of the likelihood analysis, separate reconstructions were undertaken using the AsymmMK model and rates translating to (a) gains five times as likely as losses and (b) gains 1/5 as likely as losses.

3. Results

3.1. Phylogenetic analysis

The phylogenetic dataset consisted of 117 parrot species and eight non-parrot outgroups. The mitochondrial sequences, COI and ND2, showed no insertions/deletions (indels). The COI sequences were 570 base pairs (bp) in length while the ND2 sequences were 1041 bp. The intron sequences, TROP and TGF2, were more variable in length due to the presence of 48 and 78 indels respectively. The TROP sequences were 498–533 bp with a total of 554 aligned bp. The TGF2 sequences were 611–630 bp with a total of 817 aligned bp. The maximum likelihood dataset consisted of 2982 concatenated bp. The Bayesian analysis was performed on the 2982 characters included in the concatenated dataset (partitioned by gene region) and the 126 coded indels for a total of 3108 characters. Table 4S includes the genetic details for each gene region and the concatenated dataset. All new sequences have been deposited in GenBank (see Table 1S).

The most likely tree ($-\ln L = -63846.569$) from 20 independent maximum likelihood (ML) analyses in GARLI of the concatenated dataset (Fig. 2S) and the consensus tree from the Bayesian analysis (Fig. 3) were broadly congruent, differing only in the placement of two genera, *Micropsitta* and *Graydidascalus*. The Bayesian analysis places *Micropsitta* with high support (posterior probability = 1) as sister to the clade consisting of *Alisterus*, *Aprosmictus*, *Polytelis*, *Eclactus*, *Geoffroyus*, *Psittacula*, *Psittinus*, *Tanygnathus* and *Prioniturus* while in the ML analysis *Micropsitta* is sister to the clade of *Alisterus*, *Aprosmictus* and *Polytelis* with poor support (ML bootstrap ≤ 50). The Bayesian analysis places *Graydidascalus* as sister to *Amazona* while in the ML analysis *Graydidascalus* is sister to the clade consisting of *Amazona* and *Pionus*. However, in both analyses the position of *Graydidascalus* is poorly supported. Nucleotide substitution models, priors for the Bayesian analysis, and final estimates of parameters for each analysis are listed in Table 5S.

3.2. Mitochondrial control region survey

One hundred and twelve parrot species were surveyed for the status of its mitochondrial control region by PCR of three diagnostic segments that show variation in size when a duplicated control region is present. Table 1 reports the control region status of each species surveyed and the length of each amplicon. Segment 15 was amplifiable for 96 species, while Segment 16 and Segment ND6 were amplifiable for 108 and 110 species respectively (see Table 1). *Aprosmictus erythropterus*, *Guarouba guarouba*, *Nandayus nenday* and *Rhynchopsitta pachyrhyncha* could not be scored by PCR at NMSU due to a lack of amplification. Five species (*Eos reticulata*, *Graydidascalus brachyurus*, *Hapalopsittacus pyrrhops*, *Pionites mela-*

Fig. 3. The partitioned Bayesian analysis of 117 parrot species reconstructed using two mitochondrial protein-coding genes (COI and ND2), two nuclear introns (TROP and TGF2) and coded gaps. Posterior probabilities >0.95 are given above the branches and maximum likelihood bootstraps >70 are given below the branches. Asterisks indicate a posterior probability of 1.0 or a maximum likelihood bootstrap value of 100.

Table 1
Measured PCR fragment lengths and control region status for each parrot species in the phylogeny.

Scored by PCR	Control region status	Measured fragment size (nucleotides)			
		15	16	Glu	ND6
<i>Agapornis canus</i>	1	1781	1394	708	141
<i>Agapornis roseicollis</i>	1	1912	1399	689	144
<i>Alisterus amboinensis</i>	1	1652	1253	488	141
<i>Amazona albifrons</i>	2	1042	756	609	501
<i>Amazona farinosa</i>	2	1134	769	618	137, 1943
<i>Amazona ochrocephala</i>	2	1109	779	637	1883
<i>Amazona viridigenalis</i>	2	1182	781	626	153, 508, 2146
<i>Anodorhynchus hyacinthinus</i>	1	1672	1316	645	148
<i>Aprosmictus erythropterus</i>	UN	*	*	*	144
<i>Ara ararauna</i>	1	1742	1344	627	147
<i>Ara macao</i>	1	1738	1302	685	152
<i>Aratinga canicularis</i>	1	1833	1357	648	152
<i>Aratinga finschi</i>	1	1658	1341	630	144
<i>Aratinga pertinax</i>	1	2121	1697	640	145
<i>Aratinga solstitialis</i>	1	*	1368	627	145
<i>Barnardius zonarius</i>	1	1963	1576	847	149
<i>Bolbopsittacus lunulatus</i>	1	*	1681	901	153
<i>Bolborhynchus lineola</i>	1	1580	1255	543	149
<i>Brotogeris chrysopterus</i>	1	1809	1376	667	149
<i>Brotogeris jugularis</i>	1	1717	1343	677	145
<i>Cacatua haematuropygia</i>	1	1627	1236	640	148
<i>Cacatua leadbeateri</i>	1	1722	1245	629	149
<i>Cacatua sulphurea</i>	1	*	1227	604	144
<i>Callocephalon fimbriatum</i>	1	1505	1198	606	148
<i>Calyptorhynchus banksii</i>	1	1543	1215	549	148
<i>Calyptorhynchus funereus</i>	1	1765	1320	553	150
<i>Chalcopsitta cardinalis</i>	2	990	656	602	1346
<i>Chalcopsitta duivenbodei</i>	2	943	602	680	144, 1387
<i>Chamosyna papou</i>	2	*	1049	625	141, 315, 1164
<i>Chamosyna placentis</i>	2	998	628	625	1383
<i>Coracopsis vasa</i>	1	1572	1237	562	143
<i>Cyanoliseus patagonus</i>	1	1805	1368	671	147
<i>Cyanoramphus novaezelandiae</i>	1	2047	1626	875	144
<i>Cyclopsitta diophthalma</i>	2	*	673	636	475
<i>Deropityus accipitrinus</i>	2	1261	952	642	147, 2244
<i>Diopsittaca nobilis</i>	1	*	1294	631	155
<i>Eclectus roratus</i>	1	1658	1253	561	153
<i>Eolophus roseicapillus</i>	1	1621	1187	*	152
<i>Eos histrio</i>	2	1010	622	573	1465
<i>Eos reticulata</i>	2?	*	627	575	145
<i>Eumymphicus cornutus</i>	1	1928	1583	861	150
<i>Forpus passerinus</i>	2	1200	810	670	138, 1813
<i>Forpus sclateri</i>	2	1234	650	672	2980
<i>Geoffroyus heteroclitus</i>	1	*	1498	781	152
<i>Glossopsitta porphyrocephala</i>	2	1088	720	687	1440
<i>Graydidascalus brachyurus</i>	2?	1410	889	700	154
<i>Guarouba guarouba</i>	UN	*	*	*	*
<i>Hapalopsittaca pyrrhops</i>	2?	1321	786	663	143
<i>Lathamus discolor</i>	1	1962	1577	840	147
<i>Leptosittaca branickii</i>	1	1726	1296	620	146
<i>Loriculus galgulus</i>	1	1571	1269	631	146
<i>Loriculus philippensis</i>	1	1598	1279	640	147
<i>Loriculus vernalis</i>	1	1636	1295	628	143
<i>Lorius albidinuchas</i>	2	998	640	618	1350
<i>Lorius lory</i>	2	984	645	580	1472
<i>Melopsittacus undulatus</i>	2	968	610	585	1612
<i>Micropsitta finschii</i>	1	1555	1180	502	149
<i>Micropsitta pusio</i>	1	1505	1142	479	142
<i>Myiopsitta monachus</i>	1	1639	1259	596	152
<i>Nandayus nenday</i>	UN	*	*	*	*
<i>Nannopsittaca panychlora</i>	1	1620	1254	556	151
<i>Neophema elegans</i>	1	*	1379	693	164
<i>Neophema pulchella</i>	1	1673	1288	640	160
<i>Neopssephotus bourkii</i>	1	1657	1313	628	154
<i>Neopsittacus musschenbroekii</i>	2	1004	640	584	1423
<i>Nestor notabilis</i>	1	1615	1264	596	156
<i>Northiella haematogaster</i>	1	1914	1322	592	147
<i>Nymphicus hollandicus</i>	1	1633	1200	591	146
<i>Orthopsittaca manillata</i>	1	1727	1363	640	152
<i>Pezoporus wallicus</i>	1	1900	1500	800	155
<i>Phigys solitarius</i>	2	1028	646	550	155, 1524
<i>Pionites melanocephala</i>	2?	*	971	626	154
<i>Pyrilia caica</i>	2?	1136	782	638	158

Table 1 (continued)

Scored by PCR	Control region status	Measured fragment size (nucleotides)			
		15	16	Glu	ND6
<i>Pyrilia vulturina</i>	2	982	615	600	166, 2223
<i>Pionus chalcopterus</i>	2	1150	781	614	157, 525, 2128
<i>Pionus menstruus</i>	2	1167	793	640	490
<i>Platycercus adscitus</i>	1	1919	1521	809	149
<i>Platycercus elegans</i>	1	1829	1449	771	148
<i>Poicephalus robustus</i>	2	1058	666	560	153, 1521, 1804
<i>Poicephalus senegalus</i>	2	978	639	531	1549
<i>Polytelis alexandrae</i>	1		1265	635	145
<i>Primolius couloni</i>	1	1685	1313	630	147
<i>Prioniturus luconensis</i>	2	1231	761	500	145, 2328
<i>Prioniturus montanus</i>	2	1130	795	492	148
<i>Probosciger aterrimus</i>	1	1601	1244	594	144
<i>Prosopcia tabuensis</i>	1	1878	1465	758	148
<i>Psephotus chrysopterygius</i>	1	1872	1494	858	138
<i>Psephotus varius</i>	1	1924	1517	837	148
<i>Pseudeos fuscata</i>	2	988	619	578	1370
<i>Psilopsiagon aymara</i>	1	1555	1210	546	151
<i>Psittacula brehmii</i>	1	1640	1476	604	150
<i>Psittacula columboides</i>	1	*	1211	550	150
<i>Psittacula krameri</i>	1	*	1343	582	136
<i>Psittacula roseata</i>	1	1503	1198	492	144
<i>Psittaculirostris edwardsii</i>	2	817	620	586	391, 680
<i>Psittacus erithacus</i>	2	984	617	545	144, 1589
<i>Psittacutes goldiei</i>	2	1341	638	599	1455
<i>Psittinus cyanurus</i>	1	1554	1192	486	132
<i>Psittichas fulgidus</i>	2?	1244	907	535	135
<i>Purpureicephalus spurius</i>	1	1893	1563	849	142
<i>Pyrhura albipectus</i>	1	1712	1331	626	145
<i>Pyrhura hoffmanni</i>	1	1563	1297	600	142
<i>Pyrhura lepidota</i>	1	1665	1306	640	136
<i>Pyrhura picta</i>	1	1724	1310	622	154
<i>Rhynchopsitta pachyrhyncha</i>	UN	*	*	638	137
<i>Tanygnathus lucionensis</i>	1	1585	1262	550	133
<i>Touit batavica</i>	1	1700	1200	500	137
<i>Touit purpurata</i>	1	1700	1100	500	148
<i>Trichoglossus chlorolepidotus</i>	2	842	664	*	143, 175, 772, 1501
<i>Trichoglossus haematodus</i>	2	1009	643	586	1491
<i>Vini australis</i>	2	1000	650	600	142, 1348
<i>Vini peruviana</i>	2	1000	650	600	1336
	N=112	N=96	N=108	N=107	N=110
Scored from Sequences (BR)					
<i>Cyanopsitta spixii</i>	1				
<i>Enicognathus leptorhynchus</i>	1				
<i>Guaruba guarouba</i>	1				
<i>Nandayus nenday</i>	1				
<i>Rhynchopsitta pachyrhyncha</i>	1				
<i>Triclaria malachitacea</i>	2				
	N=6				
Scored from GB Sequences or Literature					
<i>Cyanoramphus auriceps</i>	1	Boon 2000			
<i>Strigops habroptilus</i>	1	AY309456			
	N=2				

* = fragment did not amplify.

UN = unscorable by PCR.

? = ambiguous by PCR.

nocephala and *Psittichas fulgidus*) all amplified ND6 bands indicative of a single control region while the sizes of Segment 15 and Segment 16 suggested that a duplicated control region was present. These species are listed as ambiguous in Table 1.

The band sizes for each segment are plotted in Fig. 4. Segment 15 showed a bimodal distribution, with most species clearly falling into either the single or duplicated control region size categories. However, *Gradydidascalus brachyurus* fell between the expected ranges. Segments Glu and 16 from this species were subsequently sequenced. In four taxa, Segment 15 amplicons were larger than the expected size of 1896 bp for a single control region. *Lathamus discolor* and *Barnardius zonarius* were approximately 75 bp larger than expected, while *Aratinga pertinax* and *Cyanoramphus novaezelandiae* exceeded the single control region size range by 225 bp and 150 bp.

These sizes may be stochastic effects of slightly different gel conditions that affect the distance run by the size marker used to measure the bands. The other bands for these species were also larger than expected but within the range of single control region sizes.

The size distribution for Segment 16 amplicons was also bimodal with all taxa falling within the two expected range sizes. Segment ND6 showed two different patterns: a tight cluster of species from 130 to 160 bp and a scatter of species with segment sizes from 1150 bp to over 2200 bp. *Pionus menstruus* and *Amazona albifrons* had values of 450 bp and 550 bp respectively. Based upon the other band sizes, *Amazona albifrons* and *Pionus menstruus* were classified as having a duplicated control region. The presence of a duplicated control region in *Amazona albifrons* was later confirmed by sequencing.

Fig. 4. Histograms of the number of species per 50 base pair bins for each amplified segment. Black bars indicate duplicate control regions. Gray bars indicate a single control region. An asterisk indicates species that fall outside of the expected fragment size ranges based on preliminary surveys of control region lengths. (a) lengths of Segment 15, (b) lengths of Segment 16, and (c) lengths of Segment ND6.

Of the 112 species surveyed by PCR, 68 were classified as having a single control region and 35 species were classified as having a duplicated control region. Four species, *Aprosmictus erythropterus*, *Guarouba guarouba*, *Nandayus nenday* and *Rhynchopsitta pachyrhyncha* were unscorable due to a lack of PCR amplification and five species (*Eos reticulata*, *Graydidascalus brachyurus*, *Hapalopsittaca pyrrhops*, *Pionites melanocephala* and *Psittichas fulgidus*) produced ambiguous results.

3.3. Sequencing of selected taxa

In order to validate the control region classifications from the PCR survey we sequenced 44 parrot species in the phylogeny (Table 2). The common avian gene order with a single control region was confirmed in 19 species (Desjardins and Morais, 1990), while the gene order with a duplicated control region previously described by Eberhard et al. (2001) for *Amazona* and *Pionus* species was confirmed for 25 species. Of the five species that produced ambiguous results in the PCR survey, two species (*Graydidascalus brachyurus*, *Pseudoeus fuscata*) were shown to have a duplicated control region by sequencing. The aberrant size of the ND6 fragment was due to a lack of retained homology in the non-coding region. Therefore, only the functional ND6 was amplified. Sequencing of *Cyanopsitta spixii*, *Enicognathus leptorhynchus*, *Guarouba guarouba*, *Nandayus nenday*, *Rhynchopsitta pachyrhyncha* and *Triclaria malachitacea* in Brazil found that only *Triclaria malachitacea* had a duplicated control region. Three species were listed as unscorable (*Aprosmictus erythropterus*, no PCR amplification) or ambiguous (*Eos reticulata* and *Pionites melanocephala*, no sequence available to confirm status).

In the set of species with duplicated control regions, non-coding regions of various sizes were found between tRNA^{Thr} and the first control region. These non-coding regions were examined with tRNA-Scan (Lowe and Eddy, 1997) to determine if copies of tRNA^{Pro} or tRNA^{Glu} retained enough homology to be identifiable. In most cases no homology could be determined due to the extent of degeneration. The control regions were identified by the presence of conserved sequences: the goose hairpin at the 5' end of domain I and the D-Box in domain II (Eberhard et al., 2001). The F-Box sequence of *Gallus gallus* was not identified in all species. An additional eight species (*Aratinga aurea*, *Aratinga leucophthalmus*, *Brotogeris chirri*, *Forpus xanthopterygius*, *Nannopsittaca dachillae*, *Pyrrhula barrabandi*, *Pionites leucogaster*, and *Primolius auricollis*) were sequenced in Brazil for a different study (E. Tavares, C. Miyaki unpublished data), and served as additional confirmation of the distribution of control region duplications. Although these species were not included in the phylogeny, all are known from a broader phylogenetic survey to cluster with their congeners included in this study (E.E. Schirtzinger, unpublished data). GenBank sequences or published literature was used to confirm gene order for *Cyanoramphus auriceps*, *Strigops habroptilus*, *Amazona ochrocephala*, *Amazona farinosa*, *Pionus chalcopterus*, *Agapornis roseicollis*, *Melopsittacus undulatus*, and *Psittacus erithacus* (see Table 2 and Boon, 2000). The control region status of *Chalcopsitta duivenbodei* and *Chamosyna papou* could not be confirmed due to poor sequencing reactions.

Within the Neotropical parrots, a variation of the *Amazona* gene order was observed in *Deroptyus accipitrinus* and *Pionites leucogaster*. In contrast to all of the other species with control region duplications that we sequenced, *Deroptyus accipitrinus* and *Pionites leucogaster* retain a potentially functional copy of tRNA^{Pro} before the non-coding region 5' to the first control region.

3.4. Ancestral state reconstruction

To determine if mitochondrial control region duplications in parrots originated multiple times, the character states of single (0) or duplicated control region (1) were mapped onto the Bayesian tree using parsimony and maximum likelihood methods (Fig. 5). These states were assigned using our PCR classifications (68 single control region, 35 duplicated control region, three unscorable/ambiguous), sequences from selected taxa (six single control region and three duplicated control region), GenBank sequences (one single control region), or publications (one single control region). Both methods of reconstruction identified the ancestral con-

Table 2

Confirmation of PCR Classified Control Region Status by Sequencing of Selected Species or by GenBank Sequences.

Scored by PCR	Control region status	Confirmed by Sequence source	GenBank #
<i>Agapornis roseicollis</i>	1	GB	EU410486
<i>Amazona albifrons</i>	2	NMSU	JQ341164, JQ360543
<i>Amazona farinosa</i>	2	GB	AF228821
<i>Amazona ochrocephala</i>	2	NMSU/GB	AF338819, AF338820, JQ341165, JQ360544
<i>Anodorhynchus hyacinthinus</i>	1	BR	EF104124
<i>Ara ararauna</i>	1	BR	EF104127
<i>Aratinga solstitialis</i>	1	BR	EF104138
<i>Bolborhynchus lineola</i>	1	BR	EF104137
<i>Calyptorhynchus banksii</i>	1	NMSU	JQ360567
<i>Chalcopsitta cardinalis</i>	2	NMSU	JQ341170, JQ360549
<i>Chalcopsitta duivenbodei</i>	2	NMSU	JQ360545
<i>Charmosyna papou</i>	2	NMSU	JQ341166
<i>Charmosyna placensis</i>	2	NMSU	JQ341167, JQ360546
<i>Coracopsis vasa</i>	1	NMSU	JQ341168, JQ360570
<i>Cyanoliseus patagonus</i>	1	BR	EF104136
<i>Cyanopsitta spixii</i>	1	BR	EF104128
<i>Cyclopsitta diophthalma</i>	2	NMSU	JQ241169, JQ36-547
<i>Derophtys accipitrinus</i>	2	BR/NMSU	AF365437, JQ360548
<i>Diopsittaca nobilis</i>	1	BR	EF104121
<i>Enicognathus leptorhynchus</i>	1	BR	EF104139
<i>Eos histrio</i>	2	NMSU	JQ341171, JQ360550
<i>Forpus sclateri</i>	2	NMSU	JQ341172, JQ360551
<i>Glossopsitta porphyrocephala</i>	2	NMSU	JQ341173, JQ360552
<i>Graydidascalus brachyurus</i>	2	BR	EF104148
<i>Guaruba guarouba</i>	1	BR	EF104123
<i>Lorius albidinucha</i>	2	NMSU	JQ341174, JQ360553
<i>Melopsittacus undulatus</i>	2	GB	NC_009134
<i>Micropsitta pusio</i>	1	NMSU	JQ360568
<i>Myiopsitta monachus</i>	1	BR	EF104118
<i>Nandayus nenday</i>	1	BR	EF104131, EF104149
<i>Neopsittacus musschenbroekii</i>	2	NMSU	JQ341175, JQ360554
<i>Orthopsittaca manilata</i>	1	BR	EF104119
<i>Phigys solitarius</i>	2	NMSU	JQ341176, JQ360555
<i>Pyrilia caica</i>	2	NMSU	JQ341178, JQ360556
<i>Pionus chalcopterus</i>	2	NMSU/GB	AF338817, AF338818, JQ360557, JQ360558
<i>Poicephalus robustus</i>	2	NMSU	JQ341180, JQ360566
<i>Prioniturus montanus</i>	2	NMSU	JQ341183, JQ360561
<i>Pseudeos fuscata</i>	1	NMSU	JQ360569
<i>Psittacula roseata</i>	2	NMSU	JQ341181, JQ360559
<i>Psittaculirostris edwardsii</i>	2	NMSU/GB	DQ335468, JQ341182, JQ360560
<i>Psittacus erithacus</i>	1	NMSU	JQ341184, JQ360562
<i>Psitteuteles goldiei</i>	1	BR	EF104130, EF104150
<i>Pyrrhura picta</i>	1	BR	EF104135
<i>Rhynchopsitta pachyrhyncha</i>	2	NMSU	JQ341186, JQ360564
<i>Trichoglossus haematodus</i>			

Table 2 (continued)

Scored by PCR	Control region status	Confirmed by Sequence source	GenBank #
<i>Triclaria malachitacea</i>	2	BR	EF104143, EF104146
<i>Vini australis</i>	2	NMSU	JQ341187, JQ360565
Related Species Sequenced			
<i>Aratinga aurea</i>	1	BR	EF104132, EF104151
<i>Aratinga leucophthalmus</i>	1	BR	EF104133
<i>Brotogeris chirri</i>	1	BR	EF104117
<i>Primolius auricollis</i>	1	BR	EF104126
<i>Pionites leucogaster</i>	2	BR	JQ749718, JQ749719
<i>Pyrilia barrabandi</i>	2	BR/NMSU	JQ341177, EF104141, EF104144
<i>Forpus xanttopterygius</i>	2	BR	EF104140, EF104147
<i>Nannopsittaca dachillae</i>	1	BR	EF104134
<i>Psittaculirostris salvadorii</i>	2	NMSU	JQ341185, JQ360563

trol region state in parrots as a single control region, and indicated that control region duplications have originated at least six times (Clades A–F in Fig. 5). No reversions from a duplicated control region to a single control region state were reconstructed by either method. Likelihood reconstructions using the symmetric rate (MK1) model and the asymmetric rate (AsymmMK) model did not affect the number of reconstructed independent origins or result in considerable differences in likelihoods of states at interior nodes. Similarly, changing the transition rate between states in the AsymmMK model did not affect our conclusions (See Fig. 3S and Table 6S for proportional likelihood values of each reconstructed state for interior nodes for each model analyzed).

3.5. Comparison of control regions

To determine if either of the two control regions had degenerated in species with a duplicated control region, the two control region fragments were aligned and nucleotide differences were calculated. These alignments found that the two control region sequences were typically highly similar, with sequence divergences ranging from 0–10.9% between the two copies within an individual. Most nucleotide differences were found between 51 and 225 nucleotides from the goose hairpin (Fig. 6). In domain I the only conserved sequence of known function is the termination-associated sequence (Baker and Marshall, 1997; Quinn, 1997; Sbisá et al., 1997). *Graydidascalus brachyurus* was not included in these calculations because neither of its control regions contained a goose hairpin.

4. Discussion

We reconstructed the phylogenetic relationships of 117 parrot species and classified their mitochondrial control region state from PCR fragment length analysis, DNA sequences or GenBank accessions to investigate the origins and distribution of mitochondrial control region duplications within the order Psittaciformes. A total of 76 parrot species were determined to have a single control region, while 38 parrot species were determined to have a duplicated control region. One species was unscorable and two species produced ambiguous results for which no sequence confirmation was available. Mapping the control region states onto the resulting phylogeny identified at least six independent origins of the duplicated control region state. Below we discuss the implications of

Fig. 6. Means (± 1 standard error) for nucleotide differences in 25 bp non-overlapping windows of aligned duplicated control regions. The figure shows that the greatest number of nucleotide differences occurs 51–225 bp from the goose hairpin in domain I of the control region.

these results for parrot evolutionary relationships and present two alternative hypotheses for the evolution of mtDNA duplications in parrots.

4.1. Parrot phylogeny

The phylogeny reconstructed in this study is the most taxonomically comprehensive phylogeny to date of the Psittaciformes. The Bayesian tree and the maximum likelihood tree are generally well resolved and largely congruent in topology. Disagreement between the two analyses occurs solely on the location of the genus *Microspitta* and *Graydidascalus brachyurus* (Fig. 2S and Fig. 3). The Bayesian phylogeny is broadly consistent with other published studies of parrot genus level relationships, in which a clade composed of the New Zealand endemics, *Strigops* and *Nestor*, was the sister group to all other parrots, and the *Cacatuoidea* (cockatoos) was the second oldest extant clade (Tavares et al., 2006; Wright et al., 2008; Schweizer et al., 2010; Joseph et al., 2012). Other well-supported parrot clades consistently recovered across various studies include the Neotropical parrots (Arinae), the African Psittacinae, the Australasian Psittaculidae and the Platycercinae from Australia, New Zealand, Oceania and Africa (de Kloet and de Kloet, 2005; Juniper and Parr, 1998; Tavares et al., 2006; Wright et al., 2008; Schweizer et al., 2010; Joseph et al., 2012). In agreement with previous studies by Tavares et al. (2006) and Wright et al. (2008), three Neotropical clades were recovered here: the parrotlets, including *Bolborhynchus*, *Nannopsittaca*, *Touit*, and *Psilopsiagon*; amazons and allies, including *Amazona*, *Pionus*, *Pyrilia*, *Triclarina* and *Graydidascalus*; and macaws and allies, including *Ara*, *Cyanopsitta*, *Aratinga*, *Orthopsittaca*, *Pyrrhura*, *Pionites* and *Anodorhynchus*.

4.2. Mitochondrial gene order in parrots

Among the 114 parrot species for which data was available there is evidence for two of the four described avian mitochondrial gene orders. The typical avian mitochondrial gene order originally described by Desjardins and Morais (1990) was inferred for 76 species by PCR, sequencing and examination of GenBank sequences. In contrast, duplicate control regions and the gene order described in *Amazona* parrots by Eberhard et al. (2001) was found in 38 of the species surveyed and/or sequenced. In this genome arrangement, a non-coding region, that in some species has apparent similarity to ND6 and *tRNA^{Glu}*, is located between *tRNA^{Thr}* and the first control region. Two species, *Derophtus accipitrinus* and *Pionites leucogaster*, which was not in the phylogeny, were shown by sequencing

to have a variant of the *Amazona* gene order, with a functional *tRNA^{Pro}* located between *tRNA^{Thr}* and the first control region.

4.3. Evolutionary patterns of mtDNA control region duplications

Three striking patterns are apparent in our reconstructions of the evolution of control region duplications and sequencing of duplicate control regions in parrots. First, the mapping of control region duplication states onto the phylogeny of parrots identifies at least six independent origins of the control region duplications. Second, there were no reversions to a single control region state in any of these six clades. Third, although there was considerable interspecific variation, levels of sequence similarity between duplicated control regions within an individual were typically high (89–100% similarity in the species examined), at least for the first 400–500 nucleotides of domain I that was examined. This similarity would appear to be unusual due to the fact that this segment of the control region is often very different between species with many small insertions, deletions and mutations that are thought to occur as a result of the D-loop being single-stranded and accessible to mutagenic agents, such as reactive oxygen species (Shokolenko et al., 2007).

One hypothesis to explain these patterns is that a duplication of the control region and neighboring sequences occurred in the ancestors of these six clades and was retained in all descendent species of each clade. This hypothesis begs the question of what maintains the generally high degree of sequence similarity between the duplicated control regions within each taxon. The within-individual divergences that we observed between control region copies of 0–10.9% (Table 3) fall within the range of divergences observed in other taxa with duplicated control regions such as *Amazona* parrots, albatrosses, killifish, snakes, ticks, and ostracods (Abbott et al., 2005; Campbell and Barker, 1999; Eberhard et al., 2001; Kumazawa et al., 1996, 1998; Lee et al., 2001; Ogoh and Ohmiya, 2007; Tatarenkov and Avise, 2007). A high degree of sequence similarity is often interpreted as evidence for the maintenance of function in both duplicated control regions. Portions of the control region are under selection for the ability to bind with nuclear-encoded replication factors, and for functional control of replication and transcription (Doda et al., 1981; Gensler et al., 2001; He et al., 2007; Lee and Clayton, 1998; Schultz et al., 1998; Shadel and Clayton, 1997); such functionality may provide stabilizing selection on duplicated control regions. Alternatively, several studies of organisms with a duplicated control region have explained the high degree of similarity between the two control regions as evidence of gene conversion (Eberhard et al., 2001; Kumazawa et al., 1996, 1998; Ogoh and Ohmiya, 2007; Tatarenkov and Avise, 2007; Verkuil et al., 2010), but the molecular mechanisms responsible remain unclear. In either case, the maintenance of duplicated control regions concurrent with the elimination of duplicated mitochondrial genes and tRNAs suggests an advantage to having a second control region that overrides selection for compactness. Potential advantages include faster replication (Kumazawa et al., 1996) or protection against age-related deterioration of mitochondrial function (T.F. Wright and J.R. Eberhard, unpublished data).

An alternative hypothesis for the observed patterns is that a propensity for duplications to occur such as through replication slippage due to secondary structure in the control region was present in the common ancestor of each of the six clades, leading to repeated duplications of the region from cytochrome *b* to the control region with lineage specific degradation and deletion events passing to each of the descendent taxa (Verkuil et al., 2010; Zhuang and Cheng, 2010). Zhuang and Cheng (2010) found a similar pattern within *Notothenioid* fish and suggested that within each clade with control region duplications if these mutations are neutral, each descendant

Table 3
Gene order, non-coding region length and percent control region differences for parrot species with a duplicated control region.

Taxa	Source	Clade ^a	Gene order	Non-coding region length ^b	%CR differences ^c
<i>Amazona albifrons</i>	NMSU	A	Amazona	141	0.6
<i>Amazona farinosa</i>	AF338821	A	Amazona	158	0
<i>Amazona ochrocephala</i>	NMSU	A	Amazona	127	2.1
<i>Pyrilia caica</i>	NMSU	A	Amazona	155	0
<i>Pyrilia barrabandi</i>	BR/NMSU	A	Amazona	160	6
<i>Pionus chalcopterus</i>	NMSU/AF338817-18	A	Amazona	180	0.2
<i>Triclaria malachitacea</i>	BR	A	Amazona	112	2.5
<i>Derophtus accipitrinus</i>	BR	B	Variant A	319	4.7
<i>Pionites leucogaster</i>	BR	B	Variant A	325	10.9
<i>Forpus xanthopterygius</i>	BR	C	Amazona	129	0.2
<i>Forpus sclateri</i>	NMSU	C	Amazona	117	5
<i>Poicephalus robustus</i>	NMSU	D	Amazona	129	7.7
<i>Psittacus erithacus</i>	NMSU	D	Amazona	88	10.8
<i>Prioniturus montanus</i>	NMSU	E	Amazona	360	1.9
<i>Chalcopsitta cardinalis</i>	NMSU	F	Amazona	84	7.9
<i>Charmosyna placentis</i>	NMSU	F	Amazona	45	2.7
<i>Eos histrio</i>	NMSU	F	Amazona	84	2.3
<i>Glossopsitta porphyrocephala</i>	NMSU	F	Amazona	68	3.3
<i>Lorius albidinucha</i>	NMSU	F	Amazona	73	3.3
<i>Melopsittacus undulatus</i>	NC009134	F	Amazona	58	4.6
<i>Neopsittacus musschenbroekii</i>	NMSU	F	Amazona	88	3.3
<i>Phigys solitarius</i>	NMSU	F	Amazona	32	2.7
<i>Psitteuteles goldiei</i>	NMSU	F	Amazona	81	2.6
<i>Pseudeos fuscata</i>	NMSU	F	Amazona	71	3.5
<i>Trichoglossus haematodus</i>	NMSU	F	Amazona	86	3.3
<i>Vini australis</i>	NMSU	F	Amazona	58	3.5
<i>Cyclopsitta diophthalma</i>	NMSU	F	Amazona	62	5.1
<i>Psittaculirostris edwardsii</i>	NMSU	F	Amazona	60	5.5

^a Clades labeled in Fig. 5.
^b Non-coding region length was calculated as the number of nucleotides from the 3' end of tRNA^{THR} to the 5' end of the goose hairpin.
^c Percent control region differences was calculated as the number of nucleotides that differed between the 2 aligned control regions divided by the total number of overlapping nucleotides multiplied by 100. Differences were only counted if they occurred 3' of the goose hairpin.

species of a specific ancestor should have approximately the same amount of divergence between its two control regions. Table 3 shows that while species within the Australasian clade (labeled F in Fig. 5) exhibit similar levels of divergence between their duplicated control regions, the other clades show considerable variation among member species in the degree of divergence exhibited between their two control regions. Further investigation into patterns of sequence divergence over the entire length of the duplicated control region, coupled with functional studies of mitochondrial replication in species with and without control region duplications, should help distinguish between these alternative hypotheses for the evolution of duplicate control regions.

5. Conclusions

The presence of multiple mitochondrial gene orders within Psittaciformes supports the idea that the avian mitochondrial genome is a dynamic molecule. This study has shown that mitochondrial control region duplications have occurred many times in parrots, with ancestral state reconstructions suggesting six independent origins of the duplicated control region state and no reversions to a single control region state. Further investigations into the fates of duplicated mitochondrial genes, the potential costs and advantages of having a second control region, and the complex relationship between evolutionary rates, selection and time since duplication are needed to fully explain these patterns in the mitochondrial genome.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2012.04.009>.

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Anexo V

Phylogeny and biogeography of Yellow-headed and Blue-fronted Parrots (*Amazona ochrocephala* and *Amazona aestiva*) with special reference to the South American taxa

CAMILA C. RIBAS,¹* ERIKA S. TAVARES,² CELINA YOSHIHARA³ & CRISTINA Y. MIYAKI³

¹Department of Ornithology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

²Department of Natural History, Royal Ontario Museum, 100 Queen's Park, M5S 2C6, Toronto, ON, Canada

³Instituto de Biociências, Universidade de São Paulo, R. do Matão, 277, 05508-090, São Paulo, SP, Brazil

The Yellow-headed Parrot (*Amazona ochrocephala*) has a broad Neotropical distribution, ranging from Mexico to the Amazon Basin, and a history of complex taxonomy and controversial species limits. Recent molecular analyses have started to clarify the taxonomic arrangement of the complex, but have not included a representative geographical sampling from South America. These studies have shown that the Yellow-headed complex can be divided into three main lineages, and seems to be paraphyletic, due to the inclusion of the Blue-fronted Parrot (*Amazona aestiva*) that occurs in central South America. Here we present a phylogenetic analysis based on mitochondrial DNA sequences of 45 representatives of the Yellow-headed complex from South and Central America, plus 13 Blue-fronted individuals from different localities in South America. Our analyses recover the three primary lineages found previously in the Yellow-headed complex, show that there is genetic structure in the South American lineage, which can be divided into two well-supported, closely related clades, and demonstrate that Blue-fronted samples are distributed in both clades. Differentiation of South American Blue-fronted and Yellow-headed Parrot populations does not correspond to the plumage differences used to distinguish the Blue-fronted Parrot from the Yellow-headed Parrot, nor to plumage differences used to distinguish among South American Yellow-headed subspecies. This suggests that traditional taxonomy based on plumage characters needs revision, and that this may be an interesting example of ongoing divergence-with-gene-flow related to the forest/open area ecotone in southern Amazonia.

The Neotropical biota encompasses a high diversity of species with common patterns of endemism (Haffer 1969, Cracraft 1985). The assemblage of this complex biota may be better understood through the study of the patterns of diversification of its component taxa (Prance 1982). Due to their patterns of diversity and distribution (Forshaw 1989, Juniper & Parr 1998), the study of the evolutionary history of Neotropical parrots may be particularly informative for understanding the biogeographical history of this region. Recent molecular studies of this group suggest that the current taxonomic arrangement often does

not reflect phylogenetic relationships (Eberhard & Bermingham 2004, Ribas & Miyaki 2004, Russello & Amato 2004, Tavares *et al.* 2004, Ribas *et al.* 2005, 2006). A good correspondence between phylogenetic history and systematics is needed in order to reveal biogeographical patterns, as well as for conservation purposes, as parrots are among the most threatened birds in the Neotropics.

Russello and Amato (2004) presented a phylogenetic hypothesis for the genus *Amazona* based on mitochondrial and nuclear data. Although their phylogeny corroborated the majority of the traditional taxonomic arrangement of the group, an important exception was among members of the Yellow-headed species complex. This complex occurs in lowlands

*Corresponding author.
Email: ribas@amnh.org

from Mexico to the Amazon Basin and is considered by some authors as comprising only one species (*A. ochrocephala*) with nine (Forshaw 1989) or ten (Collar 1997) subspecies, defined on the basis of the distribution of yellow on the head and bill coloration. Other authors recognize three species and 11 subspecies in the complex: *A. ochrocephala* (includes the three South American subspecies – *ochrocephala*, *xantholaema* and *nattereri* – plus *panamensis* from Central America), *A. auropalliata* (with three Central American subspecies – *auropalliata*, *parvipes* and *caribaea*), and *A. oratrix* (composed of the other four Central American subspecies – *oratrix*, *tresmariae*, *belizensis* and *hondurensis*) (AOU 1998, Juniper & Parr 1998). Russello and Amato's (2004) phylogenetic results did not corroborate the subdivision of the complex into the three proposed species, but rather suggested paraphyly of the Yellow-headed complex as a result of *A. aestiva* and *A. barbadensis* being nested within it. Their findings strongly suggested that the current taxonomy is in need of revision.

Eberhard and Bermingham (2004) presented a more detailed study of the Yellow-headed complex by including five Central American and three South American subspecies. They found that the complex is divided into three primary lineages that disagree with the arrangements previously proposed. The 'Central American' lineage comprises all Central American taxa, the 'Northern South American' lineage comprises *A. o. ochrocephala* from Colombia and Venezuela, and the 'South American' lineage comprises all other South American samples (including *A. o. ochrocephala* from Brazil). Like Russello and Amato (2004), they found that the Blue-fronted Parrot (*A. aestiva*) is nested within the Yellow-headed complex, grouping with the South American lineage, whereas the Yellow-shouldered Parrot (*A. barbadensis*) appears to be the sister group to the complex. According to their results there is strong phylogenetic structure in the Central American lineage contrasting with a lack of structure in the South American lineage.

Although Eberhard and Bermingham's (2004) sampling of Central American taxa was quite complete, sampling of South American taxa was sparse: they included in their analyses individuals from only four localities representing a lineage that is distributed across the whole Amazon Basin. Furthermore, only one Blue-fronted Parrot individual was included, without information with respect to its geographical origin.

Here we present a molecular phylogenetic analysis including 22 representatives of the South American lineage of the Yellow-headed complex, one representative of the Northern South American lineage, 22 representatives of the Central American lineage, plus 13 Blue-fronted Parrot individuals from different localities, with the objective of better understanding the geographical patterns of diversity in this group. We use the resulting phylogeny to test: (1) if the lack of structure among South American taxa proposed by Eberhard and Bermingham (2004) holds even when more individuals are included in the analyses; (2) if the Amazon River may be an important barrier separating the South American from the Northern South American lineage, as proposed by Eberhard and Bermingham (2004); and (3) how Blue-fronted Parrot individuals from different localities relate to the Yellow-headed Parrot complex.

METHODS

Taxon sampling

Blood samples were obtained from 12 individual Blue-fronted Parrots and 16 South American Yellow-headed Parrots, all with known geographical origins (Appendix 1). Sequences of the following were obtained from GenBank (Appendix 1; Eberhard & Bermingham 2004): one Blue-fronted and seven South American Yellow-headed individuals; of individuals representing all the Central American taxa belonging to the Yellow-headed complex; and of the outgroups Orange-winged (*Amazona amazonica*), Red-lore (*Amazona autumnalis*) and Mealy (*Amazona farinosa*) Parrots.

DNA extraction and sequencing

DNA was extracted from blood samples through incubation overnight at 55 °C in a solution containing 0.1% SDS, 100 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA and 10 mg/mL proteinase K, and subsequently purified using the standard phenol-chloroform-isoamyl alcohol method (Bruford *et al.* 1992).

The primers used for amplification and sequencing of segments of four mitochondrial genes were: LMet (5'-GGCCCATACCCCGAAAATGA-3'; J. Groth pers. comm.) and H5766 (5'-GAGAAGCTAG-GATTTTTCGTG-3'; P. Brito pers. comm.) for NADH Dehydrogenase Subunit 2 (ND2); CO2GQL (Eberhard & Bermingham 2004) and CO3HMH

(Eberhard & Bermingham 2004) for a segment including the ATPase 8 and ATPase 6 genes, and COIF (Palumbi 1996) and COIA (Palumbi 1996) for Cytochrome Oxidase Subunit I (COI).

PCR amplifications were performed in 25 μ L reactions with 1 $\times$ buffer, 40 μ M of each dNTP, 0.2 μ M of each primer, 1 U *Taq* polymerase (GE Healthcare) and 25–50 ng of DNA. The thermocycling procedure was an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 53–55 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 7 min. A touchdown with the same incubation times was applied to amplify the ATPases 8 and 6 segment, with the annealing temperature dropping from 65 to 55 °C, one degree per cycle, followed by 23 cycles at 55 °C. PCR products were purified by incubation with 5 U of exonuclease I (USB), and 0.5 U of shrimp alkaline phosphatase (USB) at 37 °C for 1 h, followed by 10 min at 80 °C. The purified products were used as templates in sequencing reactions performed with Big Dye Terminator chemistry (Applied Biosystems) and the same primers used for the PCR amplifications. Ethanol precipitation was performed and the sequences were obtained either on an ABI377 or on an ABI3100 automated DNA sequencer (Applied Biosystems).

Sequence alignment and analysis

Sequences were assembled and checked for ambiguities using Sequencher 4.1.2 (GeneCodes Corp., Ann Arbor, MI, USA). Alignments were verified visually in MacClade 4.0 (Maddison & Maddison 2005). Base composition and transition/transversion rates were calculated in PAUP* 4.0b10 (Swofford 2002). The best-fit models of nucleotide evolution for each partition and for the combined dataset were determined through a hierarchical likelihood-ratio test in Modeltest 3.5 (Posada & Crandall 1998). Corrected genetic distances and standard errors (based on 500 bootstrap replicates) were calculated using MEGA 3.1 (Kumar *et al.* 2004). The Partition-Homogeneity Test was conducted in PAUP* to check for evidence of any conflicting phylogenetic signal among the four gene regions.

Phylogenetic tree reconstruction under maximum parsimony (MP) was performed using PAUP* 4.0b10 (Swofford 2002) for each gene independently and for the combined dataset using heuristic tree search, and tree-bisection-reconnection branch-swapping algorithm with 100 random addition sequence

replicates. All characters were equally weighted. The support for each node was estimated using 1000 bootstrap replicates.

Bayesian analysis (BI) with Markov Chain Monte Carlo (MCMC) sampling was performed in MRBAYES 3.1 (Ronquist & Huelsenbeck 2003) for the combined dataset using a partitioned likelihood approach (one partition for each gene), in which parameters were estimated separately for each data partition (*nst* = 6, *rates* = gamma). Two independent runs were executed, each for ten million generations, with one cold and four heated chains, sampling once every 1000 generations and with a burn-in time determined by the time to convergence of the likelihood scores. The posterior probabilities of each node were computed by combining the trees sampled (after burn-in, a total of 9000 trees from each run) in both runs. Another run was performed with the same parameters, but for only two million generations, unlinking the topologies of the four partitions to test if the four genes recovered the same clades that were recovered in the combined analysis.

RESULTS

Sequences of the genes ND2 (533 bp), COI (513 bp) and ATPase 6 and 8 (764 bp) were obtained from 28 individuals (Appendix 1 and Table 1). There were no indels or stop codons, and the base composition was typical of mtDNA for all regions (Table 1). When our data were analysed together with the sequences obtained from GenBank, ND2 had the highest number of informative characters for the ingroup, but for the South American taxa alone, ATPase 6 had the highest number of informative characters (Table 1). Most variation was at third codon positions in all gene regions, and the transition/transversion rate varied from 10.5 to 29.7 for the ingroup (Table 1).

Independent analysis of each gene region using both MP and BI methods recovered trees similar to those obtained in the combined analysis, but with less resolution. The Partition-Homogeneity Test failed to detect any conflict in the phylogenetic information among the four datasets (*P* = 0.72). Based on these results all subsequent analyses were conducted using the combined dataset, consisting of a matrix with 61 taxa (including three outgroups) and 1820 bp.

MP analysis recovered ten trees of 399 steps (consistency index = 0.8, retention index = 0.9). The trees differed only in the relationships among individuals within well-supported clades. The strict

Table 1. Number of base pairs sequenced, variable and informative sites, base composition and Ti/Tv ratios for each gene region analysed.

Gene	ND2	COI	ATPase 6	ATPase 8
No. of base pairs sequenced	533	513	606	168
<i>All ochrocephala/aestiva</i>				
Variable	49	31	44	12
Informative	35	19	30	8
%A	34.1	26.6	34.3	24.4
%C	35.7	28.8	38.2	36.9
%G	10.3	19.0	6.2	11.3
%T	19.9	25.6	21.3	27.4
Ti/Tv ratio	14.1	29.7	20.2	10.5
<i>South American clade only</i>				
Variable	13	12	21	5
Informative	9	7	13	1
%A	34.3	26.7	34.2	24.2
%C	35.7	28.8	38.1	36.9
%G	10.1	18.9	6.2	11.5
%T	19.9	25.6	21.5	27.4
Ti/Tv ratio	10.9	18.8	8.9	N/A

consensus of all MP trees resulted in a topology almost identical to the BI topology (Fig. 1), differing only in the position of some terminal taxa within the two South American clades.

Both analyses recovered the three primary lineages found previously by Eberhard and Bermingham (2004): the Central American (CA), South American (SA) and Northern South American lineages (NSA) (Fig. 1). The SA lineage is sister to the CA lineage with good support in both analyses (84% bootstrap support and posterior probability of 100). Within the CA lineage, several strongly supported groups were recovered (bootstrap values ranging from 91 to 100%, posterior probability values of 100), and the relationships among them are resolved (Fig. 1) and agree with the relationships found previously by Eberhard and Bermingham (2004). All Blue-fronted Parrot samples were recovered within the SA lineage, which was divided into two well-supported clades: clade 1, comprising *A. o. xantholaema*, *A. a. aestiva* from Miranda and Chapada Gaúcha (central Brazil), and *A. o. nattereri* from Santa Cruz (western Bolivia) (Appendix 1, Fig. 1), and clade 2, comprising *A. o. nattereri* from Acre (western Amazonia), Pando (northern Bolivia), Jacareacanga (southern Amazonia) and Beni (western Bolivia), *A. a. aestiva* from Miranda and Chapada Gaúcha, *A. a. xanthopteryx* and *A. o. ochrocephala* (Appendix 1, Fig. 1). These two clades were recovered with high support in the combined analyses (bootstrap support of 91 and 100%, for clades 1 and 2, respectively, and posterior

probabilities of 100) and were also recovered in the analyses of each gene independently. In the molecular data matrix, five diagnostic characters were sampled for clade 1, and six for clade 2. Within clade 2, there are four diagnostic characters for the clade containing individuals from Macapá. Corrected genetic distances (using the Tamura–Nei model, as selected by the hierarchical likelihood-ratio tests performed in Modeltest for the ingroup only) between the two SA clades were lower (0.9%) than the genetic distances between the two SA clades and the CA clade (2.1%; Table 2). The only representative of the NSA lineage was 2.3 and 2.2% divergent compared with the SA and CA clades, respectively (Table 2).

DISCUSSION

Our results corroborate the division of the Yellow-headed Parrot complex into three independent evolutionary lineages (Eberhard & Bermingham 2004, Russello & Amato 2004). Whereas Eberhard and Bermingham (2004) recovered a polytomy uniting these three lineages, in our analyses the SA lineage is sister to the CA lineage with relatively good support (bootstrap of 84% and posterior probability of 100), leaving the only representative of the NSA lineage in a basal position.

The NSA lineage, represented in our study by the Colombian sample, occurs between the ranges of the SA and CA lineages (Fig. 2). Eberhard and

Figure 1. Topology derived from Bayesian analyses based on 1820 bp of mitochondrial sequences. Numbers on the nodes are maximum parsimony bootstrap percentages and Bayesian posterior probabilities, respectively. *Indicates posterior probability smaller than 85%. Clades 1 and 2 are indicated by vertical lines. South American (SA), Central American (CA) and Northern South American (NSA) lineages are indicated by bold branches. Voucher numbers and locality codes are indicated after each taxon name (see Fig. 2 and Appendix 1).

Table 2. Genetic distances between and within groups.

	Between groups					Within groups		
	SA1 × SA2	SA1 × CA	SA2 × CA	NSA × SA	NSA × CA	SA1	SA2	CA
All genes	0.9 (0.2)	2.1 (0.3)	2.1 (0.3)	2.3 (0.3)	2.2 (0.3)	0.2 (0.0)	0.4 (0.1)	1.0 (0.2)
ND2	0.8 (0.3)	2.3 (0.5)	2.4 (0.5)	1.7 (0.5)	2.6 (0.6)	0.2 (0.1)	0.5 (0.2)	1.8 (0.3)
COI	0.6 (0.3)	2.0 (0.5)	2.0 (0.5)	2.7 (0.7)	2.0 (0.6)	0.2 (0.1)	0.3 (0.1)	0.7 (0.2)
ATPase 6/8	1.2 (0.3)	2.0 (0.4)	2.0 (0.4)	2.5 (0.5)	2.0 (0.5)	0.2 (0.1)	0.4 (0.1)	0.8 (0.2)

Values are percentage Tamura–Nei distances (standard errors, 500 bootstrap replicates). SA, South American lineage; SA1, South American clade 1; SA2, South American clade 2; CA, Central American lineage; NSA, Northern South American lineage.

Figure 2. Collection localities of South American samples. Two letter codes correspond to localities specified in Appendix 1 and Figure 1. Different circles refer to clades 1 and 2, and to the NSA lineage (see Fig. 1). The grey line delimits the distribution of Yellow-headed Parrot (*Amazona ochrocephala*). The black line delimits the distribution of Blue-fronted Parrots (*Amazona aestiva*). Distributions of subspecies are indicated with their corresponding names.

Bermingham (2004) obtained sequences of the COI gene from an additional skin from Colombia and two from Venezuela. These individuals group with the Colombian sample included in our study. This suggests an ancestral distribution of the group in northern South America with subsequent diversification to the north and to the south, with different patterns of diversification in each direction. This is consistent with *A. o. panamensis* being basal in the CA clade. Interestingly, the CA lineage shows greater geographical mtDNA differentiation than the SA lineage, despite

the larger area occupied by the latter, and the patterns of clear geographical differentiation found for other groups of Amazonian parrots (Ribas *et al.* 2005, 2006).

According to Russello and Amato (2004) and Eberhard and Bermingham (2004), the most closely related taxa to the Yellow-headed Parrot complex are *A. aurasiaca*, *A. versicolor* and *A. barbadensis*. Samples from these taxa were not available for the present study. Thus, it is important to note that although the basal nodes in our phylogeny are well

supported, the outgroups used are not the most closely related taxa to the ingroup. More closely related outgroups and additional individuals representing the NSA lineage will have to be studied in order to understand better its phylogenetic relationships and evolutionary origins.

Contrary to previous suggestions (Eberhard & Bermingham 2004) our results show a subdivision of the SA lineage into two clades. Despite the small genetic distances within the SA lineage, the two clades are well supported in all analyses with several diagnostic characters defining each one, and do not correspond to the subspecies previously recognized based on plumage coloration.

The individuals from Macapá (eastern Amazon Basin, north of the Amazon River, Fig. 2) form a well-supported clade, which is included in SA clade 2, whereas *A. o. xantholaema* from the neighbouring Marajó Island are all included in SA clade 1. This result suggests that the Amazon River may act as a barrier within the SA lineage, but is not the northern limit of the distribution of the SA lineage, as proposed by Eberhard and Bermingham (2004). More detailed sampling is needed in northern Amazonia in order to understand the geographical limits of the NSA and SA lineages.

South American clade 1 is mainly composed of individuals that occur on Marajó Island. This may indicate that these individuals originated recently from different populations on the mainland, or as a consequence of past isolation of a population on the island and subsequent introgression with mainland populations. However, this second scenario is less likely due to the very low genetic distances between SA clades 1 and 2 and marine introgressions that have probably affected Marajó island in the recent past (Klammer 1984, Marroig & Cerqueira 1997, Nores 1999). This implies that the genetic break between the two clades is a result of some other isolation event unrelated to isolation on the island. The mean genetic distance between the two clades is approximately 1%. If the evolutionary rate of 2% divergence per million years (Shields & Wilson 1987, Randi 1996) is adopted, this differentiation occurred about 500 000 years ago in the mid-Pleistocene. Glacial cycles are thought to have affected the distribution of habitats in South America during the Pleistocene (Haffer 1969, Bush 1994), and this genetic discontinuity could have originated as a consequence of barriers that are not evident today.

The lack of reciprocal monophyly between Yellow-headed and Blue-fronted Parrots has been suggested

in previous studies (Eberhard & Bermingham 2004, Russello & Amato 2004), but neither of these studies included sufficient samples of Blue-fronted Parrots to be able to detect the complex pattern that we observed here. Blue-fronted individuals from two different localities have haplotypes that are included within both clades of the SA lineage (Figs 1 & 2).

Species-level polyphyly may occur due to a number of different causes, but can be divided into two main kinds: misinterpretation of the morphological variation that gave rise to the delimitation of the species, or population-level phenomena such as incomplete lineage sorting or introgression (Funk & Omland 2003). Taxonomy of Blue-fronted and Yellow-headed Parrots is based mainly on the colour patterns of the head. In the Yellow-headed Parrot the amount of yellow on the head is the primary character used to identify the subspecies (Forshaw 1989, Lousada & Howell 1996, Juniper & Parr 1998). Some authors have reported that the amount of yellow decreases from northwest to southeast throughout the distribution range (Lousada & Howell 1996). The primary character that distinguishes the Yellow-headed Parrot from the Blue-fronted Parrot is that the latter has blue on the forehead, although the extent of the blue patch varies among individuals even within the same locality (G. Seixas pers. comm.). There are two subspecies described for the Blue-fronted Parrot: *Amazona aestiva aestiva* and *Amazona aestiva xanthopteryx*, the primary difference between them being the coloration of the shoulder, which changes from red in eastern populations to yellow in western populations, but this character also seems to vary among individuals at any particular locality (Darrieu 1983, Forshaw 1989).

In addition to the plumage characters, Blue-fronted and Yellow-headed Parrots are considered to be mostly allopatric, with just a small region of sympatry in western Brazil and northeastern Bolivia (Fig. 2). All Blue-fronted samples included in our analyses are from regions that are outside the traditionally recognized range of Yellow-headed Parrots, so that the observed mixture of haplotypes cannot be attributed to misidentification of specimens. The pattern recovered may occur because traditional taxonomy does not reflect the evolution of the group. This would mean that variation in plumage may be the result of changes related to habitat differences that are not reflected among the mtDNA lineages, or that the current interpretation of plumage characters should be reviewed, as they are highly variable in this group of parrots.

Another possibility is that the pattern recovered (gene tree) is different from the species tree due to phenomena such as introgression or incomplete lineage sorting. To test this possibility, it would be necessary to obtain sequences from nuclear genes that are inherited independently from the mitochondrial genome. The problem with this approach is the very low level of variation in nuclear genes among these closely related taxa. Eberhard and Bermingham (2004) generated a nuclear DNA (Intron XI of GADPH, 404 bp) phylogeny including one Blue-fronted and four Yellow-headed individuals. The four Yellow-headed individuals form a clade with low support (62% bootstrap support in a maximum parsimony analysis) that excludes the Blue-fronted individual, but the GADPH dataset had very low levels of variation (Eberhard & Bermingham 2004). By contrast, Russello and Amato (2004) used both mitochondrial (COI, 12S rRNA and 16S rRNA) and nuclear (β -fibrinogen Intron 7, Ribosomal Protein 40 Intron 5, and Tropomyosin α -subunit Intron 5) sequences, and in their analysis the Blue-fronted Parrot was placed within the Yellow-headed Parrot clade, even when only nuclear sequences were included. In addition, the fact that Blue-fronted individuals occur in both SA clades in our mtDNA analysis points to a genetic structure in the group that is different from the one that would be expected from morphological variation.

It is interesting to note that, whereas both Yellow-headed and Blue-fronted Parrots occupy open areas adjacent to wooded habitats, South American Yellow-headed Parrots occur in the Amazon Basin, while Blue-fronted Parrots occur in more dry and open habitats, including Cerrado, Caatinga and Chaco. If their plumage differences do reflect incipient differentiation that is not yet detectable in the mtDNA, this may be one interesting example of ongoing divergence-with-gene-flow related to the forest/open areas ecotone in southern Amazonia (Rice & Hostert 1993, Smith *et al.* 1997, Ogden & Thorpe 2002). A morphological revision of the group is needed in order to understand better the variation in plumage patterns that diagnose the two taxa.

Eberhard and Bermingham (2004) proposed two possible taxonomic arrangements for South American Yellow-headed Parrots: (1) to recognize two species, *A. ochrocephala*, including *ochrocephala* from northern South America (corresponding to the NSA lineage), and *A. nattereri*, including *ochrocephala* from Amazonia, *nattereri* and *xantholaema* (corresponding to the SA lineage); or (2) to recognize only one

species in South America, *A. ochrocephala*. The second alternative would not correspond to the phylogenetic relationships presented here, as the NSA and SA lineages are not each other's closest relatives. Our data support recognizing *ochrocephala* from Colombia and Venezuela as a separate species, *A. ochrocephala*, but the limits of its geographical distribution remain to be determined. The situation in the SA lineage is more complicated, as the three traditionally recognized subspecies are not recovered in the analysis, and are not monophyletic relative to *A. aestiva*. Additional studies are needed to determine what taxonomic arrangement should be adopted.

This study is another example of a common problem that is becoming increasingly evident with the growing number of molecular phylogenetic studies in birds: the taxonomic delimitation of taxa has to be carefully reviewed before any conclusions can be drawn with respect to biogeographical history or population dynamics. This indicates that it is not advisable to assume that nominal species or subspecies are monophyletic, and phylogenetic and phylogeographical studies must always include as many representatives of closely related taxa as possible.

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Appendix 1. Taxa sampled, sample locality, codes of the localities within South America, voucher numbers and GenBank accession numbers.

Taxon	Locality	Locality code	Institution/ voucher	ND2	ATP8	ATP6	COI
<i>A. a. aestiva</i>	Chapada Gaúcha, MG, Brazil	CG	USP 4057	DQ453646	DQ453674	DQ453674	DQ453618
<i>A. a. aestiva</i>	Chapada Gaúcha, MG, Brazil	CG	USP 4055	DQ453648	DQ453676	DQ453676	DQ453620
<i>A. a. aestiva</i>	Chapada Gaúcha, MG, Brazil	CG	USP 4056	DQ453650	DQ453678	DQ453678	DQ453622
<i>A. a. aestiva</i>	Chapada Gaúcha, MG, Brazil	CG	USP 4052	DQ453647	DQ453675	DQ453675	DQ453619
<i>A. a. aestiva</i>	Chapada Gaúcha, MG, Brazil	CG	USP 4053	DQ453657	DQ453685	DQ453685	DQ453621
<i>A. a. aestiva</i>	Miranda, MS, Brazil	MI	USP 2180	DQ453639	DQ453667	DQ453667	DQ453611
<i>A. a. aestiva</i>	Miranda, MS, Brazil	MI	USP 2183	DQ453636	DQ453664	DQ453664	DQ453608
<i>A. a. aestiva</i>	Miranda, MS, Brazil	MI	USP 2186	DQ453638	DQ453666	DQ453666	DQ453610
<i>A. a. aestiva</i>	Miranda, MS, Brazil	MI	USP 2189	DQ453645	DQ453673	DQ453673	DQ453617
<i>A. a. aestiva</i>	Miranda, MS, Brazil	MI	USP 2192	DQ453656	DQ453684	DQ453684	DQ453628
<i>A. aestiva</i>	Captive		STR1-x-97*	AY194434	AY194328	AY194295	AY194367
<i>A. a. xanthopteryx</i>	Vila Bela da Santíssima Trindade, MT, Brazil	VB	USP 1319	DQ453652	DQ453680	DQ453680	DQ453624
<i>A. a. xanthopteryx</i>	Vila Bela da Santíssima Trindade, MT, Brazil	VB	USP 1320	DQ453643	DQ453671	DQ453671	DQ453615
<i>A. o. ochrocephala</i>	Macapá, AP, Brazil	MA	USP 1556	DQ453659	DQ453687	DQ453687	DQ453631
<i>A. o. ochrocephala</i>	Macapá, AP, Brazil	MA	USP 1563	DQ453649	DQ453677	DQ453677	DQ453621
<i>A. o. ochrocephala</i>	Macapá, AP, Brazil	MA	USP 1565	DQ453653	DQ453681	DQ453681	DQ453625
<i>A. o. ochrocephala</i>	Macapá, AP, Brazil	MA	USP 1572	DQ453635	DQ453663	DQ453663	DQ453607
<i>A. o. ochrocephala</i>	Rio Xingú, Altamira, PA, Brazil	AL	NMNH B06867*	AY194435	AY194329	AY194296	AY194368
<i>A. o. ochrocephala</i>	Rio Xingú, Altamira, PA, Brazil	AL	NMNH B07034*	AY194436	AY194330	AY194297	AY194369
<i>A. o. ochrocephala</i>	Carimagua, Colombia	CA	STR1-x-61*	AY194460	AY194354	AY194321	AY194393
<i>A. o. xantholaema</i>	Ilha do Marajó, PA, Brazil	IM	USP 1587	DQ453640	DQ453668	DQ453668	DQ453612
<i>A. o. xantholaema</i>	Ilha do Marajó, PA, Brazil	IM	USP 1589	DQ453655	DQ453683	DQ453683	DQ453627
<i>A. o. xantholaema</i>	Ilha do Marajó, PA, Brazil	IM	USP 1590	DQ453632	DQ453660	DQ453660	DQ453604
<i>A. o. xantholaema</i>	Ilha do Marajó, PA, Brazil	IM	USP 1031	DQ453651	DQ453679	DQ453679	DQ453623
<i>A. o. xantholaema</i>	Ilha do Marajó, PA, Brazil	IM	USP 1042	DQ453654	DQ453682	DQ453682	DQ453626
<i>A. o. xantholaema</i>	Captive		STR1-LP1*	AY194445	AY194339	AY194306	AY194378
<i>A. o. nattereri</i>	Jacareacanga, PA, Brazil	JA	USP 2951	DQ453642	DQ453670	DQ453670	DQ453614
<i>A. o. nattereri</i>	Rio Acre, Xapuri, AC, Brazil	AC	USP 2078	DQ453637	DQ453665	DQ453665	DQ453609
<i>A. o. nattereri</i>	Rio Iaco, AC, Brazil	AC	USP 2068	DQ453644	DQ453672	DQ453672	DQ453616
<i>A. o. nattereri</i>	Rio Acre, Brasília, AC, Brazil	AC	USP 2074	DQ453641	DQ453669	DQ453669	DQ453613
<i>A. o. nattereri</i>	Assis, AC, Brazil	AC	USP 2076	DQ453634	DQ453662	DQ453662	DQ453606
<i>A. o. nattereri</i>	Rio Itimari, AC, Brazil	AC	USP 2084	DQ453633	DQ453661	DQ453661	DQ453605
<i>A. o. nattereri</i>	Pando Department, Bolivia	PA	USP 2075	DQ453658	DQ453686	DQ453686	DQ453630
<i>A. o. nattereri</i>	Pando Department, Bolivia	PA	LSU B9409*	AY194439	AY194333	AY194300	AY194372
<i>A. o. nattereri</i>	Santa Cruz Department, Bolivia	SC	LSU B12973*	AY194437	AY194331	AY194298	AY194370
<i>A. o. nattereri</i>	Beni, Bolivia	BE	LSU B-25220*	AY194438	AY194332	AY194299	AY194371
<i>A. o. panamensis</i>	Coclé, Panama		STR1-x-26*	AY194462	AY194356	AY194323	AY194395
<i>A. o. panamensis</i>	Chiriqui, Panama		STR1-x-27*	AY194463	AY194357	AY194324	AY194396
<i>A. o. panamensis</i>	Chiriqui, Panama		STR1-x-30*	AY194464	AY194358	AY194325	AY194397
<i>A. o. panamensis</i>	Chiriqui, Panama		STR1-x-34*	AY194465	AY194359	AY194326	AY194398
<i>A. o. auropalliata</i>	Guanacaste, Costa Rica		STR1-x-98*	AY194444	AY194338	AY194305	AY194377
<i>A. o. auropalliata</i>	Chiapas, Mexico		STR1-x-56*	AY194449	AY194343	AY194310	AY194382

Appendix 1. Continued.

Taxon	Locality	Locality code	Institution/ voucher	ND2	ATP8	ATP6	COI
<i>A. o. oratrix</i>	Tamaulipas, Los Colorados, Mexico		STRI-x-44*	AY194451	AY194345	AY194312	AY194384
<i>A. o. oratrix</i>	Tamaulipas, Los Colorados, Mexico		STRI-x-45*	AY194452	AY194346	AY194313	AY194385
<i>A. o. oratrix</i>	Tamaulipas, Los Colorados, Mexico		STRI-x-46*	AY194453	AY194347	AY194314	AY194386
<i>A. o. oratrix</i>	Tamaulipas, Los Colorados, Mexico		STRI-x-47*	AY194457	AY194351	AY194318	AY194390
<i>A. o. oratrix</i>	Tamaulipas, Los Colorados, Mexico		STRI-x-48*	AY194450	AY194344	AY194311	AY194383
<i>A. o. oratrix</i>	Veracruz, Tempoal, Mexico		STRI-x-54*	AY194447	AY194341	AY194308	AY194380
<i>A. o. oratrix</i>	Veracruz, Tempoal, Mexico		STRI-x-55*	AY194448	AY194342	AY194309	AY194381
<i>A. o. belizensis</i>	Belize Zoo		STRI-x-37*	AY194440	AY194334	AY194301	AY194373
<i>A. o. belizensis</i>	Belize Zoo		STRI-x-38*	AY194441	AY194335	AY194302	AY194374
<i>A. o. belizensis</i>	Belize Zoo		STRI-x-39*	AY194442	AY194336	AY194303	AY194375
<i>A. o. belizensis</i>	Belize Zoo		STRI-x-41*	AY194443	AY194337	AY194304	AY194376
<i>A. o. tresmariae</i>	Nayarit, Isla Maria Madre, Mexico		STRI-x-49*	AY194454	AY194348	AY194315	AY194387
<i>A. o. tresmariae</i>	Nayarit, Isla Maria Madre, Mexico		STRI-x-50*	AY194455	AY194349	AY194316	AY194388
<i>A. o. tresmariae</i>	Nayarit, Isla Maria Madre, Mexico		STRI-x-51*	AY194456	AY194350	AY194317	AY194389
<i>A. o. tresmariae</i>	Nayarit, Isla Maria Madre, Mexico		STRI-x-52*	AY194458	AY194352	AY194319	AY194391
<i>A. o. tresmariae</i>	Nayarit, Isla Maria Madre, Mexico		STRI-x-53*	AY194459	AY194353	AY194320	AY194392
<i>A. amazonica</i>	Sucumbios, Ecuador		ANSP 3307*	AY194466	AY194360	AY194327	AY194399
<i>A. autumnalis</i>	Tamaulipas, Los Colorados, México		STRI-x-42*	AY194446	AY194340	AY194307	AY194379
<i>A. fatinosa</i>	Captive		STRI-x-21*	AY194461	AY194355	AY194322	AY194394

USP, Universidade de São Paulo; STRI, Smithsonian Tropical Research Institute; NMNH, National Museum of Natural History; LSU, Louisiana State University; ANSP, Academy of Natural Sciences of Philadelphia.

*Sequences from Eberhard and Bermingham (2004).

Anexo VI

Molecular systematics in *Aratinga* parakeets: species limits and historical biogeography in the ‘*solstitialis*’ group, and the systematic position of *Nandayus nenday*

Camila C. Ribas and Cristina Y. Miyaki*

Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, R. do Matão 277, 05508-090 São Paulo, SP, Brazil

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Abstract

The parrot genus *Aratinga* comprises 20 species that can be separated, based on morphological characters, in at least three distinct groups. We performed a phylogenetic analysis based on mtDNA sequences of individuals belonging to the *solstitialis* group with the objectives of: (1) assessing the genetic differences among individuals in order to clarify their specific status; (2) testing the monophyly of the group and establishing its phylogenetic position relative to other *Aratinga* species; (3) making inferences about temporal and geographical patterns of diversification in the Neotropics. As a result of the analysis, the three taxa belonging to the *Aratinga solstitialis* complex were found to be diagnosable phylogenetic species, the monotypic genus *Nandayus* was found to be included in the *solstitialis* group and the non-monophyly of the genus *Aratinga* was confirmed. Most of the speciation events occurred during the Pliocene and Pleistocene and may be related to habitat shifts associated to climate oscillation during these periods.

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1. Introduction

The study of the distribution of organisms in South America has revealed areas of endemism that are common to different groups of animals (Brown, 1977; Cracraft, 1985; Haffer, 1969; Vanzolini and Williams, 1970). The origin of these areas as well as the origin of the high species diversity found in some of them has been the subject of many investigations, and different hypotheses have been proposed to account for these observations. Some of them postulate that the high diversity is due to the tropics being old and stable and having had low extinction rates, so that endemic species accumulated over time (Fjeldsø, 1994). Other hypotheses are based on factors that promote speciation in tropical faunas and focus on the geographic context of speciation and the physical causes of isolation. These hypotheses try to deduce historical biogeographic events that promoted speciation based on distributions and phylogenetic relationships of extant taxa, and different models have

been proposed (see Bush, 1994; Haffer, 1997; Moritz et al., 2000 for reviews). Among them, the most discussed has been the Pleistocene refugia model (Haffer, 1969), which proposes that climatic changes during the Pleistocene generated contractions and expansions of rainforests and savannas, promoting speciation. Alternatively, an emerging view of diversification in the Neotropics is that no single model can explain it, but instead several of the current models might be integrated to give a better picture of the complex processes producing biological diversity (Bush, 1994; Marroig and Cerqueira, 1997).

Despite all the proposed hypotheses, the origin and history of Neotropical patterns of endemism and diversity are still poorly understood (Bates et al., 1998; Cracraft and Prum, 1988) and comparisons among patterns of distribution and phylogenetic relationships of closely related, allopatric groups of taxa can provide important information to address this issue (Cracraft, 1985; Cracraft and Prum, 1988; Crisci, 2001).

There are 148 recognized species of parrots (Psittacidae) in South America (Forshaw and Cooper, 1989). Most of these species contain subspecies and many of

* Corresponding author. Fax: +55-11-30917553.

E-mail address: cymiyaki@usp.br (C.Y. Miyaki).

them are endemic. This high degree of geographical differentiation complicates the systematic arrangement of the group, mainly at the specific and subspecific levels, but at the same time makes it a very informative group for biogeographical studies. Detailed systematic studies of the various complexes of subspecies existing today are needed, as the current taxonomy may be hiding a great amount of the diversity that exists in the group.

The genus *Aratinga* comprises 20 species distributed throughout South America, Central America, and the West Indies (Forshaw and Cooper, 1989). Molecular systematic studies of Neotropical genera of psittacids fail to give good support to the monophyly of this genus (Tavares, 2001). Based on plumage and osteological characters several groups of taxa within the genus can be defined. One such group, from now on referred to as the 'solstitialis group,' comprises *Aratinga weddelli* and three allopatric taxa whose taxonomic status is uncertain: *A. solstitialis* (Linnaeus) 1758, *A. jandaya* (Gmelin) 1788, and *A. auricapilla* (Kuhl) 1820. Peters (1937) listed these as separate species but noted without qualification that they were 'probably conspecific'; this treatment is followed by Forshaw and Cooper (1989). Sick (1997) considers the three taxa 'geographic races (semispecies)' of *A. solstitialis*. Recent treatments recognize the three species but note that they are 'allospecies' (Juniper and Parr, 1998) forming a 'superspecies' (Collar, 1999). The

plumage characters that unite *A. solstitialis*, *A. jandaya*, *A. auricapilla*, and *A. weddelli* include: dark blue on the apex of the primaries, dark blue in the primary coverts, ventral side of the rectrices dark, and apex of the dorsal face of the rectrices dark blue. These characters are found only in these taxa among all *Aratinga* species (Silveira, L.F., Lima, F.T., Höfling, E., in preparation. A new species of *Aratinga* Spix, 1824 (Psittacidae) from Brazil, with a revision of the *Aratinga Solstitialis* complex).

Aratinga weddelli occurs in 'terra firme' forest in southwestern Amazonia, *A. solstitialis* in open savannas and savanna woodland, várzea forest and secondary vegetation in eastern Amazonia, *A. jandaya* in north-eastern Brazil, where it is restricted to wooded cover, including humid forest edge and adjacent open areas and avoids more arid vegetation, such as caatinga (Fig. 1). *A. auricapilla* occurs in forest edges and cerrado regions in southeastern Brazil (Fig. 1), and two subspecies have been described: *A. a. auricapilla* in the northern part of its range, and *A. a. aurifrons* in the southern part of its range, but the validity of the morphological differences that led to this distinction is being questioned (Silveira et al. in prep.). Individuals of the three taxa are known to hybridize in captivity, but hybridization has never been confirmed in nature (Joseph, 1992).

The taxonomic status of *A. solstitialis*, *A. jandaya*, and *A. auricapilla* as subspecies or distinct species remains uncertain. This has led to uncertainties about

Fig. 1. Distribution of *Aratinga solstitialis*, *A. jandaya*, *A. auricapilla*, and *A. weddelli* (modified from Forshaw and Cooper, 1989; Sick, 1997; Collar, 1999). Numbers show approximate locations for all samples used in this study. Circles: collection locality; squares: region from which sample originated, exact location not available.

the degree of vulnerability of these taxa and to errors when referring to individuals of the three taxa simply as *A. solstitialis*. Defining the limits of the geographical patterns of variation is important for both taxonomy and conservation, as it allows the recognition of discrete evolutionary and taxonomic units in nature (Cracraft, 1987). Also, the determination of the phylogenetic relationships among these taxa, which present allopatric patterns of distribution that roughly correspond to different areas of endemism described for South America (Cracraft, 1985; Haffer, 1985), will contribute, together with other such studies, to test hypotheses of area relationships (Bates et al., 1998; Cracraft and Prum, 1988; Marks et al., 2002) and to clarify the history of the diversification in the continent.

The aim of this study was to perform a molecular systematic investigation of the relationships and genetic differences among individuals belonging to each of the taxa included in the *solstitialis* group in order to clarify their specific status. Also, by comparing these taxa with other *Aratinga* species and with other psittacine genera we intend to test the monophyly of the *solstitialis* group and establish its phylogenetic position relative to some other *Aratinga* species. Finally, by determining the phylogenetic relationships and the degree of differentiation among taxa in the *solstitialis* group, we try to understand the historical biogeography of the group and to make inferences about the historical processes that originated the present patterns of geographical variation and diversity.

Here we present partial sequences of the mitochondrial 12S and 16S ribosomal subunits, cytochrome *b* gene, as well as the control region for individuals of the four *Aratinga* species mentioned above. The data produced were analyzed in conjunction with a broader data set including 12 species of psittacids. Sequence divergence analysis and molecular clock dating were performed and the divergence dates are related to biogeographical models.

2. Materials and methods

2.1. Taxon sampling

In order to assess patterns of evolution and species limits, sequences were obtained from *Aratinga solstitialis* (four individuals), *A. jandaya* (5), *A. auricapilla* (7), and *A. weddelli* (4) (Table 1, GenBank accession numbers for 12S rDNA, 16S rDNA, cytochrome *b*, and control region, respectively). Blood samples were collected in the field or in captivity, whenever reliable information about geographical origin was available (Fig. 1, Table 1). All the birds were identified by plumage characters and their geographical origins agreed with known species distributions.

In order to test for the monophyly of the *solstitialis* group and to establish how it relates to other psittacid genera, the sequences obtained in the preset study were analyzed in conjunction with a broader data set that included sequences obtained from the GenBank. The potential outgroup sequences used, followed by their GenBank accession numbers for 12S rDNA, 16S rDNA, cytochrome *b*, and control region, respectively, are presented in Table 1. Sequences from two individuals per species were used for *Aratinga cactorum*, *A. aurea*, *A. leucophthalmus*, and *Nandayus nenday*. Nomenclature follows Forshaw and Cooper (1989) and Penhallurick (2001).

2.2. DNA extraction, amplification, and sequencing

Total DNA was isolated from blood samples stored in 95% ethanol using proteinase K, and the phenol-chloroform procedure according to Bruford et al. (1992).

Fragments of four mitochondrial regions were amplified via the polymerase chain reaction (PCR) using the following primers: rDNA12S: L1735 (GGATTAGA TACCCCACTATGC); H2170 (AGGGTGACGGGCG GTATGTACG), rDNA16S: L2702 (CCTACCGAGC TGGGTGATAGCTGGTT), H3309 (TGCGCTACC TTCGCACGGT) (Miyaki et al., 1998); cytochrome *b* L15298 (TGAGGCCAAATATCATTCTGAGGGGC) (Cheng et al., 1994), H15764 (CCTCCTAGTTTGTG GGGATTGA) (Miyaki et al., 1998); control region: Lglu 16737 (GCCCTGAAAARCCATCGTTG); H522 (TGG CCCTGACYTAGGAACCAG) (Eberhard et al., 2001); and L420 (CACGAGAGATCAYCAACCCGGTGT), H1032 (AAGTGTAACAAAGTGCATCAGGGT) (Tavares, 2001).

The amplifications were performed in 10 µl reactions that contained 0.8 mM dNTP, 1 µM of each primer, 0.5 U *Taq* polymerase (Pharmacia), buffer 1× (Pharmacia), and 1 µl of template DNA. Thermal cycling conditions were: an initial denaturation step of 95 °C for 5 min followed by 30 cycles of 95 °C (1 min), 53 °C (40 s), 72 °C (40 s), and a final incubation of 72 °C for 5 min. PCR products were visualized by electrophoresis on agarose gels and purified using Shrimp alkaline phosphatase (0.5 U, USB) and Exonuclease I (5 U, USB). Purified PCR products were used as templates for Big Dye (Perkin-Elmer) terminator cycle sequencing reactions following the manufacturer's recommendations. The same primers used for the first amplification were used in the sequencing reaction. Sequences were read with an ABI 377 automated sequencer. Both strands were sequenced for each region studied.

2.3. Sequence alignment and statistics

The sequences obtained were determined by comparing the heavy and light strands using the programs

Table 1

Individuals studied, their origin and their sequences' GenBank accession numbers (12S, 16S, cytochrome *b*, and control region, respectively)

Taxon	# ^a	Locality	GenBank Accession No.
Ingroup (sequences obtained in the present study)			
<i>Aratinga solstitialis</i> 1	3460	Alenquer, Pará, Brazil	AY208190, AY208210, AY208230, AY208250
<i>Aratinga solstitialis</i> 2	0941	Monte Alegre, Pará, Brazil	AY208193, AY208213, AY208233, AY208253
<i>Aratinga solstitialis</i> 3	0940	Monte Alegre, Pará, Brazil	AY208191, AY208211, AY208231, AY208251
<i>Aratinga solstitialis</i> 4	2807	Pará, Brazil	AY208192, AY208212, AY208232, AY208252
<i>Aratinga jandaya</i> 1	1312	Rio Gurupi, Pará, Brazil	AY208204, AY208224, AY208244, AY208264
<i>Aratinga jandaya</i> 2	0877	Rio Gurupi, Pará, Brazil	AY208205, AY208225, AY208245, AY208265
<i>Aratinga jandaya</i> 3	1314	Rio Gurupi, Pará, Brazil	AY208202, AY208222, AY208242, AY208262
<i>Aratinga jandaya</i> 4	4027	Santa Ines, Maranhão, Brazil	AY208203, AY208223, AY208243, AY208263
<i>Aratinga jandaya</i> 5	4026	Imperatriz, Maranhão, Brazil	AY208201, AY208221, AY208241, AY208261
<i>Aratinga auricapilla</i> 1	0662	São Paulo, Brazil	AY208199, AY208219, AY208239, AY208259
<i>Aratinga auricapilla</i> 2	0659	São Paulo, Brazil	AY208196, AY208216, AY208236, AY208256
<i>Aratinga auricapilla</i> 3	0145	São Paulo, Brazil	AY208200, AY208220, AY208240, AY208260
<i>Aratinga auricapilla</i> 4	0036	Rio Pardo, Bahia, Brazil	AY208197, AY208217, AY208237, AY208257
<i>Aratinga auricapilla</i> 5	1144	Adamantina, São Paulo, Brazil	AY208194, AY208214, AY208234, AY208254
<i>Aratinga auricapilla</i> 6	1142	Adamantina, São Paulo, Brazil	AY208195, AY208215, AY208235, AY208255
<i>Aratinga auricapilla</i> 7	4249	Aracatuba, São Paulo, Brazil	AY208198, AY208218, AY208238, AY208258
<i>Aratinga weddellii</i> 1	2065	Acre, Brazil	AY208208, AY208228, AY208248, AY208268
<i>Aratinga weddellii</i> 2	2085	Acre, Brazil	AY208206, AY208226, AY208246, AY208266
<i>Aratinga weddellii</i> 3	2086	Acre, Brazil	AY208207, AY208227, AY208247, AY208267
<i>Aratinga weddellii</i> 4	1318	Pontes e Lacerda, Mato Grosso, Brazil	AY208209, AY208229, AY208249, AY208269
Outgroup (sequences obtained from previous studies ^b)			
<i>Anodorhynchus hyacinthinus</i>	0114	Mato Grosso do Sul, Brazil	U70741, U70752, U70763, AF430809
<i>Anodorhynchus leari</i>	0410	Raso da Catarina, Brazil	AF362914, AF365407, AF370763, AF430808
<i>Orthopsittaca manilata</i>	2385	Mato Grosso, Brazil	AF362924, AF365417, AF370770, AF430828
<i>Nandayus nenday</i> 1	0143	Mato Grosso do Sul, Brazil	U70746, U70757, AY219914, AF430833
<i>Nandayus nenday</i> 2	0377	Mato Grosso do Sul, Brazil	AF 362937, AF365428, AY219915, AF430834
<i>Aratinga cactorum</i> 1	0837	Tocantins, Brazil	AF362920, AF365413, AF370768, AF430823
<i>Aratinga cactorum</i> 2	0992	Tocantins, Brazil	AF362921, AF365414, AY281254, AF430824
<i>Aratinga aurea</i> 1	0346	Goias, Brazil	U70740, U70751, U70762, AF438818
<i>Aratinga aurea</i> 2	0461	Mato Grosso, Brazil	AY286200, AY286217, AY286208, AF438819
<i>Aratinga leucophthalmus</i> 1	1625	Ilha do Marajo, Para, Brazil	AF362918, AF365411, AF370767, AF430822
<i>Aratinga leucophthalmus</i> 2	2090	Acre, Brazil	AF362919, AF365412, AY281253, AF365434
<i>Ara ararauna</i>	0013	Goias, Brazil	U70739, U70750, U70761, AF430811
<i>Primolius couloni</i>	3142	Acre, Brazil	AF362940, AF365431, AF370780, AF430835
<i>Primolius auricolis</i>	1742	Mato Grosso, Brazil	AF362926, AF365419, AF370772, AF430829
<i>Diopsittaca nobilis</i>	1126	Para, Brazil	AF362923, AF365416, AF370769, AF430826
<i>Guarouba guarouba</i>	0004	Maranhao, Brazil	U70744, U70755, U70766, AF430813

^a Catalog number at the genetic resources collection, LGEMA, Universidade de São Paulo.^b Miyaki et al. (1998), Tavares (2001), and Tavares, E.S., Yamashita, C.Y. (submitted). Phylogenetic relationships among some Neotropical parrot genera (Psittacidae; Aves) based on mitochondrial sequences.

Sequence Navigator and Sequence Analysis (Applied Biosystems). The alignments were performed in Clustal X (Thompson et al., 1997) and manually corrected using MacClade 4.0 (Maddison and Maddison, 1999). The alignments were used to construct character matrices for each studied gene region as well as for combined data. Aligned sequences were examined in order to verify if there were fixed molecular markers characteristic of each of the taxa studied.

Base composition, T_s/T_v ratios, percent sequence divergence (*p*-distance; Nei, 1987) and Kimura's two-parameter distances (K2-P; Kimura, 1980) among sequences were calculated using MEGA (Kumar et al., 2001) and PAUP* (version 4.0b10; Swofford, 1998). Saturation was evaluated for each region by plotting the absolute numbers of transitions against *p*-distance and

against K2-P distances for all pairwise comparisons. *p*-Distances were also plotted against K2-P distances for all pairwise comparisons. In both analyses, deviations from linearity would indicate saturation effects.

2.4. Phylogenetic analyses

All phylogenetic analyses were conducted in PAUP* (version 4.0b10; Swofford, 1998). Sequences from the different regions were analyzed both independently and combined using the methods of maximum parsimony (MP) and maximum likelihood (ML). MP analyses were performed with heuristic tree searches, using unordered and equally weighted characters, tree bisection–reconnection (TBR) branch swapping, and 10 random-addition sequence replications. Bootstrap

(1000 replications) and decay indices (Bremer, 1994) were used to determine the relative support for inferred monophyletic groupings.

The likelihood-ratio test as implemented in MODELTEST (Posada and Crandall, 1998) was used to select the simplest model of molecular evolution yielding a significantly higher likelihood than others. The model selected was used for the ML analyses, which were performed using heuristic tree search, TBR branch swapping and 10 random addition replicates. The robustness of the trees found was determined by 100 bootstrap replications.

2.5. Molecular clock and divergence dates estimates

Due to the absence of a reliable psittacid fossil record it was not possible to calibrate a local substitution rate for our data. For this reason, rates of substitution that have been calculated for other avian orders were used. Two different substitution rates were applied to the data: one derived from geological dating of island formation (1.6%/Ma; Fleischer et al., 1998) and the other estimated from fossil records (2.0%/Ma; Randi, 1996; Shields and Wilson, 1987). As both these rates were calculated for cytochrome *b* sequences and the dataset obtained in the present work includes other mitochondrial regions as well, we applied these rates to estimate the date of the most basal split in the ingroup (*A. weddelli* × all other taxa) using the branch lengths of the ML tree constructed from a matrix containing only cytochrome *b* sequences. We then used this date to calculate an internal rate for the ingroup based on the ML tree constructed from the combined dataset. This rate was then applied to the branch lengths of the linearized ML tree constructed using the combined dataset to estimate the divergence dates. For these analyses, matrices containing only the ingroup (22 terminals, see Results) and one outgroup (*Aratinga aurea*) were used. A likelihood ratio test assuming a χ^2 distribution with number of taxa minus two degrees of freedom (Huelsenbeck and Rannala, 1997) was applied to these two matrices (cy-

tochrome *b* only and combined) in order to compare the log-likelihood values from ML trees constructed with and without a molecular clock constraint.

3. Results

3.1. Sequence characteristics

Nuclear copies of mitochondrial genes in birds have been previously reported (Quinn, 1997). In the present study, no contaminants were detected in the PCR products; the base composition found was typical of avian mitochondrial DNA; the primers used were designed to amplify avian mitochondrial regions; and all the sequences obtained aligned easily to other psittacine mitochondrial sequences. These facts strongly suggest that the sequences obtained are of mitochondrial origins.

Eberhard et al. (2001) found that the control region is duplicated in the mitochondrial genome of parrots belonging to the genera *Amazona* and *Pionus*. Tavares et al. (Tavares, E.S., Lourenço, R., Miyaki, C.Y., in preparation. Mitochondrial gene arrangements around the control region in some Neotropical Psittacidae genera) have found, through PCR and sequencing analyses of the control region and its flanking genes, that this duplication does not exist in *Anodorhynchus hyacinthinus*, *Ara ararauna*, *Diopsittaca nobilis*, *Guarouba guarouba*, and *Cyanopsitta spixii*, so that these species have the same mitochondrial gene order found in *Gallus gallus*. Through the analysis of the size of PCR amplified fragments (data not shown) we found that the *Aratinga* species studied here also do not present this duplication, so that the control region sequences could be used without concern about wrong hypotheses of homology.

A total of 2010 base pairs (bp) from four different mitochondrial regions (Table 2), were obtained from individuals of *Aratinga solstitialis* ($n = 4$), *A. jandaya* ($n = 5$), *A. auricapilla* ($n = 7$), and *A. weddelli* ($n = 4$). When these sequences were aligned with sequences from

Table 2
Variable sites, informative sites, nucleotide composition, and T_s/T_v ratios of the mitochondrial regions studied

	12S rDNA	16S rDNA	Cytochrome <i>b</i>	Control region
Number of base pairs	295	461	340	914
Number variable (%)	14 (4.7)	45 (9.8)	45 (13.2)	137 (15.0)
Number informative (%)	13 (4.4)	42 (9.1)	40 (11.7)	123 (13.5)
%A	31.4	35.2	28.2	22.9
%C	29.2	31.6	34.4	25.5
%G	22.0	16.4	10.7	19.3
%T	17.3	16.7	26.7	32.2
T_s/T_v ratio	7.36 (SE ^a = 3.26)	10.41 (SE = 4.49)	10.21 (SE = 5.80)	5.59 (SE = 1.47)

All 20 *Aratinga* individuals were included in this analysis.

^aSE, standard error.

the other psittacine genera, a matrix containing 2029 characters was obtained. This length difference is due to the indels that were introduced in the alignment when the more divergent sequences were added. The statistics shown in Table 2 refer only to the sequences obtained in the present work. The alignments are available upon request to the authors.

The alignment of the *A. solstitialis*, *A. jandaya*, *A. auricapilla*, and *A. weddelli* sequences (ingroup) showed that a small number of indels were present in the 12S and 16S rDNAs and in the control region. There were no indels in the cytochrome *b* region. Base composition analysis of the ingroup sequences showed a higher frequency of cytosine and adenine in all regions with the exception of the control region in which a higher frequency of thymine was found (Table 2). No base composition bias was detected in the dataset, including variable sites only for all the sequences ($X^2 = 10.6734$, $df = 105$, and $P = 1.000$). The number of transitions observed was higher than the number of transversions in all regions (Table 2).

Among the mitochondrial regions sequenced, the rDNAs were the least variable and had the lowest phylogenetic signal (Table 2). Cytochrome *b* had higher variability, mainly in third positions. Translation of the cytochrome *b* sequences showed that there were no differences in amino acid sequence among all *A. solstitialis*, *A. jandaya*, and *A. auricapilla* individuals. The control region was the most variable region and also had the highest phylogenetic information content for the taxa analyzed (Table 2). This confirms the utility of this region for the study of closely related taxa.

Saturation plots for each studied region did not indicate saturation when comparing the number of transitions with the uncorrected distances for each pairwise comparison (data not shown). A linear relationship between *p*-distances and K2-P distances for all pairwise comparisons was also found for all regions (data not shown). Transition/transversion ratios were high among *A. solstitialis*, *A. jandaya*, and *A. auricapilla* individuals, which accumulated a small number of transversions.

3.2. Phylogenetic analyses

Phylogenetic analyses included all the sequences obtained in the present work (20 individuals) and data from 16 other psittacines obtained from GenBank (Table 1). This matrix contained 2029 characters and 36 terminals. The inclusion of 16 sequences as putative outgroups had the objective of testing for the monophyly of the ingroup, analyzing the relationship of the ingroup with the other *Aratinga* species included, and placing the new taxa in a phylogenetic context among the other Neotropical psittacine for which sequences were available. As the relationships among Neotropical psittacine genera are not well established and the

alignment of the control region among 'long' and 'short' tailed genera is problematic because of a duplication of this region in some short tailed genera (Eberhard et al., 2001), we decided to root the trees obtained with the most distantly related outgroup taxa, the two species belonging the genus *Anodorhynchus*. This procedure did not affect the relationships inside the ingroup, which are the main focus of the present analysis.

In the MP analysis there were 527 parsimony-informative characters, and six most parsimonious trees were generated having 1465 steps, a consistency index of 0.566, and a retention index of 0.786. The strict consensus tree is shown in Fig. 2, along with the values of bootstrap support (1000 replications) and decay indices (Bremer, 1994) (heuristic search, random addition of taxa with 100 replications, TBR). The independent analysis of each region (data not shown) yielded very poor resolution among the outgroup sequences. All four isolated regions datasets recovered the clade composed of *A. solstitialis*, *A. jandaya*, *A. auricapilla*, and *N. nenday*. Control region and 16S rDNA analysis also included *A. weddelli* in this clade.

For the ML analysis, the evolutionary model chosen by MODELTEST was Tamura–Nei with a proportion of invariable sites of 0.3969 and the gamma distribution shape parameter of 0.6395, the tree obtained had log likelihood of 9895.80761 (Fig. 3). One hundred bootstrap replications were performed, and bootstrap values are shown in Fig. 3. Independent analysis of the different regions (data not shown) generated many polytomies, with the exception of the control region, which did recover all the main clades found in the total evidence analysis.

MP and ML analyses of the combined dataset resulted in similar topologies (Figs. 2 and 3), with some small differences at the tips. In all trees, individuals of *A. solstitialis*, *A. jandaya*, *A. auricapilla*, and *A. weddelli* formed distinct, reciprocally monophyletic clades with high bootstrap and Bremer supports. *A. jandaya* and *A. auricapilla* appeared as sister groups with *A. solstitialis* external to them. *Nandayus nenday* always appeared as being the sister taxon to the (*A. solstitialis*, *A. auricapilla*, and *A. jandaya*) clade with high bootstrap support, and *A. weddelli* was the next most closely related species to this group. The branch lengths inside this group were very short in both analyses. These results show that *N. nenday* is included in the ingroup. The node uniting *Aratinga cactorum* and *Aratinga aurea* to the ingroup had low bootstrap and Bremer supports in the MP analysis.

3.3. Genetic distances and diagnostic characters

Measures of uncorrected pairwise sequence divergence (*p*-distances) among individuals of *A. solstitialis*, *A. jandaya*, *A. auricapilla*, *N. nenday*, and *A. weddelli* were obtained for each region independently. Individuals

Fig. 2. Phylogenetic reconstruction based on 2029 bp of mitochondrial sequences using MP assuming equal weighting of characters (strict consensus of six trees, $L = 1465$ steps). Bootstrap values (1000 replications) greater than 50% are shown above branches and Bremer decay indices are shown below branches. Bar is located at the base of the ingroup.

of each taxon were grouped and the distances and standard errors for each region were measured within and between groups (Tables 3 and 4). Distances among individuals of the same species ranged from 0.0 to 1.4%, while distances among individuals belonging to different species ranged from 0.0 to 10.7%. The greatest distances were found when comparing control region sequences.

There were 70 variable characters among *A. solstitialis*, *A. jandaya*, and *A. auricapilla*. Forty eight of them were phylogenetically informative for parsimony (three in the 12S rDNA sequence, four in 16S rDNA, seven in cytochrome *b*, and 34 in the control region). Among these, 17 were common to all *A. solstitialis* and differentiate them from *A. jandaya* and *A. auricapilla*, five of these are shared with the outgroup and thus are primitive retentions, and the other 12 are diagnostic

for the *A. solstitialis* haplotypes, being ten transitions and two transversions. Eight characters are diagnostic for *A. jandaya* haplotypes, one of them (1147) has a reversion in one *A. auricapilla* sequence (individual 1142) and another (1279) is shared with the outgroup and may be interpretable as a primitive retention depending on the optimization used. This also happens with two (1236 and 1833) of the four diagnostic characters for *A. auricapilla*. The remaining phylogenetically informative characters were common to some individuals belonging to the same species. All the differences involving *A. auricapilla* and *A. jandaya* were transitions.

A MP analysis of the 16 ingroup haplotypes using *N. nenday* as outgroup was performed with the branch and bound option in PAUP*, resulting in four equally

Fig. 3. Phylogenetic reconstruction based on 2029 bp of mitochondrial sequences using Maximum Likelihood ($Tr N + I + F$; $I = 0.3841$, $\alpha = 0.6459$), bootstrap values (100 replications) shown on branches. Bar is located at the base of the ingroup.

Table 3
Average p -distances (%) and standard errors (within brackets) for 12S (295 bp, below diagonal) and 16S (461 bp, above diagonal) between groups of individuals belonging to each taxon. Average p -distances within groups (%) shown in the diagonal for 12S and 16S, respectively

	N^a	1	2	3	4	5
1. <i>A. auricapilla</i>	7	0.0/0.1	0.7 (0.4)	0.4 (0.3)	2.7 (0.7)	4.5 (0.8)
2. <i>A. jandaya</i>	5	0.0 (0.0)	0.0/0.0	0.6 (0.3)	2.9 (0.8)	4.7 (0.9)
3. <i>A. solstitialis</i>	4	0.7 (0.4)	0.7 (0.4)	0.5/0.2	2.6 (0.7)	4.3 (0.8)
4. <i>N. nenday</i>	2	0.9 (0.5)	0.9 (0.5)	1.6 (0.6)	0.3/0.2	5.8 (1.0)
5. <i>A. weddellii</i>	4	3.4 (1.0)	3.4 (1.0)	3.1 (0.9)	3.6 (1.0)	0.0/0.1

^a Number of analyzed individuals from each species.

parsimonious trees of 37 steps (data not shown). The support values were obtained from 200 bootstrap replicates. Individuals belonging to each species cluster to-

gether with high bootstrap support (100% for *A. solstitialis* individuals, 97% for *A. jandaya*, and 92% for *A. auricapilla*).

Table 4

Average *p*-distances (%) and standard errors (within brackets) for cytochrome *b* (340 bp, below diagonal) and control region (914 bp, above diagonal) between groups of individuals belonging to each taxon. Average *p*-distances within groups (%) shown in the diagonal for cyt *b* and CR, respectively

	N ^a	1	2	3	4	5
1. <i>A. auricapilla</i>	7	0.6/0.8	1.9 (0.4)	2.3 (0.4)	4.7 (0.7)	10.5 (0.9)
2. <i>A. jandaya</i>	5	0.4 (0.2)	0.0/0.6	2.5 (0.5)	5.0 (0.7)	10.1 (0.9)
3. <i>A. solstitialis</i>	4	1.7 (0.7)	1.3 (0.6)	0.2/0.3	5.1 (0.7)	10.5 (0.9)
4. <i>N. nenday</i>	2	3.5 (0.9)	3.1 (0.9)	3.2 (0.9)	0.3/1.4	10.7 (0.9)
5. <i>A. weddelli</i>	4	9.4 (1.5)	9.2 (1.5)	8.5 (1.5)	10.5 (1.6)	0.1/0.4

^a Number of analyzed individuals from each species.

3.4. Molecular clock

Two different approaches may be used to estimate divergence times from sequence data: a fixed rate of sequence evolution found for other groups of organisms may be applied to the data, or the calibration used may be derived from the data itself, when there is a node that can be dated by the fossil record or some other means. It was not possible to apply this second approach to our data due to the absence of good fossil calibration, and only the rates of sequence evolution were used. Two matrices were constructed containing only the ingroup (*Aratinga solstitialis*, *A. jandaya*, *A. auricapilla*, *Nandayus nenday*, and *A. weddelli*) and one outgroup (*Aratinga aurea*). One matrix contained only the cytochrome *b* sequence (340 bp), whereas the other contained the combined dataset (2012 bp). ML trees were constructed using the models selected by MODELTEST for each matrix: HKY, $T_i/T_v = 7.54$, $I = 0$, equal rates for all sites for the cytochrome *b* data, and TrN + Γ ($\alpha = 0.153$) for the combined dataset. ML scores for both trees with or without a molecular clock enforced

did not show significant differences (LRT: $X^2 = 31.84$, $df = 21$, and $P > 0.05$ for cytochrome *b*, and $X^2 = 28.52$, $df = 21$, and $P > 0.10$ for the combined dataset).

Two different rates were applied to the ML tree derived from the cytochrome *b* data: 1.6%/Ma (Fleischer et al., 1998) and 2.0%/Ma (Randi, 1996; Shields and Wilson, 1987), and two time estimates were obtained for the split between *A. weddelli* and all other taxa (Fig. 4). Using these two time estimates, two new rates were calculated on the ML tree constructed with the combined dataset (1.97% and 2.46%). These new rates refer only to the ingroup sequences and were used to estimate the divergence dates for the other three nodes (Fig. 4).

4. Discussion

4.1. Systematic relationships within *Aratinga*

The analysis of the 36 psittacine sequences, comprising 16 taxa, showed that *Aratinga solstitialis*, *A.*

Fig. 4. Area cladogram showing the distribution of the ingroup taxa studied, the areas of endemism where each one occurs and the divergence times estimated for each node using the rates of sequence divergence of 2.0 and 1.6% per million years, respectively.

jandaya, and *A. auricapilla* are closely related, and that individuals belonging to the same taxon cluster together into reciprocally monophyletic groups. Both MP and ML analyses showed that *Nandayus nenday*, initially included as one of the outgroup taxa, is more closely related to (*A. solstitialis*, *A. jandaya*, and *A. auricapilla*) than *A. weddelli*. This close relationship between these taxa has been suggested before only by Miranda-Ribeiro (1920), who proposed the inclusion of *Aratinga solstitialis*, *A. jandaya*, and *A. auricapilla* in the genus *Nandayus*. Our results show that *N. nenday*, presently classified in a monotypic genus, is imbedded in a closely related group of species. *Aratinga cactorum* and *Aratinga aurea* form the sister group to the ingroup in both analyses, but this is not well supported by the MP analysis. *Aratinga leucophthalmus* is distantly related to the other *Aratinga* species in all analyses. Its phylogenetic position among the other psittacines is not well defined with the current taxon sampling, but it is clear that it does not form a monophyletic group with the other *Aratinga* species.

As *A. solstitialis* is the type species of the genus *Aratinga*, the results suggest that *N. nenday* should be included in the genus *Aratinga* and that *A. leucophthalmus* should be assigned to another genus. The analyses presented here also suggest that *A. aurea* and *A. cactorum* may group with the other *Aratinga*, but a better taxon sampling will be necessary to test this issue. Further analyses will also be necessary to determine which other species, presently included in the genus *Aratinga*, group with *A. leucophthalmus*.

4.2. Sequence divergence and diagnosable units

The divergence between *A. jandaya* and *A. auricapilla* individuals is very small and the control region sequence is the only one to show a significantly larger distance between these taxa ($1.9\% \pm 0.4$) when compared to the intra-taxon distances ($0.6\% \pm 0.2$ for *A. jandaya* individuals and $0.8\% \pm 0.2$ for *A. auricapilla* individuals). The divergences found when comparing control region sequences of *A. jandaya* and *A. auricapilla* with *A. solstitialis* are similar ($2.5\% \pm 0.5$ for *A. jandaya* and $2.3\% \pm 0.4$ for *A. auricapilla*) and still quite small when compared to the divergences found between other congeneric pairs of psittacine species, such as *Anodorhynchus leari* and *A. hyacinthinus*, which have a divergence of 5.2% in this same region of the mtDNA. The small amount of genetic divergence found among *A. solstitialis*, *A. jandaya*, and *A. auricapilla* individuals indicates a recent origin for these three forms, but the results presented here suggest that they are distinct and give no indication of interbreeding in nature.

Despite having diagnostic plumage characters, *A. solstitialis*, *A. jandaya*, and *A. auricapilla* are still considered by some authors as subspecies within *A. sol-*

stitialis (e.g., Sick, 1997). Our analyses show that these three taxa have diagnostic characters and form reciprocally monophyletic groupings in the phylogenetic analyses, meeting the two criteria—diagnosability (Cracraft, 1983; Eldredge and Cracraft, 1980) and monophyly (de Queiroz and Donoghue, 1988; Donoghue, 1985; Moritz, 1994)—required to be considered distinct species under the Phylogenetic Species Concept (Cracraft, 1987). This concept recognizes basal and diagnosably distinct taxa as species, avoiding further subdivision of species in subspecies, and thus is appropriate, in this case, to define the evolutionary units.

The sequences analyzed here provide evidence that *A. solstitialis*, *A. jandaya*, and *A. auricapilla* can be distinguished by exclusive nucleotide differences and cannot be further subdivided. Under the Phylogenetic Species Concept they are three separate and well-defined species. It is relevant to note that despite the great similarity among their DNA sequences and osteology, differences in plumage are very distinctive. This is evidence that changes in plumage patterns can occur very rapidly after isolation when compared to genetic divergence, and thus plumage variation may be an important factor driving speciation in these birds. This lack of correspondence between genetic and plumage divergence has been observed in other groups of birds (Driskell, 2002; Greenberg et al., 1998; Zink and Dittmann, 1993), showing that plumage differentiation may sometimes occur so rapidly that there is no time for corresponding genetic differentiation to arise.

4.3. Tempo and mode of diversification

The adoption of a molecular clock to estimate divergence dates based on sequence data is subject to many sources of errors, including the possibility that the molecular data may not evolve in a clock-like manner (Mindell et al., 1996) and that the calibration may be poor. On the other hand, the possibility of adding a temporal framework to phylogenetic analyses allows inferences about the past history of taxa. In this way, despite the many errors that time estimates may incorporate, they have been widely used in phylogenetic analyses (Dimcheff et al., 2002; Groombridge et al., 2002), and different tests and methodologies have been developed to estimate, correct, and reduce errors (Huelsenbeck and Crandall, 1997).

Many authors have estimated rates for mitochondrial sequence divergence and most of them range around 2% sequence divergence per million years (Fleischer and McIntosh, 2001). These rates seem to be good predictors of time for divergences up to 5 Ma. Simulations have shown that predictions of older dates are generally underestimated (Moore et al., 1999). According to our estimates, *A. solstitialis*, *A. jandaya*, and *A. auricapilla* diverged during the Pleistocene, their split from *N.*

nenday occurred during the end of the Pliocene, and *A. weddelli* separated from this group in the end of the Miocene.

The end of the Pliocene and the Pleistocene in South America are marked by the climatic cycles of temperature and humidity change (Hooghiemstra and Ran, 1994). The responses of the vegetation to these changes from hot and humid to dry and cold climate are still poorly understood, and the extent to which these changes influenced the distribution of forests and savannas in the continent is the subject of continuous debate (Bush et al., 1990; Colinvaux and De Oliveira, 2001; van der Hammen, 1974; van der Hammen and Hooghiemstra, 2000). One of the most common critics to the Pleistocene refugia hypothesis is that most species studied so far seem to be older than the Pleistocene, and thus their diversification could not have been caused by the refuges.

The pattern of diversification described here, with at least two speciation events dating to the Pleistocene and another one to the end of the Pliocene (Fig. 4) shows that there are speciation events that may have been related to Pleistocene climatic changes and is one example of species that originated recently enough to have been affected by the Pleistocenic ice ages and the changes in vegetation and climate caused by them. Many other examples of this kind may be found if sufficiently young divergences are investigated. Perhaps in other groups of birds plumage differentiation has been slower, and thus diversification is still not reflected in external characters (and these taxa are still considered conspecific). Detailed population genetic studies may reveal a diversity that is still not known in Neotropical species.

The pattern of phylogenetic relationships found also indicates a shift in habitat preference corresponding to the divergence between the forestal species *A. weddelli* and its sister group. The diversification of *A. solstitialis*, *A. jandaya*, and *A. auricapilla* occurred in open areas associated with forest edges and was probably driven by isolation of the open area habitats of eastern Brazil during more humid periods. These species are still more associated with forests than *N. nenday*, which occurs in the habitats of the open vegetation Chaco area. These habitat shifts may have been important mechanisms of speciation during the climate oscillations that occurred in South America before and during the Pleistocene.

4.4. Historical biogeography

The species studied here have distributions that roughly correspond to one or more areas of endemism delimited by Cracraft (1985), Haffer (1985), and Ridgely and Tudor (1989). *A. weddelli* is the only one strictly associated to forests and occurs in the Inambari and Rondônia areas of endemism. Among the other four species, *N. nenday* is strictly associated with open areas,

occurring in the Chaco area of endemism. The other three species occur in dry vegetation areas, but are associated with forest edges. *A. solstitialis* occurs in the Guianas area of endemism, but is found only in the Amazonian savannas. *A. jandaya* occurs in the Cerrado and Belém/Pará areas, occupying the edges of the south eastern Amazonian forest. *A. auricapilla* occurs mainly in regions of transition between the Cerrado and the Atlantic Forest areas (Fig. 1).

Some hypotheses, based on the phylogenetic relationships within different groups of birds (Cracraft and Prum, 1988; Prum, 1988; Marks et al., 2002) or on analysis of distributional data of bird species (Bates et al., 1998), have been proposed to explain the relationships among South American areas of endemism. The Atlantic Forest appears in some hypotheses as basal to the Amazonian areas (Bates et al., 1998) and in others it appears as closely related to the southeastern Amazon Belém/Pará area of endemism (Marks et al., 2002). Cracraft and Prum (1988) have suggested that this region is a composite, or biogeographic 'hybrid' area having component taxa with different biogeographic histories.

The sister relationship between *A. jandaya* and *A. auricapilla* is another example of the close association between southeastern Amazonian and southeastern Brazilian faunas (Fig. 4). The barriers isolating these taxa may be related to the arid vegetation that occurs in northeastern Brazil. According to the molecular clock estimations, their separation occurred around 500,000 years ago. There's much debate about the extent to which the climatic changes that occurred during the Pleistocene affected the distribution of forests and open areas, but many authors agree that the climate became significantly cooler during glacial periods (van der Hammen and Hooghiemstra, 2000). As these two species are associated with lowland forest edges and open areas, their differentiation may be related to the development and expansion, in glacial periods, of the very dry vegetation of the caatinga.

Aratinga solstitialis, distributed in the Guiana area, appears as a basal sister-group to the (*A. jandaya* and *A. auricapilla*) clade, thus showing an older split between northern and southern Amazonia than between northeast and southeast Brazil. The history of the Amazonian arid lands is closely related to the interplay between forest and savanna vegetation during the Pleistocene ice ages. If corridors of open vegetation were created in the Amazon forest, the fauna currently associated to the savannas could have been isolated by the re-expansion of the forest and by the marine introgressions postulated to have occurred during the interglacial periods (Haffer, 1969; Haffer, 1997; Marroig and Cerqueira, 1997). The origin of *A. solstitialis* dates to 770,000 to 960,000 years ago and may be related to the 'Amazonian lagoon' postulated to have been formed around 750,000 years ago (Klammer, 1984; Marroig and Cerqueira, 1997).

The split between *N. nenday* and the (*A. solstitialis*, *A. jandaya*, and *A. auricapilla*) clade occurred at the end of the Pliocene. *N. nenday* is part of the Chaco avifauna. This avifauna has low endemism and its components are derived from other habitats, including forest edges (Short, 1975). The lineage that gave origin to *N. nenday* may have originated from a forest ancestor, inasmuch as *A. weddelli* is found in forested habitats (Fig. 4).

Aratinga weddelli from the 'terra firme' forest in western Amazonia is basal to all four species above, indicating that the earliest speciation event separated the western Amazonian species from species associated with more arid vegetation in central and eastern South America and a posterior diversification separated the open area species *N. nenday* from the three *Aratinga* species, associated to forest edges (Fig. 4). This pattern suggests a closer relationship of the Amazonian aridlands with the eastern Brazilian open areas than with western Amazonia. This group of taxa is also an example of a close relationship between forest and arid land species, contradicting the hypothesis of an old split between biotas associated with these two habitats (Haffer, 1985).

The pattern of diversification described here for the *Aratinga* species included in the *solstitialis* group is one more piece of information about the systematic of this complex genus and about the processes of diversification that originated the diversity of species found in South America. Other studies, sampling more species presently included in the genus *Aratinga* will be needed in order to understand the evolutionary relationships among them, and define the monophyletic groupings. The factors that affected speciation in the *Aratinga* species studied here may have been important for many other groups as well and, as phylogenetic studies of South American groups accumulate, the patterns will become clearer making it easier to understand the processes of diversification in the continent.

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Anexo VII

ORIGINAL
ARTICLE

Historical biogeography and diversification within the Neotropical parrot genus *Pionopsitta* (Aves: Psittacidae)

Camila C. Ribas^{1,2*}, Renato Gaban-Lima¹, Cristina Y. Miyaki¹ and Joel Cracraft²

¹Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil and ²Department of Ornithology, American Museum of Natural History, New York, NY, USA

ABSTRACT

Aim We investigate spatial and temporal patterns of diversification within the Neotropical avifauna using the phylogenetic history of parrots traditionally belonging to the genus *Pionopsitta* Bonaparte 1854. This genus has long been of interest for those studying Neotropical biogeography and diversity, as it encompasses species that occur in most Neotropical forest areas of endemism.

Location The Neotropical lowland forests in South and Central America.

Methods Phylogenetic relationships were investigated for all species of the genus *Pionopsitta* and five other short-tailed parrot genera using complete sequences of the mitochondrial genes *cyt b* and ND2 as well as 26 plumage characters. The resulting phylogeny was used to test the monophyly of the genus, investigate species limits, and as a framework for reconstructing their historical biogeography and patterns of diversification.

Results We found that the genus *Pionopsitta*, as previously defined, is not monophyletic and thus the Chocó, Central American and Amazonian species will now have to be placed in the genus *Gypopsitta*. The molecular and morphological phylogenies are largely congruent, but disagree on the position of one of the Amazon basin taxa. Using molecular sequence data, we estimate that species within *Gypopsitta* diversified between 8.7 and 0.6 Ma, with the main divergences occurring between 3.3 and 6.4 Ma. These temporal results are compared to other taxa showing similar vicariance patterns.

Main conclusions The results suggest that diversification in *Gypopsitta* was influenced mainly by geotectonic events, marine transgressions and river dynamics, whereas Quaternary glacial cycles of forest change seem to have played a minor role in the origination of the currently recognized species.

Keywords

Amazon, biogeography, diversification, *Gypopsitta*, mitochondrial DNA, molecular systematics, parrots, *Pionopsitta*, speciation.

*Correspondence: Camila Cherem Ribas, Department of Ornithology, American Museum of Natural History, Central Park West at 79th street, New York, NY, USA. 10024. E-mail: ribas@amnh.org

INTRODUCTION

The South American lowlands harbour the richest vertebrate biota on Earth and thus have deservedly attracted considerable interest from those seeking to understand the origin of biological diversity. This region includes tropical wet forests, Amazonian and Atlantic, separated by a belt of more open, drier vegetation – the Chaco, Cerrado and Caatinga. The history of spatial change among these habitats has been

proposed as one of the most important causes of diversification in the region (the Pleistocene Refugia hypothesis of Haffer, 1969, 1993, 1997). Other theories and hypotheses to explain the great Neotropical diversity have been primarily ecological (Endler, 1977; Bush, 1994; Tuomisto *et al.*, 1995) or they have invoked landscape changes due to tectonics that lead to isolation and diversification (Wallace, 1852; Platnick & Nelson, 1978; Salo *et al.*, 1986; Cracraft & Prum, 1988). Some of these hypotheses have been tested for some groups (Patton

et al., 1994), but none is widely accepted, and all workers recognize that different explanations may not be mutually exclusive.

Patterns of diversification and historical biogeography within the lowland Neotropical biota have been investigated in a number of studies, but the history of the region and the processes that originated its great diversity remain poorly understood. Different taxa often show apparent similarities in distributions and areas of endemism, but phylogenetic patterns of the included species frequently reveal that area relationships vary from group to group, with some historical commonality but major differences as well (Cracraft & Prum, 1988; Cracraft, 1988; Aleixo, 2002; Marks *et al.*, 2002; Cortés-Ortiz *et al.*, 2003). At the same time, some authors have attempted to reconstruct the history of the lowland biota using methods that do not rely explicitly on knowledge of phylogenetic relationships, and not unexpectedly these too have revealed similarities and differences in biogeographical pattern (e.g. Bates *et al.*, 1998; Ron, 2000). Nevertheless, a general conclusion that emerges from all these studies – those explicitly phylogenetic and those not – is that the biotic elements (clades) inhabiting the tropical lowlands do not all share a single common pattern but that multiple histories are involved (Cracraft, 1988). Just how those histories might exhibit congruence in spatial vicariance, and what the relative or absolute timing of vicariance events might be are crucial questions that have been the subject of frequent debate. All workers agree, however, that additional phylogenetic studies of groups with species exhibiting congruence in their distributions (i.e. in shared areas of endemism) will be required if the complex evolutionary history of this biota is to be unravelled (e.g. Bates *et al.*, 1998; Marks *et al.*, 2002).

***Gypopsitta*: an exemplar of Amazonian diversification**

Some groups of parrots are especially characteristic of tropical wet forest and have long been of interest to those investigating lowland forest biogeography in that they have species distributed in long-recognized areas of endemism (e.g. Haffer, 1969, 1985; Cracraft, 1985). In particular, parrots in the genus *Gypopsitta* Bonaparte 1856 (traditionally classified in the genus *Pionopsitta* Bonaparte 1854; see below) occur exclusively in forest areas in South and Central America, and their geographically disjunct distributions have made this an especially suitable group for biogeographical inference (Haffer, 1985; Cracraft & Prum, 1988). In this paper, relationships among the eight phylogenetic species of *Gypopsitta* are investigated: one of these, *G. aurantiocephala*, is the most recently described parrot species (Gaban-Lima *et al.*, 2002). In addition to *G. aurantiocephala*, three species – *G. barrabandi* (Kuhl 1820), *G. caica* (Latham 1790) and *G. vulturina* (Kuhl 1820) – are distributed in the Amazon basin, whereas three others – *G. haematotis* (Sclater and Salvin 1860), *G. pulchra* (Berlepsch 1897) and *G. coccinollaris* (Lawrence 1862) – occur west of the Andes in the forests of north-western South America (Chocó area of endemism) and Central America

(Fig. 1). The eighth species, *G. pyrrhia* (Bonaparte 1853), occurs in northern South America (Fig. 1). Another species, *Pionopsitta pileata* (Scopoli 1769), formerly thought to be congeneric with these species, is also discussed below.

Cracraft & Prum (1988) undertook the only phylogenetic analysis of the group to date and included all species except the recently described *G. aurantiocephala*. Their findings were based on a cladistic analysis of plumage characters using a species in the genus *Hapalopsittaca* Ridgway 1912 as an outgroup. These authors found that the species included in *Gypopsitta sensu* this paper were grouped into two clades corresponding to taxa distributed east and west of the Andes (Fig. 2). As they noted, knowledge about the spatial and temporal history of *Gypopsitta* (= their *Pionopsitta*) is particularly important because it can provide evidence bearing on several broad questions about the history of the Amazon Basin that have interested numerous investigators (e.g. Haffer, 1967, 1969; Cracraft & Prum, 1988; Bates *et al.*, 1998; Marks *et al.*, 2002; Cortés-Ortiz *et al.*, 2003; Costa, 2003; Aleixo, 2004; Burns & Naoki, 2004): (1) whether *cis/trans* (east/west) Andean (i.e. Chocó-Central America and Amazonia) disjunctions are due to orogenic events in the northern Andes or to ecological processes of more recent origin, (2) whether their diversification within the Amazon Basin might be due to climatic oscillations that occurred mainly during the Quaternary, as postulated by the Refugia hypothesis, or to pre-Pleistocene events.

This study was designed to reanalyse the relationships postulated by Cracraft & Prum (1988) using a newly generated molecular data set and broader taxon sampling, and to explore the implications of these data for reconstructing patterns of historical biogeography within the group. We soon discovered, however, that *Pionopsitta* is not monophyletic and that the type species of the genus, *P. pileata*, is phylogenetically distant from the other species. This means that the genus name *Pionopsitta* Bonaparte 1854 is no longer available for the eight remaining species without expanding that genus to include a number of other disparate genera and species. Thus, rather than perpetuate taxonomic confusion by using *Pionopsitta* for a large clade of parrots, a name change was in order. As first revisor, we designate the name *Gypopsitta* Bonaparte 1856, the type species of which is *G. vulturina*, and we accordingly apply *Gypopsitta* to these species throughout this paper. The name *Gypopsitta* is equally as old as other available names, and because it has previously been used for a species in this clade, it maintains a semblance of nomenclatural stability.

MATERIALS AND METHODS

Samples and geographical distribution

Blood and tissue samples were obtained from 29 individuals belonging to *P. pileata* as well as to seven of the eight species of *Gypopsitta*. For the remaining species (*G. pyrrhia*), skin samples (toe pads) were obtained from two museum specimens (Table 1, specimens 21 and 22). Skin samples were also

Figure 1 Distribution of species of *Gypopsitta* (determined from locality data obtained from museum collections, see Materials and methods). Numbers correspond to locations of the samples used in this study (see Table 1).

obtained from two individuals of *G. vulturina* (specimens 8 and 9), as only two tissue samples were available. We also used one sample from a formalin-fixed specimen of *G. vulturina* (specimen 5) in order to increase the sampling.

Because the monophyly of *Pionopsitta* (including *Gypopsitta* and *P. pileata*) has not been rigorously tested by molecular data, we included in the analysis seven individuals belonging to closely related genera: *Pionus* Wagler 1832 [*P. menstruus* (Linnaeus 1766) and *P. fuscus* (Müller 1776)], *Graydidascalus* Bonaparte 1854 [*G. brachyurus* (Kuhl 1820)], *Triclaria* Wagler 1832 [*T. malachitacea* (Spix 1824)], *Hapalopsittacus* Ridgway 1912 [*H. amazonina* (Des Murs 1845)] and *Amazona* Lesson 1830 [*A. xanthops* (Spix 1824)] (but see Russello & Amato, 2003). Three individuals belonging to three different genera of

more distantly related 'long-tailed' parrots, *Pyrrhura picta* (Müller 1776), *Aratinga weddelli* (Deville 1851) and *Anodorhynchus leari* Bonaparte 1856 (see Miyaki *et al.*, 1998; Ribas & Miyaki, 2004; Tavares *et al.*, 2004), were also included as distant outgroups.

Collection locality records for all species belonging to the genus *Gypopsitta* were gathered from museum collections (Museu de Zoologia da Universidade de São Paulo, Museu Nacional do Rio de Janeiro, American Museum of Natural History, Yale Peabody Museum of Natural History, Field Museum of Natural History and Museum of Vertebrate Zoology, Berkeley) in order to construct the distribution map shown in Fig. 1. Localities corresponding to individuals included in this study are given in Table 1.

Figure 2 Relationships among species of *Gypsopsisitta* (= *Pionopsitta*) found by Cracraft & Prum (1988) based on 26 plumage characters. The tree has 29 steps: the number of character changes supporting each node is shown.

DNA extraction, amplification and sequencing

DNA extraction from blood samples was performed through incubation of a small amount of blood at 54 °C with proteinase K for c. 4 h followed by the phenol-chloroform procedure. Extraction from tissue, skin and formalin-fixed tissue samples was performed using the DNeasy tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations. Samples were incubated in proteinase K for 4 h for tissues and overnight for skin and formalin-fixed samples. DTT was added to the incubation buffer for all samples. Formalin-fixed tissue samples were extensively washed in sterile water prior to extraction. All the materials used for handling the skin and formalin-fixed tissue samples were irradiated with ultraviolet light prior to use and extra care was taken to avoid contamination. Negative controls were used during all procedures.

The entire genes that code for cytochrome *b* (hereafter *cyt b*) and for the subunit 2 of the NADH dehydrogenase (ND2) were sequenced for all samples. Sequencing two separate genes allowed us to compare the patterns of sequence evolution in this avian group and to assess the utility of each gene in resolving relationships at different phylogenetic levels. DNA extracted from fresh tissue and blood samples was amplified using 40 µL reaction volumes containing: 2.0 µL DNA solution, 1x PCR buffer (Promega, Madison, WI, USA), 2.5 mM MgCl₂, 0.8 mM dNTPs, 0.5 µM of each primer and 2 U of Taq DNA polymerase (Promega). DNA extracted from skin and formalin-fixed tissue samples was amplified in 40 µL reaction volumes containing: 2.0 µL DNA solution, 1x PCR buffer (Qiagen), 1 mM sterile dNTPs (Takara Biomedicals, OTSU, Shiga, JP), 1 µM of each primer

and 1 U of HotStarTaq DNA polymerase (Qiagen). Primer sequences are shown in Table 2.

Amplifications of the entire genes (*cyt b* and ND2) using external primers were performed and the products of these reactions were visualized by electrophoresis in 1.3% low-melting-agarose gels run in TAE (Tris-acetate low-EDTA buffer, pH 7.8). The single amplification products were cut, dissolved in 200 µL of sterile water, heated at 70 °C for 15 min and stored at room temperature. These products were then used as templates for a re-amplification reaction using internal primers, under the same conditions described above, but with higher annealing temperatures.

For amplification from DNA extracted from skins and from formalin-fixed tissues, reactions were performed using six pairs of primers for each gene (Table 2) designed to amplify fragments of c. 250 base pairs (bp) with overlap of at least 50 bp between adjacent fragments.

Amplification products were visualized through electrophoresis in agarose gels. Purification of products was performed with the QIAquick 96 PCR BioRobot kit (Qiagen) using the BioRobot 9600 (Qiagen). One to three microlitres of the purified PCR product were used as template for the 11 µL sequencing reaction using dRhod (Applied Biosystems, Foster City, CA, USA). Sequencing primers were the same used for the amplifications and re-amplifications. Ethanol precipitation of the cycle sequencing product was performed and the samples were run on a 3100 Automated DNA Sequencer following the ABI protocol.

Sequence alignment and analysis

Chromatograms were edited, aligned and confirmed using Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Nucleotide sequences were determined by comparing the heavy and light strands and visually checked for miscalls. The alignment was performed manually using MacClade 4.0 (Maddison & Maddison, 1999) and sequences were translated to confirm the correct reading frame positions and to check for the presence of stop codons.

MEGA (Kumar *et al.*, 2001) was used to calculate *p*-distances (Nei, 1987) and standard errors within and among taxa. Maximum likelihood (ML) distances using parameters selected by MODELTEST (Posada & Crandall, 1998) were calculated using PAUP*4.0b10 (Swofford, 1998). The number of transition and transversion substitutions at each codon position was plotted against *p*-distances to test for evidence of saturation due to multiple substitutions in the two genes. Evolutionary rates in the two genes were compared by plotting pairwise percentage sequence divergences in *cyt b* against those in ND2. To determine if *cyt b* and ND2 contain similar phylogenetic signal, phylogenetic congruence was evaluated via the partition homogeneity test (Farris *et al.*, 1995) with 100 replicates in PAUP* and also through comparison of separate analyses of the two data sets. Another partition homogeneity test compared the three codon positions. The uniformity of base composition at each codon

Table 1 Taxa, identification number in the present study, collection numbers, institutions and collection locality

Taxon	No.	Voucher	Institution*	Collection locality
<i>Gypopsitta aurantiocephala</i>	1	3668	USP	Cururu River, Jacareacanga, Para, Brazil
<i>Gypopsitta aurantiocephala</i>	2	2943	USP	Cururu River, Jacareacanga, Para, Brazil
<i>Gypopsitta aurantiocephala</i>	3	2944	USP	Cururu River, Jacareacanga, Para, Brazil
<i>Gypopsitta aurantiocephala</i>	4	2949	USP	Cururu River, Jacareacanga, Para, Brazil
<i>Gypopsitta vulturina</i>	5	A4955	MPEG	Tocantins River, Sacunda, Para, Brazil
<i>Gypopsitta vulturina</i>	6	B6888	NMNH	Xingu River, Altamira, Para, Brazil
<i>Gypopsitta vulturina</i>	7	B6905	NMNH	Xingu River, Altamira, Para, Brazil
<i>Gypopsitta vulturina</i>	8	429164	AMNH	Xingu River, Para, Brazil
<i>Gypopsitta vulturina</i>	9	288269	AMNH	Limoatuba, Tapajos River, Para, Brazil
<i>Gypopsitta caica</i>	10	7587	ANSP	Iwokrama Reserve, Guyana
<i>Gypopsitta caica</i>	11	7638	ANSP	Iwokrama Reserve, Guyana
<i>Gypopsitta caica</i>	12	389173	FMNH	Branco River, Boa Vista, Roraima, Brazil
<i>Gypopsitta caica</i>	13	B09175	NMNH	North-western Baramita, Guyana
<i>Gypopsitta caica</i>	14	B11726	NMNH	Upper Essequibo River, Guyana
<i>Gypopsitta caica</i>	15	B10779	NMNH	Acari Mountains, Guyana
<i>Gypopsitta barrabandi</i>	16	B9596	LSUMNS	Nicolas Suarez, Pando Department, Bolivia
<i>Gypopsitta barrabandi</i>	17	B10823	LSUMNS	Shesha River, Pando Department, Peru
<i>Gypopsitta barrabandi</i>	18	389690	FMNH	Jiparana River, Rondonia, Brazil
<i>Gypopsitta barrabandi</i>	19	389691	FMNH	Jiparana River, Rondonia, Brazil
<i>Gypopsitta barrabandi</i>	20	389693	FMNH	Jiparana River, Rondonia, Brazil
<i>Gypopsitta pyrilia</i>	21	475573	AMNH	Remedios, South-western Colombia
<i>Gypopsitta pyrilia</i>	22	133049	AMNH	Cauca River, Puerto Valdivia, Colombia
<i>Gypopsitta pulchra</i>	23	B1448	LSUMNS	Zoo/Captive
<i>Gypopsitta pulchra</i>	24	B1449	LSUMNS	Zoo/Captive
<i>Gypopsitta coccincolaris</i>	25	B2185	LSUMNS	Cerro Pire, Darien Province, Panama
<i>Gypopsitta coccincolaris</i>	26	B2201	LSUMNS	Cerro Pire, Darien Province, Panama
<i>Gypopsitta coccincolaris</i>	27	B26852	LSUMNS	Gamboa, Colon Province, Panama
<i>Gypopsitta haematotis</i>	28	5769	ANSP	Cascajilloso, Veraguas, Panama
<i>Gypopsitta haematotis</i>	29	5770	ANSP	Cascajilloso, Veraguas, Panama
<i>Pionopsitta pileata</i>	30	494/SOB396	USP	Captive
<i>Pionopsitta pileata</i>	31	3466	USP	Captive/Sorocaba Zoo
<i>Pionopsitta pileata</i>	32	3467	USP	Captive
<i>Pionopsitta pileata</i>	33	3850/SOB96787	USP	Captive
<i>Pionopsitta pileata</i>	34	3851/SOB97484	USP	Captive
<i>Pionus menstruus</i>	35	2087	USP	Acre, Brazil
<i>Pionus fuscus</i>	36	5017	KUMNH	Guyana
<i>Graydidascalus brachyurus</i>	37	1623	USP	Rio Busutuba, Ilha Caviana, Pará, Brazil
<i>Graydidascalus brachyurus</i>	38	286592	FMNH	Loreto, Peru
<i>Amazona xanthops</i>	39	1876	USP	Captive
<i>Hapalopsittaca amazonina</i>	40	32099	LSU	Cajamarca Department, Peru
<i>Trichilaria malachitacea</i>	41	415	USP	Captive
<i>Aratinga weddelli</i>	42	2085	USP	Acre, Brazil
<i>Pyrrhura picta</i>	43	5039	NMNH	Baramita, Guyana
<i>Anodorhynchus leari</i>	44	410	USP	Captive

*USP, Universidade de São Paulo; MPEG, Museu Paraense Emílio Goeldi; NMNH, National Museum of Natural History; AMNH, American Museum of Natural History; ANSP, Academy of Natural Sciences of Philadelphia; FMNH, Field Museum of Natural History; LSUMNS, Louisiana State University Museum of Natural History; KUMNH, Kansas University Museum of Natural History.

position was evaluated through a chi-square test of homogeneity.

Phylogenetic analysis was undertaken for each gene independently (cyt *b*, 1140 bp and ND2, 1041 bp) and in combination (2181 bp), employing maximum parsimony (MP) and ML using PAUP* as well as with Bayesian analysis using MrBayes v3.0 b4 (Huelsenbeck & Ronquist, 2001).

MP analyses were implemented with heuristic tree searches, tree bisection reconnection (TBR) branch swapping, and 100 random addition sequence replications. Searches were performed using equally weighted characters and including all substitutions. The relative support for inferred monophyletic groupings was determined using 1000 bootstrap replications and decay indices (Bremer, 1994) calculated with Autodecay

Table 2 Primers used to amplify *cyt b* and ND2

Gene	Primers	Sequence 5'–3'	Reference
<i>Cyt b</i> (tissues)	N5L14750	GGACCAGAAGGACTTGCCGACCTA	This paper
	CBH15422	GGTGGGGTTGTCTACGGAGAA	This paper
	CBL 15298	TGAGGCCAAATATCAITCTGAGGGGC	Cheng <i>et al.</i> (1994)
	CBH 15764	CCTCCTAGTTTGTGGGATTGA	Miyaki <i>et al.</i> (1998)
	CBL 15507	AACCTACTAGGAGACCCAGA	J. Feinstein (pers. comm.)
	HB20	TTGGTTCACAAGACCAATGTT	J. Feinstein (pers. comm.)
<i>Cyt b</i> (skins and formalin-fixed tissue)	N5L 14839	GATCGTTCGCACTATCCACC	This paper
	CBH 15120	GGAGGTTCGGATTAGTCAACCG	
	CBL 15062	CTGCAGACACCTCCCTAGCC	
	CBH 15315	GTGATAACTGTGGCTCCTCAG	
	CBL 15249	CCTCCTACTCACCTCATAGC	
	CBH 15516	TTAGATGAAATGCCTAGGGGG	
	CBL 15462	CACCAGCCTAGTCCTCGTCC	
	CBH 15708	GCGTATGCGAATAGGAAGTATCAT	
	CBL 15660	CTTCACCCCAGCAAACCCCTAG	
	CBH 15902	ACTGGTTGACTTCCTACTCATG	
	CBL 15845	TCCGCCCGCCTCACAACCTCC	
	ThrH 16058	GTTCACAAGACCAATGTT	
ND2 (tissues)	LMet	GGCCCATACCCGAAAATGA	J. Groth (pers. comm.)
	H5764	GAGAAGCTAGGATTTTCGTG	P. Brito (pers. comm.)
	L5602	GATTCCCAGAAGTTCTTCAAGG	P. Brito (pers. comm.)
	H6313	CTCTATTTAAGGCITTGAAGGC	Sorenson <i>et al.</i> (1999)
ND2 (skins and formalin-fixed tissue)	Met L5215	TGATGGTTCAACCCCTTCCCT	This paper
	H5485	CCTGTGTGTTGTGCGTTGATTGTGC	
	L5394	GCCATTGAAGCAGCAACCAAATA	
	H5636	TGTTGAAAGGAGTATGGCAGT	
	L5574	CACITTTGATTTCCAGAAGTACTTC	
	H5823	CAATGGTGATTATTATTCA	
	L5745	GGATTAACCAAACACAAACACG	
	H6005	AGGGAGGCCCGATAATGATAG	
	L5932	TAACCTCATGAACCAAGGCCCC	
	H6180	GCTTTATTTTGCTGAGGGGTT	
	L6108	CTGTTCTTCTACCTACGCCTAGC	
	Trp H6313	GAAGGCCCTTTGGTTTGTTATCCTAAG	

version 4.0 (Eriksson, 1999; 100 random-addition replicates per tree).

The likelihood ratio test as implemented in *MODELTEST* (Posada & Crandall, 1998) was used to select the simplest model of molecular evolution yielding a significantly higher likelihood than others. The model selected was used for the ML analyses, which were implemented using heuristic tree search, TBR branch swapping and 10 random addition replicates. Branch support on the ML tree was assessed by 100 bootstrap replications using a starting tree obtained by neighbour-joining and SPR (subTree pruning-regrafting) branch swapping. In order to understand better the sensitivity of data to the different ML parameters, Bayesian inference of phylogeny was implemented using *MrBayes* v3.0 b4 (Huelsenbeck & Ronquist, 2001). Following the ML models that were selected by *MODELTEST* for each data set (*cyt b* and ND2), we chose a GTR + I + Γ model of sequence evolution. The analysis was run using partitioned likelihood, so that the parameters could vary independently for the two genes in the

combined analysis. Four simultaneous chains were run for 1,000,000 generations with trees being sampled every 100 generations for a total of 10,000 trees. The ML scores became stable (stopped improving) around the 1000th tree, so that burn-in was completed by the 1000th tree, and 9000 trees were kept in each analysis. Three independent analyses were performed for the combined data set and the 27,000 sampled trees were used to compute the posterior probabilities of each node.

Morphological analysis

External character variation among the species of *Gypopsitta* was analysed using specimens housed in the American Museum of Natural History (New York). As described below, the tree was rooted using outgroup comparison. The characters defined previously by Cracraft & Prum (1988) were re-examined and scored again, and some were discarded and new ones were added to the analysis. The character matrix was

constructed in MacClade 4.0 and analysed in PAUP* using MP with unordered characters.

Tests for comparisons of different topologies

Different tests have been proposed to compare a topology obtained in a phylogenetic analysis with another topology derived from a different data set or different method of phylogenetic reconstruction. Templeton's test (Templeton, 1983) and the Kishino–Hasegawa test (Kishino & Hasegawa, 1989) were the first ones used for this purpose, but both have been criticized as being inappropriate for comparing trees that have not been defined *a priori* (Goldman *et al.*, 2000). Alternative tests that have been proposed include the Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999), the SOWH test (Goldman *et al.*, 2000), and the parametric bootstrap test (Huelsenbeck *et al.*, 1996), and we used these to compare the topologies obtained in the molecular and morphological analyses. All three tests were performed using a reduced molecular data set containing only one representative of each species of *Gypopsitta* and two outgroups (matrix with 11 taxa, 2181 bp), with the morphological topology being used as the null hypothesis. Analysis of the reduced data set resulted in the same relationships among taxa as were found using all individuals.

The Shimodaira–Hasegawa test was performed in PAUP*. The SOWH test was performed following the protocol suggested by Anderson, Goldman and Rodrigo (Guidelines for performing the SOWH test, <http://www.ebi.ac.uk/goldman/tests/SOWHinstr.html>): 100 simulated data sets were generated via parametric bootstrapping (REV, GTR model) using the program Seq-Gen 1.2.6 (Rambaut & Grassly, 1997); for each simulated data set the ML topology was obtained and its log likelihood compared to the log likelihood of the null hypothesis (the topology obtained from morphological data). The distribution of the differences in log likelihood was used to determine the null distribution for a one-tailed test at a significance level of 5% (Goldman *et al.*, 2000). The test was performed with full optimization, using the reduced data set, TBR branch swapping, and with a starting tree obtained by neighbour-joining. For the parametric bootstrap test, another set of simulated data sets was obtained using Seq-Gen 1.2.6 (REV, GTR) with parameters estimated from the ML topology. The differences in length between the trees obtained from each data set and the null hypothesis were used to determine the null distribution, and a one-tailed test at a significance level of 5% was used to test for the rejection of the null hypothesis.

Molecular dating of divergence times

A likelihood ratio test assuming a chi-square distribution with number of taxa minus two degrees of freedom (Huelsenbeck & Rannala, 1997) was applied to the *cyt b*, ND2, and combined matrices containing only the species belonging to *Gypopsitta* and *H. amazonina* as outgroup (see Results) in order to

compare the log-likelihood values from ML trees constructed with or without a molecular clock constraint. In addition, branch length and two cluster tests were applied to these matrices using Lintree (Takezaki *et al.*, 1995).

The absence of a reliable psittacid fossil record or geological calibration point prevents a calculable estimate of a local substitution rate. As an alternative solution, a range of divergence times was estimated using the branch lengths of the ML tree and applying substitution rates that have been estimated for other bird species (see below). Branch lengths and standard errors for the ML tree were calculated using Lintree (Takezaki *et al.*, 1995). Two different rates of *cyt b* sequence divergence were used, one based on a geological calibration of island formation (1.6% Myr⁻¹, Fleischer *et al.*, 1998) and the other locally calibrated for other bird species using fossil records (2.0% Myr⁻¹, Shields & Wilson, 1987; Randi, 1996).

RESULTS

Molecular characterization

Sequences from the entire *cyt b* (1140 bp) and ND2 (1041) genes were obtained for all individuals studied (GenBank accession numbers: AY669400–AY669487). All sequences were easily aligned manually and no indels were present. Gene sequences began with the ATG (*cyt b*) or ATA (ND2) start codons and both terminated with TAA stop codon for all individuals. Although nuclear copies of mitochondrial genes in birds have been reported before (Quinn, 1997), there is evidence that we have sequenced functional mtDNA genes: (1) we performed long amplifications of both genes for most individuals studied; (2) when using internal primers to perform the amplifications from skin and formalin-fixed tissue samples, we utilized specially designed, and very specific, primers; we sequenced fragments that overlapped by at least 50 bp and found no ambiguities or differences in these regions; (3) there were no unexpected stop codons; (4) no contaminants were detected in the PCR products; and (5) all sequences obtained aligned easily to other psittacine mitochondrial sequences.

Variable and parsimony informative sites, base composition and transition/transversion (Ti/Tv) ratios are shown for each gene and for the combined data in Table 3. Base composition analyses showed a higher frequency of adenine and cytosine in both genes, mainly at third positions (Table 3). No base composition bias was detected among taxa in the data sets, including variable sites only ($P = 0.99$ for *cyt b* and $P = 0.72$ for ND2), and considering each codon position independently ($P = 1$ for first and second positions in *cyt b* and ND2; $P = 0.96$ for third position in *cyt b* and $P = 0.54$ for third position in ND2). The results of the partition homogeneity test (Farris *et al.*, 1995) on the combined data set revealed significant congruence between genes ($P = 0.53$) and also among codon positions for both genes ($P = 0.91$ for ND2 and $P = 0.72$ for *cyt b*).

Gene	Cyt <i>b</i>	ND2	Combined
Number of base pairs	1140	1041	2181
Number of individuals	44	44	44
Variable first/second/third	108/32/314	141/78/287	249/110/601
Informative first/second/third	82/18/269	104/50/235	186/68/504
%A first/second/third	0.266/0.204/0.378	0.391/0.161/0.419	0.325/0.184/0.397
%C first/second/third	0.289/0.280/0.480	0.280/0.368/0.407	0.285/0.322/0.445
%G first/second/third	0.205/0.127/0.044	0.132/0.089/0.046	0.170/0.109/0.045
%T first/second/third	0.239/0.388/0.098	0.197/0.381/0.127	0.219/0.385/0.112
Ti/Tv ratio	9.95	6.53	7.85
rAC	1.0822	0.3295	0.5167
rAG	24.6281	11.0585	16.3688
rAT	1.2988	0.3012	0.5076
rCG	0.3497	0.2059	0.2328
rCT	24.6281	4.6667	8.1367
α	1.9249	1.5953	1.8819
pinv	0.5417	0.4067	0.4838
Tree length (ML)	1.64	1.89	2.79
-ln L	6627.3698	6688.57024	7290.31981
No. trees (MP)	12	36	3
Tree length (MP)	1123	1172	1578

Table 3 Variable and informative sites and base composition for each gene and for the combined data set

Comparisons of pairwise distances between the two genes showed a similar pattern of rates of sequence divergence, with *cyt b* evolving slower than ND2 at divergence levels $c. > 5\%$ (overall slope 0.667) (Fig. 3). All transversion substitutions (data not shown) and transition substitutions at first and second positions increased linearly with percentage sequence divergence in both genes (Fig. 3). Third position transitions showed evidence of multiple hits only when sequences from other genera (other than *Gypopsitta*) were included in the comparisons (Fig. 3). Multiple hits in *cyt b* were evident at levels of sequence divergence above 8% and in ND2 multiple hits occurred only above 12% divergence.

For the measures of uncorrected pairwise sequence divergence, individuals belonging to each taxon were grouped and the distances and standard errors were measured within and between groups (Tables 4 and 5). Intra-specific divergence ranged from 0.1% to 1.2% while inter-specific divergences ranged from 1% to 16% (excluding the long-tailed outgroups). Distances between *Gypopsitta* species and *P. pileata* ranged from 10.8% to 11.9% in *cyt b* and from 15.1% to 16.0% in ND2 sequences. Comparisons among *Gypopsitta* species never exceeded 8.1% in *cyt b* and 10.9% in ND2.

The smallest inter-specific genetic distances were found between individuals of *G. vulturina* and *G. aurantiocephala* (1.0% in *cyt b* and 1.5% in ND2). There were 20 variable sites in the *cyt b* sequences of individuals of *G. aurantiocephala* ($n = 4$) and *G. vulturina* ($n = 5$). Of these 20, only two were exclusive to *G. vulturina* and thus diagnostic, whereas five were diagnostic for *G. aurantiocephala*. There was one amino acid replacement (position 199) that was exclusive to all individuals of *G. aurantiocephala*. In the ND2 sequences, there were 28 variable sites, with six being diagnostic for each species. There were also two amino acid differences (positions 91 and

310) exclusive to *G. aurantiocephala* and one (position 193) exclusive to *G. vulturina*.

Phylogenetic analyses

Independent MP analyses with all characters unweighted were performed on 44 terminals for *cyt b* (1140 bp), ND2 (1041 bp), and the combined data set (2181 bp). These analyses resulted in 12 most-parsimonious trees for the *cyt b* data, 36 trees for ND2, and three trees for the combined analysis (see Table 3 for trees characteristics). Within each data set trees were congruent, with the only differences being in the relationships among individuals of the same species-level taxon. Topologies obtained for the three different data sets were also largely congruent, the only difference being a basal polytomy when *cyt b* data were analysed independently. The topology found for the combined data is shown in Fig. 4, along with bootstrap values (1000 replicates using heuristic searches) and decay indices (Bremer, 1994). *Hapalopsittaca amazonina* is the sister group to the eight *Gypopsitta* species with high support. This clade, in turn, appears as sister to the *Pionus/Graydidascalus/Amazona* clade but with low support, and *P. pileata*, is sister to *Triclaria malachitacea*.

The results from MODELTEST indicated that the most complex model of sequence evolution (general time reversible with a proportion of invariant sites and rates at variable sites following a Γ frequency distribution, GTR + I + Γ) offered a significant increase in likelihood over less complex models for all three data sets (details in Table 3). ML analyses of each data set resulted in similar topologies, the only difference, again, was a basal polytomy in the *cyt b* analysis. The topology found in the combined analysis is shown in Fig. 5, along with ML bootstrap values and Bayesian posterior probabilities as

Figure 3 Saturation analyses. (a, b) Pairwise transition sequence distance (absolute number of changes) plotted against total sequence distance (p -distance) for first (diamonds), second (squares) and third (triangles) codon positions for cytochrome *b* and ND2. Solid symbols indicate comparisons inside *Gypopsitta*. (c) Pairwise p -distances for cytochrome *b* plotted against those for ND2.

measures of support. Both ML and Bayesian analyses found the same topology, and those were nearly congruent with that found using MP, the only difference being that in MP *T. malachitacea* groups with *P. pileata*, while in ML analysis this does not happen (Fig. 4). The relationships among the species of *Gypopsitta* were the same in all analyses.

All analyses discovered the non-monophyly of the genus *Pionopsitta* as defined in the traditional taxonomy. *Hapalopsittaca* groups with the eight *Gypopsitta* species with high support. The node uniting *Gypopsitta* and *Hapalopsittaca* with the clade composed of *Pionus*, *Graydidascalus* and *Amazona* had low support in all analyses. The node uniting all these taxa

Table 4 p -distances within groups of individuals belonging to the same taxon

	<i>n</i>	p -distance (SE)	
		Cyt <i>b</i>	ND2
<i>G. aurantiocephala</i>	4	0.002 (0.001)	0.001 (0.001)
<i>G. vulturina</i>	5	0.003 (0.001)	0.005 (0.001)
<i>G. caica</i>	6	0.004 (0.001)	0.008 (0.002)
<i>G. barrabandi</i>	5	0.008 (0.002)	0.004 (0.001)
<i>G. pyrilia</i>	2	0.007 (0.003)	0.003 (0.002)
<i>G. pulchra</i>	2	0.010 (0.003)	0.005 (0.002)
<i>G. coccincolaris</i>	3	0.012 (0.003)	0.001 (0.001)
<i>G. haematotis</i>	2	0.009 (0.003)	0.003 (0.002)
<i>P. pileata</i>	5	0.005 (0.002)	0.001 (0.001)

n, Number of individuals; SE, standard error.

to *Triclaria* had high support in ML and Bayesian analyses. *Pionopsitta pileata* always appeared in a basal position. Also, in all analyses, *Gypopsitta* was divided into two well-supported clades showing strong *cis/trans* Andean distributions (see below).

Morphological analysis

The relationships revealed by the new molecular data (Figs 4 & 5) are not entirely congruent with those proposed by Cracraft & Prum (1988) on the basis of morphology (Fig. 2), especially among the species taxa of the Amazon basin. In the 1988 morphological analysis of Cracraft & Prum, however, relationships within *Pionopsitta* were examined assuming that the genus as traditionally defined (including *P. pileata*) was monophyletic. *Hapalopsittaca melanotis* was then used to root that tree. As in the present molecular analyses, that morphological analysis found *P. pileata* to lie outside the other species, which were divided into two major *cis/trans* Andean clades. Because the molecular data strongly support removing *P. pileata* from the genus, it is possible that morphological character optimizations in the original study were compromised due to the assumption of *Pionopsitta* monophyly. We therefore undertook a new analysis of the morphological data in order to understand better phenotypic evolution within *Gypopsitta* (see Table 6).

Morphological comparisons using a range of taxa included in the molecular analyses (Figs 4 & 5) supported the hypothesis that *Hapalopsittaca* is closely related to *Gypopsitta*. Thus, the two genera share a number of characters not generally found in the other distantly related genera used in the molecular analysis, including: (1) a diffuse upper breast patch that is green and tinged brownish or yellowish, (2) under-tail coverts that have lost red pigment, (3) the loss of a strong, distinct notch along the edge of upper mandible, and (4) the loss of dark blue feathers on the upper breast and/or belly. Although additional work on generic interrelationships will be necessary using increased taxon sampling, molecular and

Table 5 *p*-distance among groups of individuals belonging to different taxa, *cyt b* below diagonal, ND2 above

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 <i>G. aurantiocephala</i>	—	0.015	0.043	0.055	0.063	0.095	0.098	0.103	0.155	0.150	0.148	0.141	0.152	0.148	0.177
2 <i>G. vulturina</i>	0.010	—	0.045	0.056	0.063	0.093	0.095	0.103	0.153	0.151	0.150	0.141	0.148	0.151	0.181
3 <i>G. caica</i>	0.047	0.042	—	0.064	0.070	0.092	0.097	0.106	0.152	0.148	0.146	0.141	0.151	0.144	0.178
4 <i>G. barrabandi</i>	0.061	0.058	0.063	—	0.048	0.094	0.092	0.099	0.151	0.146	0.149	0.146	0.154	0.140	0.175
5 <i>G. pyrrhura</i>	0.049	0.050	0.066	0.058	—	0.105	0.101	0.109	0.160	0.139	0.148	0.142	0.149	0.145	0.180
6 <i>G. pulchra</i>	0.071	0.071	0.072	0.081	0.080	—	0.052	0.059	0.155	0.144	0.141	0.137	0.148	0.151	0.177
7 <i>G. coccincolaris</i>	0.071	0.071	0.067	0.074	0.075	0.048	—	0.026	0.153	0.142	0.137	0.135	0.136	0.150	0.180
8 <i>G. haematotis</i>	0.069	0.070	0.071	0.077	0.073	0.061	0.046	—	0.156	0.146	0.145	0.139	0.141	0.151	0.180
9 <i>P. pileata</i>	0.116	0.116	0.112	0.118	0.112	0.119	0.108	0.110	—	0.140	0.165	0.123	0.142	0.140	0.169
10 <i>Pionus</i>	0.090	0.090	0.087	0.093	0.092	0.098	0.087	0.087	0.105	—	0.141	0.097	0.124	0.130	0.157
11 <i>Hapalopsittaca</i>	0.114	0.115	0.109	0.108	0.115	0.114	0.111	0.107	0.138	0.110	—	0.142	0.150	0.161	0.183
12 <i>Graydidascalus</i>	0.107	0.106	0.107	0.112	0.109	0.116	0.110	0.102	0.125	0.103	0.135	—	0.083	0.111	0.147
13 <i>Amazona</i>	0.098	0.096	0.094	0.108	0.105	0.111	0.099	0.096	0.116	0.080	0.122	0.090	—	0.129	0.164
14 <i>Triclaria</i>	0.106	0.106	0.105	0.105	0.105	0.114	0.108	0.098	0.110	0.095	0.120	0.103	0.097	—	0.160
15 Outgroups	0.127	0.126	0.123	0.124	0.125	0.142	0.124	0.127	0.130	0.110	0.145	0.135	0.122	0.129	—

Figure 4 Maximum parsimony analysis of combined data (2181 bp): one of the three topologies found in PAUP. MP bootstrap proportions and decay indices, respectively, are shown on nodes.

morphological data currently available support the hypothesis that *Hapalopsittaca* is the likely sister group to *Gypopsitta* and therefore is an appropriate outgroup taxon.

Twenty-six plumage characters were scored for *H. amazonina* and the eight taxa of *Gypopsitta* (Table 6). *Hapalopsittaca*

amazonina was included instead of *H. melanotis*, which was used originally by Cracraft & Prum (1988), because tissue samples for DNA analysis were only available for the former species. Phylogenetic analysis was conducted using MP implemented in PAUP* and the resulting topology is shown in

Figure 5 Maximum likelihood analysis of combined data (2181 bp): only topology found in PAUP. ML bootstrap values (100 replications) and Bayesian posterior probabilities, respectively, are shown on nodes.

Fig. 6. This pattern of relationships differs from the one found by Cracraft & Prum (1988) in that *G. barrabandi* was found to be the sister group to *G. pyrilia* instead of *G. vulturina* (compare Figs 2 & 6), but *G. caica* remains as the basal species of the Amazonian clade. Both morphological topologies (Figs 2 & 6) differ from the molecular results, in which *G. caica* appears as the sister group to *G. vulturina* and *G. aurantiocephala* (Figs 4 & 5).

Combined analysis

In order to investigate the phylogenetic position of *G. caica* further, we undertook a combined analysis. The morphological characters for all *Gypopsitta* species as well as for *H. amazonina* were added to the molecular data, thus creating a matrix of 2207 characters for 30 terminals. An MP analysis of these data (not shown) resulted in three most-parsimonious trees of 951 steps (consistency index excluding uninformative characters = 0.627), with the trees only differing by rearrangements among individuals of the same species. All three trees recovered *G. caica* as the sister group of [(*barrabandi* + *pyrilia*) + (*vulturina* + *aurantiocephala*)], a topology

like that for morphology alone (also as in Cracraft & Prum, 1988). In the molecular analyses, *G. caica* grouped with *G. vulturina* and *G. aurantiocephala* with moderate to strong support (Figs 4 & 5), whereas with morphological data *G. caica* was the sister group of the Amazonian taxa with strong support (Fig. 6). In the combined analysis, *G. caica* is the sister group to the other Amazonian taxa with very weak support (< 50% bootstrap for 1000 replicates). Although the relationships found by the molecular data have moderate to strong support, it required only a few conflicting morphological characters to move *G. caica* to a basal position within the Amazonian clade.

Parametric bootstrap tests were used to test if the molecular data set rejected the topology found in the morphological analysis. Both the parametric test based on MP and the Shimodaira–Hasegawa test did not reject the null hypothesis (the morphological topology) ($P > 0.05$, Table 7), agreeing with the results found in the combined (molecular + morphology) analysis, thus suggesting uncertainty in both the molecular and morphological data regarding the position of *G. caica*. On the other hand, the likelihood-based SOWH test did reject the null hypothesis ($P < 0.001$, Table 7), thus showing that there is phylogenetic signal in the molecular data

Table 6 Morphological character matrix for 26 characters for eight *Gypopsitta* species and for the outgroup taxon, *Hapalopsittaca amazonina*

Taxa	Characters*																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
<i>H. amazonina</i>	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
<i>G. coccincolaris</i>	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	2	0	0	0	1	0	0	1	1	1
<i>G. haematotis</i>	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	2	0	0	0	1	0	0	1	1	1
<i>G. pulchra</i>	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	1	0	1	0	0	1	0	0
<i>G. barrabandi</i>	1	1	1	0	1	0	1	1	0	1	0	1	0	0	0	0	0	1	0	1	0	1	1	0	0	0
<i>G. vulturina</i>	1	1	1	0	1	0	1	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0
<i>G. pyralia</i>	1	1	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	1	0	0	0
<i>G. caica</i>	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0
<i>G. aurantiocephala</i>	1	1	1	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0

*Characters and character states are defined as follows: (1) red pigmentation on the lesser wing coverts and leading edge of wing: 0 (absent), 1 (present); (2) bright red pigmentation on greater underwing coverts: 0 (absent), 1 (present); (3) shoulder patch with distinct yellow/red/orange (carotenoid) pigmentation: 0 (absent), 1 (present); (4) upper breast dark red-tinged brownish-green: 0 (absent), 1 (present); (5) upper breast light yellowish-green: 0 (absent), 1 (present); (6) red on upper breast: 0 (absent), 1 (present); (7) belly with iridescent blue sheen to green feathers: 0 (absent), 1 (present); (8) tibial feathering with extensive yellow or orange: 0 (absent), 1 (present); (9) head feathers reduced on anterior portion of crown: 0 (absent), 1 (present); (10) forehead, crown, and nape moderately to heavily melanized: 0 (absent), 1 (present); (11) forehead, crown, and nape dark grey-green: 0 (absent), 1 (present); (12) crown and nape black: 0 (absent), 1 (present); (13) crown, nape and hindneck yellow: 0 (absent), 1 (present); (14) yellow collar on hindneck: 0 (absent), 1 (present); (15) ill-defined yellow-orange band at juncture of neck and back: 0 (absent), 1 (present); (16) red on auriculars: 0 (absent), 1 (present); (17) size of red patch on auriculars (character 16): 0 (absent, inapplicable), 1 (large), 2 (small); (18) deep yellow-orange malar patches: 0 (absent), 1 (present); (19) face extensively pinkish-red: 0 (absent), 1 (present); (20) basal portion of inner web of tail yellow (ventral view): 0 (absent), 1 (present); (21) red on inner web of tail (ventral view): 0 (absent), 1 (present); (22) lores only partly feathered: 0 (absent), 1 (present); (23) trailing edge of primaries green (ventral view): 0 (absent), 1 (present); (24) trailing edge of primaries blue or blue-green (ventral view): 0 (absent), 1 (present); (25) crown and nape dark olive in tone, forming well-defined border posteriorly with green of upper back: 0 (absent), 1 (present); (26) malar and throat dark greyish: 0 (absent), 1 (present).

that conflicts with the morphological data regarding the position of *G. caica*.

Molecular clock

For the molecular clock analyses, matrices containing only *Gypopsitta* species and *H. amazonina* were constructed (30 terminals). The likelihood ratio test showed that there was no significant difference between the $-\ln L$ values for the unconstrained cyt *b* ML tree (3803.1933) vs. the clock-enforced tree (3819.4637) ($P > 0.30$), but did show significant differences for the ND2 and combined data sets, and thus only cyt *b* data will be used here for inference of divergence times. The branch length and two cluster tests implemented in Lintree (Takezaki *et al.*, 1995) indicated no relative rate differences among the sequences included in the cyt *b* matrix.

The branch lengths of the ML tree constructed using the parameters chosen by MODELTEST for the corresponding matrix (TrN + Γ , $\alpha = 0.1747$) were used to estimate the divergence times. Two estimates of rates of cyt *b* sequence evolution (1.6% Myr⁻¹, Fleischer *et al.*, 1998; 2.0% Myr⁻¹, Shields & Wilson, 1987; Randi, 1996) were applied in order to encompass the range of rates reported for the gene. From these, two divergence dates and standard errors (corresponding to the errors associated with the distance measures) were obtained for each node (Fig. 7).

DISCUSSION

Saturation analyses and comparative utility of mtDNA genes for phylogenetic reconstruction

Both genes showed signs of saturation at third positions when the pairwise comparisons included outgroups (outside *Gypopsitta*). As reported for other groups of birds (Griffiths, 1997), cyt *b* sequences become saturated at distances above 9% divergence. Variable sites were more concentrated at third positions in cyt *b* sequences (69.2% of the variable sites for the complete matrix) than in ND2 sequences (56.7%). ND2 was more variable in general: this gene has also been found to be more variable than cyt *b* in other groups of birds (Hackett, 1996) and this is related, in our data set, to a greater accumulation of substitutions in first and second positions in ND2.

In their study of waterfowl Johnson & Sorenson (1998) concluded that ND2 and cyt *b* evolve at similar rates if the time since divergence has been relatively recent, and that a large part of their sequence evolution is governed by base composition bias and transition/transversion bias. They also found that the transition/transversion bias was similar in the two genes. In these parrots we found differences in rates of evolution (Fig. 3) and in transition/transversion rates (Table 3). These results may be due to greater evolutionary divergence among the sequences studied here, but may also

Figure 6 Topology resulting from the MP analysis of the revised morphological matrix, containing 26 plumage characters. The tree has 32 steps. The number of character changes supporting each node is shown above branches, bootstrap support values are shown below branches.

be an indication that these two protein-coding genes present some differences in their patterns of sequence evolution and thus may have different utilities in reconstructing phylogenies.

Molecular systematics

The phylogenetic results strongly confirm that the genus conventionally recognized as *Pionopsitta* is not monophyletic. The type species of the genus, *P. pileata*, does not group with the other species traditionally included in the genus and instead is separated from them by at least two branching events. Our results strongly identify *H. amazonina* as the sister group to *Gypopsitta*. Lying outside these taxa, moreover, is a well-supported clade containing the genera *Pionus*, *Graydidascalus* and *Amazona xanthops*, a relationship found independently by Russello & Amato (2004). Together, *Pionus*, *Graydidascalus* and *Amazona xanthops* are resolved as the sister group of *H. amazonina* + *Gypopsitta*, but the branch support

for this relationship is low. *Triclaria malachitacea* appears as sister group to *P. pileata* in unweighted MP analyses with medium support (69%, see Fig. 4) but in the ML analysis *P. pileata* lies outside *T. malachitacea* and the other taxa (Fig. 5).

Relationships among species

In our analyses, mtDNA haplotypes in the ingroup revealed seven clearly defined clusters, supported by high bootstrap values (79–100%), that correspond to six of the seven species recognized by Cracraft & Prum (1988). The seventh species, *G. aurantiocephala*, was only described recently and appears as the sister species to *G. vulturina*. Both *G. vulturina* and *G. aurantiocephala* individuals are reciprocally monophyletic in all analyses. These results provide additional strong support for the validity of the new species as well as for its relationship to *G. vulturina*. The phylogeny also reveals that *cis/trans* Andean clades represent monophyletic groups, as postulated by Cracraft & Prum (1988), and both these clades have high support.

In both MP and ML analyses (Figs 4 & 5) *G. vulturina* and *G. aurantiocephala* were found to be sister to *G. caica* with moderate to strong support, and *G. pyrilia* and *G. barrabandi* were moderately supported as sister taxa. This arrangement contradicts the phylogeny proposed by Cracraft & Prum (1988) in which *G. vulturina* is sister to *G. barrabandi*, *G. pyrilia* is their sister group, and *G. caica* has a basal position (Fig. 2). In the Cracraft and Prum analysis, the *G. barrabandi* + *G. vulturina* clade was supported by five exclusive characters and one parallelism shared with *G. caica*. The reanalysis of the morphological data presented here recovers a clade composed of [(*G. barrabandi*, *G. pyrilia*), (*G. vulturina*, *G. aurantiocephala*)], with *G. caica* appearing as their sister group (Fig. 6). Although this arrangement has strong support in the morphological data, and is also recovered in the combined analysis, the basal position of *P. caica* has extremely weak support in this last analysis (see Results).

The mtDNA phylogeny is well-resolved and nearly all nodes are strongly supported. Thus, assuming that *G. caica* is the sister group to (*G. vulturina*, *G. aurantiocephala*), its basal position in the morphological and combined trees could be explained either by its having retained multiple ancestral character states or by having regained them, depending on how character changes are optimized. In either case *G. caica* would have the primitive condition for these characters relative to the other four species. Although we judge the molecular topology to be our best estimate of relationships, additional data will be required to resolve *G. caica*'s position more completely.

Table 7 Tests of the null hypothesis (H_0) of the topology resulting from the morphological analyses

Test	Critical value for difference at 5% confidence level	Observed difference	P-value	Rejection of H_0
SOWH (ML)	0.97	9.59	< 0.001	Yes
Parametric bootstrap (MP)	3.2	2.0	> 0.05	No
Shimodaira–Hasegawa (ML)	–	9.90	0.075	No

Figure 7 Maximum likelihood analysis of *cyt b* data for the ingroup only, with the molecular clock enforced. The accompanying table shows, for each node, the geographical disjunction, the corresponding distance with standard error, and the estimated divergence dates using the rates of 2.0% and 1.6% divergence Myr^{-1} . The minimum and maximum estimated age of divergence for each node (in parentheses) were calculated based on the standard error for the distance measure associated with that node.

Node	Geographical separation	ML distance (%)	Date 2.0% (Ma)	Date 1.6% (Ma)
1	Cis/trans Andes	13.91 $\pm$ 0.21	6.95 (6.84–7.06)	8.69 (8.56–8.82)
2	E/W Amazonia	10.18 $\pm$ 0.19	5.09 (4.99–5.19)	6.36 (6.24–6.48)
3	W Amazonia/NW Venezuela	9.86 $\pm$ 0.59	4.93 (4.63–5.23)	6.16 (5.79–6.53)
4	Guianas/Pará	6.66 $\pm$ 0.18	3.33 (3.24–3.42)	4.16 (4.05–4.27)
5	Belém-Pará/Rondonia	1.14 $\pm$ 0.08	0.57 (0.53–0.61)	0.71 (0.66–0.76)
6	Chocó/Central America	9.32 $\pm$ 0.57	4.66 (4.38–4.94)	5.83 (5.47–6.19)
7	N/SCentral America	7.86 $\pm$ 0.62	3.93 (3.62–4.24)	4.91 (4.52–5.30)

Genetic distances and species limits

The genetic distance between *G. vulturina* and *G. aurantiocephala* was significantly smaller than the distances among the other species (1.0% in *cyt b* and 1.5% in ND2). Nevertheless, the existence of diagnostic morphological (Gaban-Lima et al., 2002) and molecular characters (see Results) supports their recognition as different species under the Phylogenetic Species Concept *sensu* Cracraft (1983), Nixon & Wheeler (1990) and Davis & Nixon (1992). Better sampling will be needed to refine distributional limits of these species and confirm the absence of intermediate forms in nature.

Gypopsitta coccincolaris has been considered a subspecies of *G. haematotis* by some authors (Haffer, 1970; Forshaw, 1989), while Cracraft & Prum (1988) consider these two taxa as separate species. These species occur in southern and northern Central America and there seems to exist a region of sympatry in Panama. In the present study, one of the sampled *G. coccincolaris* individuals (number 27) is from a locality that is closer to those of the two *G. haematotis* specimens from Veraguas than it is to the other two *G. coccincolaris* collected in Darien Province (see Fig. 1). Despite the geographical proximity and phenotypic similarity between these two species, interspecific distances were markedly higher (2.6% in ND2, 4.6% in *cyt b*) than the intraspecific distances (0.1–1.2%), indicating the existence of an effective barrier separating these two species in the past.

Spatial and temporal patterns of diversification of *Gypopsitta*: testing biogeographical hypotheses

The biogeographical interpretations discussed below are based on the molecular phylogenetic results as well as on

the range of divergence times estimated from the ML analysis and molecular clock calculations (Fig. 7). Drawing correlations, let alone causal relationships, between biogeographical patterns such as area relationships, on the one hand, and Earth history, on the other, is difficult. Events of Earth history that result in vicariance, such as mountain uplift or the initiation of river barriers, generally are established over long periods of time (relative to taxonomic differentiation) and determining when those geological changes might be sufficient to isolate populations of organisms will often be uncertain. Likewise, a description of the biogeographical history of a single group, such as the parrots discussed here, cannot lead by itself to biogeographical generalizations without comparison to other groups (Nelson & Platnick, 1980). Consequently inferences about common causation, such as vicariance events, are limited.

Cis/trans (east/west) Andean disjunction

The two major lineages of *Gypopsitta* are distributed on opposite sides of the Andes (Fig. 1), with the west Andean *haematotis* species group occurring from the Chocó area of endemism in Colombia, through Central America to southern Mexico, whereas the remaining species are distributed within Amazonia and northern South America. For decades investigators have recognized a small number of explanations to account for taxonomically differentiated disjuncts west of the Andean mountains (e.g. Chapman, 1917; Haffer, 1967, 1974; Cracraft & Prum, 1988; Brumfield & Capparella, 1996). The first hypothesis proposes that the *trans*-Andean avifauna originated from populations distributed throughout an ancestral northern Amazonian-Pacific lowland forest that was subsequently fragmented by Andean uplift. Thus, the

Andean uplift hypothesis has generally made the prediction that the divergence dates between these groups should be roughly coincident in time with, or slightly younger than, the main uplift of the Andean Cordillera (but see below).

Haffer (1967, 1974, pp. 159–161) championed a second hypothesis, namely that the Chocó avifauna resulted from the expansion of Amazonian populations into north-western Colombia and the Chocó during Pleistocene humid periods and were then isolated in a wet Chocó 'refuge' during drier interglacial periods. Although the *forest refugia hypothesis* makes no direct prediction about the dates of divergence among species – refuge formation is assumed to have been possible at multiple times (and not only during the Pleistocene; see Haffer, 1993) – it has been most commonly interpreted as applying to periods of high amplitude cyclical climate change in the Pleistocene. The refuge hypothesis, moreover, assumes that mountain uplift was not the primary barrier causing disjunction (Haffer, 1967, 1974) and that climatic/ecological vicariance would have taken place *after* mountain building (e.g. Haffer, 1967; Brumfield & Capparella, 1996, p. 1611). Adopting these assumptions, however, does not allow for a critical test of the two hypotheses, inasmuch as isolation by climatic fluctuation could be invoked either before or after mountain uplift.

The northern Andes are divided into three ranges with different tectonic histories: the Western, Central, and the Eastern Cordillera and its adjacent ranges. The Western Cordillera is formed from accretion of oceanic terranes (volcanic island arc) that were emplaced from the Early Cretaceous into the Cenozoic (Case *et al.*, 1990), and to the west, the Chocó block was accreted to South America beginning in the Middle Miocene (Duque-Caro, 1990). As a consequence, the region experienced significant uplift from Miocene to Holocene times (Case *et al.*, 1990).

The Central and Eastern Cordilleras are mostly underlain by continental crust (Case *et al.*, 1990). The Central Cordillera is the older of the two and had its initial uplift in the Late Cretaceous into the Paleogene (Gómez *et al.*, 2003), apparently in response to accretion of terranes to the west (Cooper *et al.*, 1995). Uplift of the Eastern Cordillera began approximately at 12.9 Ma, and a continuous mountain range, probably low-lying, was created by 11.8 Ma since westward sediment-fill into the Magdalena Valley indicates increased elevation (Hoorn *et al.*, 1995; Guerrero, 1997). This onset of uplift appears causally related to the accretion of the Chocó terrain to the Western Cordillera, beginning *c.* 12 Ma (Duque-Caro, 1990; Dengo & Covey, 1993; Cooper *et al.*, 1995), which resulted in compression across the Colombian Andes. To the north and west, the Santander massif followed an uplift history similar to that of the Eastern Cordillera (Hoorn *et al.*, 1995; Gregory-Wodzicki, 2000), but it and the Sierra de Perijá, the Santa Marta massif, and the Mérida Andes experienced their main uplift in the Late Miocene–Pliocene (Kellogg, 1984; Hoorn *et al.*, 1995).

Within this broad geological context, how might the history of *Gypopsitta* and other *cis/trans* Andean disjuncts be interpreted? Using the standard error estimates for our molecular clock dates, the separation between the east and west Andean clades of *Gypopsitta* (Fig. 7, node 1) occurred between 8.82 and 6.84 Ma, during the Late Miocene. This age is more consistent with vicariance resulting from Andean uplift (Eastern Cordillera) than with the traditional refuge hypothesis. At this time the Chocó terrane was being implaced, creating for the first time significant lowland habitat west of the Western Cordillera (Duque-Caro, 1990). It seems unlikely that vicariance of these disjuncts would have been created by Western or Central Cordilleran uplift, as these ranges were already in place and were being raised even further. In contrast, the north-eastern Andes, including the Eastern Cordillera, and probably also the Santander and Sierra de Perijá ranges, were relatively low in elevation prior to 8 Ma and experienced their greatest uplift after *c.* 5–6 Ma (Hoorn *et al.*, 1995; Gregory-Wodzicki, 2000; Hooghiemstra & Van der Hammen, 2004). This temporal history of uplift is consistent with our estimated age of divergence between the east and west Andean clades of *Gypopsitta*, but it does not completely explain the current distribution of *G. pulchra* in the Chocó.

The molecular data suggest that the three west Andean species of *Gypopsitta* are old and arose in the Early Pliocene or perhaps the latest part of the Late Miocene (Fig. 7, nodes 6 and 7). This was a time of high global temperatures, especially around a closing Panamanian Isthmus (Crowley, 1991), and regional temperature fluctuations during this period would have been less than at higher latitudes (Dowsett *et al.*, 1996). It is not likely, therefore, that climate oscillations were important for driving species formation at this time. Instead, sea-level fluctuations may have played a major role in subdividing lowland and lower-montane populations as Norens (2004) has previously suggested. Over the period of time that the *G. haematotis* species-group presumably was diversifying, beginning as early as 6.19 Ma (Fig. 7, node 6) until as late as 3.62 Ma (Fig. 7, node 7) there were at least three major episodes of sea-level rise and fall (Krantz, 1991) that probably would have been sufficient to isolate populations to the west in the Chocó and Nechí regions as well as to the north in Central America.

Finally, our results for the timing of the east vs. west Andean disjunction are very similar to those of Cortés-Ortiz *et al.* (2003) for howler monkeys (*Alouatta*) and for several lineages of curassows (Pereira & Baker, 2004). Using several mitochondrial genes, including *cyt b*, Cortés-Ortiz *et al.* (2003) estimated the *cis/trans* Andean disjunction at around 6.8 Ma. In contrast to our results (Fig. 7), differentiation within the Chocó-Central American monkey taxa began later, *c.* 3.0 Ma, but most of that took place within Central America. Any disjunction between Chocó and Central American populations of *A. palliata*, which was not investigated with their sampling, would have been still younger. The estimated ages of the *cis/trans* Andean disjunctions in both *Alouatta* and *Gypopsitta* are older than that postulated for *Bufo marinus* at 2.7 Ma (Slade &

Moritz, 1998). Likewise, Pereira & Baker (2004), using six mitochondrial regions, estimated the mean age of *cis/trans* Andean disjuncts in curassows to be c. 5.5–8.2 Ma. All of these ages, it should be noted, are older than the onset of Pliocene glacial climates beginning c. 2.4 Ma.

Eastern Andean taxa: diversification in the Amazon basin

The previous analysis of *Gypopsitta* by Cracraft & Prum (1988) postulated a sequence of vicariant events that partitioned the species within Amazonia. Their first proposed vicariance event isolated the Guyanan centre of endemism, the second segregated south-eastern Amazon, and the last subdivided the western Amazon region (Cracraft & Prum, 1988). The molecular results obtained in this study point to a different order for these events, and the dates obtained from the molecular clock estimates suggest that much of the diversification in this group took place as early as Late Miocene and Pliocene times.

Using the molecular tree as a basis for inference, the first vicariance event within the east Andean species was an east/west break that separated the lineage leading to *G. barrabandi* and *G. pyrilia* from that of (*G. caica* [*G. vulturina*, *G. aurantiocephala*]). Depending on the rate of molecular evolution in cytochrome *b* that is applied, this event is estimated to have occurred in the Late Miocene/Early Pliocene (between 6.48 and 4.99 Ma; Fig. 7, node 2). Moreover, except for the divergence of *G. aurantiocephala* and *G. vulturina* (see below), all speciation events in Amazonian *Gypopsitta* pre-date the Pleistocene. For the reasons articulated in our discussion about the west Andean species, it is also not likely that vicariance among the east Andean species was driven by cyclical climate change, although admittedly very little is known about Amazon basin climate and vegetation during the relevant time period. Sea-level fluctuations may have played a role (Nores, 1999), although it is not clear how those might have affected the biogeographical pattern exhibited by *Gypopsitta*. If *G. caica* and *G. vulturina*/*G. aurantiocephala* are related, as indicated by the molecular data (Fig. 7, node 4), then their distribution lies east of the Negro and Madeira rivers, whereas *G. barrabandi* + *G. pyrilia* are distributed to the west. The Amazon River system, however, apparently cut through the Purus and Monte Alegre arches to become transcontinental in the Late Miocene, perhaps as early as 11 Ma (Hoorn, 1994) or as late as 8.5–8.0 Ma (Lundberg *et al.*, 1988), too early, it would seem, to have directly caused divergences within *Gypopsitta*. It is possible, however, that later the evolving Amazon did play a role in the vicariance between *G. caica* and *G. vulturina*/*aurantiocephala*.

It seems likely that speciation of *G. pyrilia* and *G. barrabandi* (Fig. 7, node 3) are related to Pliocene tectonic events in northern Amazonia. Either mountain building or possibly marine transgressions could have partitioned off *G. pyrilia* from an ancestral population that extended south through western Amazonia. We estimate that event took place between 6.53 and 4.63 Ma, which as discussed earlier, is coincident with

a period of major uplift in the Eastern Cordillera and associated ranges.

Thus, the evidence suggests that most speciation events in *Gypopsitta* can be related to palaeogeographical events that took place in South America before the Quaternary. The only divergence that occurred during the Quaternary was the *G. vulturina*/*G. aurantiocephala* split, between 0.76 and 0.53 Ma (Fig. 7, node 5). These two taxa currently exhibit distributions that are congruent with proposed areas of endemism (Belém/Pará area and between the Tapajós and Xingú rivers in the Rondônia area, respectively; Cracraft, 1985; Haffer, 1985), and they are sympatric along the middle and lower Tapajós and lower Madeira rivers (Gaban-Lima *et al.*, 2002). Glacial cycles may have had an influence on this speciation event, but the fine-grained evidence to test this is currently lacking.

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BIOSKETCHES

Camila C. Ribas received her PhD from Universidade de Sao Paulo. She is currently a Postdoctoral Fellow at the Department of Ornithology, American Museum of Natural History. Her research interests include historical biogeography of the Neotropical region, molecular systematics and patterns of diversification.

Renato Gaban-Lima is a doctoral candidate at the Zoology Department at the Universidade de Sao Paulo. His research focuses on morphology, systematics and biogeography of parrots.

Cristina Y. Miyaki is Associate Professor at the Biology Department at the Universidade de Sao Paulo. Her research focuses on molecular systematics, biogeography and phylogeography of birds.

Joel Cracraft is Lamont Curator of Birds and Curator-in-Charge, Department of Ornithology, American Museum of Natural History in New York. His research interests include avian systematics, historical biogeography and biological diversification.

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Anexo VIII

MOLECULAR SYSTEMATICS AND PATTERNS OF DIVERSIFICATION IN *PYRRHURA* (PSITTACIDAE), WITH SPECIAL REFERENCE TO THE *PICTA*–*LEUCOTIS* COMPLEX

CAMILA C. RIBAS,^{1,3} LEO JOSEPH,^{2,4} AND CRISTINA Y. MIYAKI¹

¹Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo,
Rua do Matão, 277, São Paulo, SP 05508-090, Brazil;

²Department of Ornithology, Academy of Natural Sciences, 1900 Benjamin Franklin Parkway,
Philadelphia, Pennsylvania 19103, USA

ABSTRACT.—Parakeets in the genus *Pyrrhura* occur in Amazonia and in almost all other major Neotropical forests. Their uneven distribution (with some widespread and several geographically restricted endemic taxa) and complex patterns of plumage variation have long generated a confused taxonomy. Several taxonomically difficult polytypic species are usually recognized. Here, we present a mitochondrial DNA (mtDNA) phylogenetic analysis of *Pyrrhura*, with emphasis on the especially problematic *picta*–*leucotis* complex, to provide a more robust basis for interpreting the systematics and historical biogeography of the group. Our main findings are that (1) *Pyrrhura* can be divided into three main evolutionary lineages, one comprising *P. cruentata*, an Atlantic Forest endemic, the second comprising the *picta*–*leucotis* complex, and the third comprising the remaining species; (2) the traditionally recognized species *P. picta* and *P. leucotis* are not monophyletic; and (3) most of the species recognized by Joseph (2000, 2002) are diagnosable as independent evolutionary units, with the exception of the following species pairs: *P. snethlageae* and *P. amazonum*, *P. leucotis* and *P. griseipectus*, and *P. roseifrons* and *P. peruviana*. Other than *P. cruentata*, the two clades that constitute *Pyrrhura* appear to have radiated and evolved their present mtDNA diversity over short periods during the Plio-Pleistocene. Received 30 September 2004, accepted 30 September 2005.

Key words: Amazonia, diversification, mitochondrial DNA, molecular systematics, Neotropics, parakeets, *Pyrrhura*.

Sistemática Molecular y Patrones de Diversificación en *Pyrrhura* (Psittacidae), con Énfasis en el Complejo *Picta*–*Leucotis*

RESUMEN.—Los pericos del género *Pyrrhura* se encuentran en la Amazonía y en la mayoría de los demás bosques neotropicales principales. Su distribución desigual (con algunos taxa de amplia distribución y otros endémicos a regiones geográficas estrechas) y los patrones complejos de variación en el plumaje, han conducido a que la taxonomía del grupo sea confusa. Usualmente se reconocen varias especies politípicas y taxonómicamente difíciles. Con el fin de proveer información básica robusta para interpretar la sistemática y la biogeografía histórica del grupo, en este estudio presentamos un análisis filogenético de *Pyrrhura* con énfasis en el complejo de *picta*–*leucotis* basado en ADN mitocondrial (ADNmt). Encontramos que *Pyrrhura* puede dividirse en tres linajes evolutivos principales: *P. cruentata* (una

³Present address: Department of Ornithology, American Museum of Natural History, Central Park West at 79th Street, New York, New York 10024, USA. E-mail: ribas@amnh.org

⁴Present address: Australian National Wildlife Collection, Box 284, Canberra ACT, Australia 2601.

especie endémica del bosque atlántico), el complejo de *picta*–*leucotis* y las especies restantes. Las especies reconocidas tradicionalmente como *P. picta* y *P. leucotis* no son monofiléticas. La mayoría de las especies reconocidas por Joseph (2000, 2002) pueden diagnosticarse como unidades evolutivas independientes, a excepción de los siguientes pares de especies: *P. snethlageae* y *P. amazonum*, *P. leucotis* y *P. griseipectus*, y *P. roseifrons* y *P. peruviana*. Aparte de *P. cruentata*, los dos clados que conforman el género parecen haberse diversificado y haber evolucionado su diversidad actual en el ADNmt durante períodos cortos en el Plio-Pleistoceno.

THE ORIGIN OF the high species diversity in lowland Neotropical forests has long been a major topic in evolutionary biology (Wallace 1852; Haffer 1969, 1993; Endler 1977; Salo et al. 1986; Cracraft and Prum 1988; Bush 1994; Tuomisto et al. 1995). Most authors agree that a fruitful way to address this issue is through linking phylogenetic relationships and geographic distributions of extant taxa (Moritz et al. 2000). Accordingly, numerous papers have studied patterns of phylogenetic relationships of species complexes found in Neotropical forests. Some examples in which molecular approaches have been taken concern snakes (Zamudio and Greene 1997), amphibians (Slade and Moritz 1998, Loughheed et al. 1999), rodents (Patton et al. 1994, 1996, 2000; da Silva and Patton 1998; Matocq et al. 2000; Costa 2003), cats (Eizirik et al. 1998), birds (Cracraft and Prum 1988, Marks et al. 2002, Aleixo 2004, Pereira and Baker 2004, Ribas and Miyaki 2004, Cheviron et al. 2005), and primates (Cortés-Ortiz et al. 2003). Clearly, this approach to understanding species diversification and distribution patterns depends on a sound systematic framework. Here, we provide such a framework for the genus *Pyrrhura*.

Pyrrhura occur in all Neotropical forests, including the Amazon, the Atlantic, and the west Andean and Central American forests. Though distinctive and easily recognizable at the generic level, several species of *Pyrrhura* show subtle, taxonomically difficult patterns of variation, typically involving a small number of plumage characters. Further, taxa within a given species complex are often narrowly distributed geographic endemics. As a result, species-level systematics has long been problematic (Forshaw 1989; Collar 1997; Juniper and Parr 1998; Joseph 2000, 2002).

The Painted Parakeet (*Pyrrhura picta*) and Maroon-faced Parakeet (*P. leucotis*) complex, in particular, highlights these problems, some of which arise from the group's taxonomic

history. Most authors have followed Peters (1937), who reduced the group to two species with no accompanying arguments. Thus, it has been conventional to recognize two polytypic species: *P. picta* (subspecies: *picta*, *eisenmanni*, *roseifrons*, *lucianii*, *subandina*, *caeruleiceps*, *pantchenko*, and *amazonum*) and *P. leucotis* (subspecies: *leucotis*, *emma*, *auricularis*, *pfrimeri*, and *griseipectus*). Joseph (2000, 2002) and Joseph and Stockwell (2002) reviewed geographic variation and taxonomy in the group and recognized 13 different species in the complex, including two new taxa for the east and west Amazon basin (*P. snethlageae* and *P. peruviana*, respectively; see Fig. 1). Joseph (2000, 2002) highlighted several problems needing further study. One of them concerns relationships among taxa within the complex. Another concerns finer-scale relationships among eastern and western Amazonian taxa recognized by Joseph (2002).

The genus *Pyrrhura* also presents a useful opportunity to examine another, more general problem arising in avian molecular systematics. Molecular systematic studies of birds have shown that many traditionally accepted taxonomic units are paraphyletic assemblages and that, in particular, many subspecies have been found to represent deeply divergent, monophyletic mitochondrial DNA (mtDNA) clades (Bates et al. 1999, Aleixo 2002, Marks et al. 2002, Ribas and Miyaki 2004, Russello and Amato 2004, Ribas et al. 2005). These findings indicate that traditional taxonomic treatments may conceal much of the diversity that exists in natural populations of birds. Thus, the combination of molecular systematic studies with accurate descriptions of morphological variation is the best way for understanding the evolution of the different groups and for developing a taxonomic arrangement that reflects the evolutionary history. *Pyrrhura*, which has long been a taxonomically difficult genus because of limited morphological diversity, provides an excellent

FIG. 1. Collection localities of the samples used and geographic distribution of the 13 taxa recognized by Joseph (2000, 2002) in the *picta-leucotis* complex. Limits of *P. amazonum* and *P. snethlageae* distributions are not well defined (see text). Note that distributional limits of *P. peruviana* and *P. roseifrons* are especially tentative; the southern population of *P. peruviana* is currently known only from between the northern and southern sectors of the range of *P. roseifrons* (see Joseph [2002] for details). Numbers correspond to samples used in the study. Circles indicate exact collection localities, whereas squares indicate approximate collection localities (see Table 1).

opportunity for exploring relationships between genetic and morphological diversity.

Here, we have set out to clarify relationships within *Pyrrhura* generally, and more specifically within the *picta-leucotis* complex, to provide a more robust basis for interpreting the group's systematics and historical biogeography. We obtained sequences of the mitochondrial cytochrome *b* and control region for individuals representing 10 of the 13 taxa recognized by Joseph (2000, 2002) in the *picta-leucotis* complex and 11 of the remaining 16 species recognized in the genus *Pyrrhura*. With this phylogenetic analysis, we had the objectives of (1) determining whether the *picta-leucotis* complex is monophyletic within *Pyrrhura*; (2) determining whether the species *P. picta* and *P. leucotis* (*sensu* Peters [1937] and

later treatments based on it) are monophyletic; (3) determining whether the different species recognized by Joseph (2000, 2002) are diagnosable as independent evolutionary units in a molecular analysis; and (4) making preliminary inferences about when the diversification of the group occurred and how it can be related to diversification processes in the Neotropics. We acknowledge renewed concern (e.g., Weckstein et al. 2001, Ballard et al. 2002) about making taxonomic changes solely on the basis of mtDNA and so have addressed our aims with this in mind.

METHODS

Taxon sampling.—We obtained blood and tissue samples from 54 individuals belonging

to 10 of the 13 species of the *picta-leucotis* complex and from 24 individuals belonging to the other 11 species of the genus *Pyrrhura* (Table 1). There were no samples available for three taxa (*P. lucianii*, *P. caeruleiceps*, and *P. subandina*) from the *picta-leucotis* complex and for five taxa outside it (*P. viridicata*, *P. calliptera*, *P. devillei*, *P. egregia*, and *P. hoematotis*). Collection localities were obtained for most individuals (Fig. 1 and Table 1). Four different genera closely related to *Pyrrhura* (*Aratinga* sp., *Derophtus* sp., *Guarouba* sp., and *Anodorhynchus* sp.; Tavares et al. 2004) were included as outgroups. Because outgroup choice did not affect the position of the root, final analyses were performed using only *Anodorhynchus leari* as the outgroup.

DNA extraction, amplification and sequencing.—We extracted DNA from blood samples through incubation of a small amount of blood at 54°C with proteinase K for ~4 h followed by a phenol-chloroform procedure. Extraction from tissue samples was done with the DNeasy tissue kit (Qiagen, Valencia, California), following the manufacturer's recommendations.

We sequenced the whole cytochrome-*b* gene and mitochondrial control region for all samples. We sequenced the latter because of the low levels of diversity found in the cytochrome-*b* data and the higher rate of evolution known for the control region (Baker and Marshall 1997). Also, sequencing two separate mtDNA regions allowed us to compare the patterns of sequence evolution and to assess the use of each one in resolving phylogenetic relationships.

Amplifications were done with 40 µL reaction volumes, containing 2.0 µL DNA solution, 1× PCR buffer (Promega, Madison, Wisconsin), 2.5 mM MgCl₂, 0.8 mM dNTPs, 0.5 µM of each primer, and 2 U of *Taq* DNA polymerase (Promega). Amplifications of the entire regions (cytochrome-*b* and control region) using external primers were performed, and the products of these reactions were visualized by electrophoresis in 1.3% low-melting-agarose gels run in TAE (Tris-acetate low-EDTA buffer, pH 7.8). The single amplification products were cut, dissolved in 200 µL of sterile water, heated at 70°C for 15 min, and stored at room temperature. These products were then used as templates for reamplification reactions using internal primers, under the same conditions described above, but with higher annealing temperatures.

We used the following external primers to amplify cytochrome-*b* gene (all given as 5' to 3'): N5L (GGACCAGAAGGACTTGCCGACCTA; Ribas et al. 2005) and Hthr16082 (TCTTTGGTTTACAAGACCAATG; E. S. Tavares pers. comm.); internal primers were CBH15422 (GGTGGGGTTGTCTACGGAGAA; Ribas et al. 2005), CBL15298 (TGAGGCCAAATATCATTCTGAGGGG; Cheng et al. 1994), CBH15764 (CCTCC TAGTTTGTGGGGATTGA; Miyaki et al. 1998), CBL15507 (AACCTACTAGGAGACCCAGA; J. Feinstein pers. comm.), and HB20 (TTGGTTCACAAGACCAATGTT; J. Feinstein pers. comm.). For the control region amplifications, we used the external primers LGlu 16737 (GCCCTGAAAARCCATCGTTG; Eberhard et al. 2001) and HPhe (TCTTGGCAKCTTCAGTACCA TGCTTT; Tavares 2001); internal primers were CRH522 (TGGCCCTGACYTAGGAACCAG; Eberhard et al. 2001), CRL478 (CAGGAGAGATCA YCAACCCGGTGT; Tavares 2001), CRH 1020 (ACCCTGATGCACTTTGTTTTACACCT; Tavares 2001), and CRL846 (TCATTTTCGCACT GATGCACTTG; Tavares 2001).

We purified polymerase chain reaction (PCR) products with the QIAquick 96 PCR BioRobot kit (Qiagen), using the BioRobot 9600 (Qiagen). One to three microliters of the purified PCR product were then used as template for the sequencing reaction using dRhod (Applied Biosystems, Foster City, California). Sequencing primers were the same used for the amplifications and reamplifications. Ethanol precipitation of the cycle sequencing product was performed, and the samples were run on a 3100 Automated DNA Sequencer following the ABI protocol (Applied Biosystems).

Sequence alignment, genetic distances, and phylogenetic analyses.—We used SEQUENCHER, version 4.1 (Gene Codes Corporation, Ann Arbor, Michigan) to compare sequences of heavy and light strands and to edit and visually correct sequences. We used CLUSTAL_X (Thompson et al. 1997) to align the sequences and MACCLADE, version 4.0 (Maddison and Maddison 2000), to manually correct the alignment. Nucleotide sequences obtained for cytochrome *b* were translated to confirm the correct reading frame positions and to check for the presence of unexpected stop codons.

Uncorrected *p*-distances (Nei 1987) and standard errors within and among taxa were calculated using MEGA (Kumar et al. 2001).

TABLE 1. *Pyrrhura* spp. tissue samples used in the study and respective GenBank accession numbers.

Taxon	Voucher institution	Voucher number	Collection locality	GenBank accession number (cytochrome <i>b</i> / control region)
1 <i>P. roseifrons</i>	FMNH	397723	Madre de Dios, Peru	AY751585 / AY751669
2 <i>P. roseifrons</i>	LSUMNS	B10802	Pucallpa, Peru	AY751586 / AY751671
3 <i>P. roseifrons</i>	LSUMNS	B10804	Pucallpa, Peru	AY751587 / AY751661
4 <i>P. roseifrons</i>	LSUMNS	B10847	Pucallpa, Peru	AY751588 / AY751662
5 <i>P. roseifrons</i>	LSUMNS	B10849	Pucallpa, Peru	AY751589 / AY751663
6 <i>P. roseifrons</i>	LSUMNS	B11085	Pucallpa, Peru	AY751590 / AY751664
7 <i>P. roseifrons</i>	LSUMNS	B11281	Pucallpa, Peru	AY751591 / AY751665
8 <i>P. roseifrons</i>	LSUMNS	B11282	Pucallpa, Peru	AY751592 / AY751666
9 <i>P. roseifrons</i>	LSUMNS	B27438	Contamana, Peru	AY751595 / AY751672
10 <i>P. roseifrons</i>	LSUMNS	B27441	Contamana, Peru	AY751596 / AY751667
11 <i>P. roseifrons</i>	LSUMNS	B27648	Contamana, Peru	AY751597 / AY751668
12 <i>P. roseifrons</i>	LSUMNS	B27652	Contamana, Peru	AY751584 / AY751670
13 <i>P. peruviana</i>	USP	5084	Cordillera del Condor, southeast Ecuador	AY751582 / AY751660
14 <i>P. peruviana</i>	USP	5085	Cordillera del Condor, southeast Ecuador	AY751583 / AY751659
15 <i>P. eisenmanni</i>	ANSP	5758	Veraguas, Panama	AY751598 / AY751686
16 <i>P. eisenmanni</i>	ANSP	5759	Veraguas, Panama	AY751599 / AY751687
17 <i>P. picta</i>	FMNH	395728	Vila Surumu, RO, Brazil	AY751600 / AY751690
18 <i>P. picta</i>	KUMNH	1196	Iwokrama Reserve, Guyana	AY751601 / AY751689
19 <i>P. picta</i>	KUMNH	1198	Iwokrama Reserve, Guyana	AY751602 / AY751691
20 <i>P. picta</i>	NMNH	B09287	Baramita, Guyana	AY669400 / AY751688
21 <i>P. picta</i>	NMNH	B09631	Baramita, Guyana	AY751603 / AY751692
22 <i>P. picta</i>	NMNH	B10941	Acari, Guyana	AY751604 / AY751693
23 <i>P. picta</i>	NMNH	B10944	Baramita, Guyana	AY751605 / AY751694
24 <i>P. snethlageae</i>	LSUMNS	B12781	Santa Cruz, Bolivia	AY751593 / AY751677
25 <i>P. snethlageae</i>	LSUMNS	B12782	Santa Cruz, Bolivia	AY751594 / AY751678
26 <i>P. snethlageae</i>	USP	4976	Porto Velho, RO, Brazil	AY751606 / AY751681
27 <i>P. snethlageae</i>	FMNH	389694	Cach. Nazaré, RO, Brazil	AY751607 / AY751679
28 <i>P. snethlageae</i>	FMNH	389695	Cach. Nazaré, RO, Brazil	AY751608 / AY751680
29 <i>P. snethlageae</i>	FMNH	389696	Cach. Nazaré, RO, Brazil	AY751609 / AY751682
30 <i>P. snethlageae</i>	USP	2930	Alta Floresta, MT, Brazil	AY751610 / AY751683
31 <i>P. snethlageae</i>	USP	2931	Alta Floresta, MT, Brazil	AY751611 / AY751684
32 <i>P. snethlageae</i>	USP	2933	Alta Floresta, MT, Brazil	AY751612 / AY751685
33 <i>P. snethlageae</i>	USP	2934	Jacareacanga, PA, Brazil	AY751613 / AY751674
34 <i>P. amazonum</i>	NMNH	B06897	Altamira, PA, Brazil	AY751614 / AY751673
35 <i>P. amazonum</i>	NMNH	B07027	Altamira, PA, Brazil	AY751615 / AY751675
36 <i>P. amazonum</i>	NMNH	B07033	Altamira, PA, Brazil	AY751616 / AY751676
37 <i>P. pfrimeri</i>	USP	3912	Captive bird	AY751617 / AY751696
38 <i>P. pfrimeri</i>	USP	3913	Captive bird	AY751618 / AY751697
39 <i>P. pfrimeri</i>	USP	4041	Terra Ronca, GO, Brazil	AY751619 / AY751698
40 <i>P. pfrimeri</i>	USP	4044	Terra Ronca, GO, Brazil	AY751620 / AY751695
41 <i>P. pfrimeri</i>	USP	4045	Terra Ronca, GO, Brazil	AY751621 / AY751699
42 <i>P. emma</i>	USP	3513	Captive bird	AY751622 / AY751700
43 <i>P. emma</i>	USP	3514	Captive bird	AY751623 / AY751701
44 <i>P. emma</i>	USP	3515	Captive bird	AY751624 / AY751702
45 <i>P. emma</i>	USP	4247	Captive bird	AY751625 / AY751703
46 <i>P. emma</i>	USP	4248	Captive bird	AY751626 / AY751704

TABLE 1. Continued.

Taxon	Voucher institution	Voucher number	Collection locality	GenBank accession number (cytochrome <i>b</i> / control region)
47 <i>P. griseipectus</i>	USP	1069	Captive bird	AY751627 / AY751705
48 <i>P. griseipectus</i>	USP	1070	Captive bird	AY751628 / AY751706
49 <i>P. griseipectus</i>	USP	3914	Captive bird	AY751629 / AY751707
50 <i>P. griseipectus</i>	USP	3915	Captive bird	AY751630 / AY751708
51 <i>P. griseipectus</i>	USP	3916	Captive bird	AY751631 / AY751709
52 <i>P. leucotis</i>	USP	3921	Captive bird	AY751632 / AY751712
53 <i>P. leucotis</i>	USP	3922	Captive bird	AY751633 / AY751710
54 <i>P. leucotis</i>	USP	3923	Captive bird	AY751634 / AY751711
55 <i>P. orcesi</i>	LSU	B7803	El Oro, Ecuador	AY751635 / AY751713
56 <i>P. orcesi</i>	LSU	B7818	El Oro, Ecuador	AY751636 / AY751714
57 <i>P. rhodocephala</i>	USP	3871	Captive bird	AY751637 / AY751716
58 <i>P. rhodocephala</i>	USP	3872	Captive bird	AY751638 / AY751715
59 <i>P. albipectus</i>	ANSP	4439	Zamora-Chinchi, Ecuador	AY751639 / AY751729
60 <i>P. albipectus</i>	ANSP	4490	Zamora-Chinchi, Ecuador	AY751640 / AY751728
61 <i>P. molinae</i>	AMNH	CJV140	La Paz, Bolivia	AY751641 / AY751717
62 <i>P. molinae</i>	KUMNH	1526	Argentina (Confiscated bird)	AY751642 / AY751718
63 <i>P. frontalis</i>	LSUMNS	B25884	Caazapa Dept., Paraguay	AY751643 / AY751723
64 <i>P. lepida</i>	NMNH	B07007	Altamira, Pará, Brazil	AY751644 / AY751719
65 <i>P. lepida</i>	NMNH	B07028	Altamira, Pará, Brazil	AY751645 / AY751721
66 <i>P. perlata</i>	USP	848	Jacareacanga, Pará, Brazil	AY751646 / AY751722
67 <i>P. perlata</i>	USP	860	Jacareacanga, Pará, Brazil	AY751647 / AY751720
68 <i>P. melanura</i>	AMNH	SC759	Amazonas, Venezuela	AY751648 / AY751733
69 <i>P. melanura</i>	AMNH	SC888	Amazonas, Venezuela	AY751649 / AY751730
70 <i>P. melanura</i>	AMNH	SC889	Amazonas, Venezuela	AY751650 / AY751731
71 <i>P. melanura</i>	ANSP	5111	Sucumbios, Ecuador	AY751651 / AY751734
72 <i>P. melanura</i>	ANSP	5112	Sucumbios, Ecuador	AY751652 / AY751732
73 <i>P. hoffmani</i>	NMNH	B05272	Chiriqui, Panama	AY751653 / AY751724
74 <i>P. hoffmani</i>	NMNH	B05447	Chiriqui, Panama	AY751654 / AY751725
75 <i>P. rupicola</i>	KUMNH	1525	Bolivia (Confiscated bird)	AY751655 / AY751726
76 <i>P. rupicola</i>	KUMNH	653	Madre de Dios, Peru	AY751656 / AY751727
77 <i>P. cruentata</i>	USP	2228	Bahia, Brazil	AY751657 / AY751735
78 <i>P. cruentata</i>	USP	2230	Bahia, Brazil	AY751658 / AY751736

Museum and collection acronyms: AMNH = American Museum of Natural History; ANSP = Academy of Natural Sciences; FMNH = Field Museum of Natural History; KUMNH = University of Kansas Museum of Natural History; LSUMNS = Louisiana State University Museum of Natural Science; MPEG = Museu Paraense Emílio Goeldi; NMNH = United States National Museum of Natural History, Smithsonian Institution; and USP = Universidade de São Paulo.

Maximum-likelihood (ML) distances using parameters selected by MODELTEST (Posada and Crandall 1998) were calculated using PAUP*, version 4.0b10 (Swofford 1998). The number of transition and transversion substitutions at each codon position was plotted against *p*-distances and ML distances to test for evidence of saturation in the two regions. Phylogenetic congruence was evaluated via the partition homogeneity test (Farris et al. 1995), with 100 replicates in PAUP*, and also through comparison of separate analyses of the

two data sets. Another partition homogeneity test compared the three codon positions. The uniformity of base composition at each codon position was evaluated through a chi-square test of homogeneity.

We analyzed sequence data matrices with maximum parsimony (MP) and ML using PAUP* and Bayesian methods using MRBAYES, version 3.0 b4 (Huelsenbeck and Ronquist 2001). We implemented MP analyses with heuristic tree searches, tree bisection-reconnection (TBR) branch swapping, and 10 random-

addition sequence replications. Relative support for inferred monophyletic groupings was determined using 1,000 bootstrap replications and decay indices (Bremer 1994) calculated with AUTODECAY, version 4.0 (Eriksson 1999; 100 random-addition replicates per tree).

We used the likelihood ratio test as implemented in MODELTEST (Posada and Crandall 1998) to select the simplest model of molecular evolution that yielded significantly higher likelihood than others. The model selected was used for the ML analyses, which were performed using heuristic tree search, TBR branch swapping, and 10 random-addition replicates. The robustness of the tree was determined by 100 bootstrap replications, with a starting tree obtained by neighbor-joining and SPR branch swapping.

Bayesian inference used ML models selected by MODELTEST for each data set (cytochrome *b* and control region). A Bayesian analysis partitioned according to the two different regions was performed assuming six classes of DNA substitution. The proportion of invariable sites and the shape parameter of the gamma distribution for each partition were estimated. The analysis was run for 1 million generations, with one cold and three heated chains. The ML scores stabilized around the 1,000th tree, so that burn-in was completed by the 1,000th tree, and 9,000 trees were kept in each analysis. Three independent analyses were performed for the combined data set, and the 27,000 sampled trees obtained in each analysis were used to compute the posterior probabilities of each node.

Because of the large number of terminal taxa and consequent increase of computational time required for ML and Bayesian analyses, these analyses were done in two parts: (1) one with only one individual representing each studied species (10 *picta-leucotis* taxa plus 11 other species of *Pyrrhura* and the outgroup) and (2) one containing all individuals of the *picta-leucotis* complex and only one individual not belonging to the complex, as an outgroup.

Molecular dating of divergence times.—To test the assumption of rate constancy of DNA substitution, we used a likelihood ratio test (LRT) assuming a chi-square distribution with degrees of freedom equal to the number of taxa minus two (Huelsenbeck and Rannala 1997). We compared the log-likelihood values from ML trees constructed with or without a molecular clock constraint. This test was applied to the

cytochrome *b*, control region, and combined matrices containing all taxa and containing only the species belonging to the genus *Pyrrhura*.

Because only cytochrome-*b* data were found to evolve in a clock-like manner (see below), only those sequences could be used for estimating divergence times. Divergence times were calculated using the ML branch lengths and applying two different rates of cytochrome-*b* sequence divergence: 1.6% Ma⁻¹, based on Fleischer et al.'s (1998) analysis of Hawaiian passerines; and 2.0% Ma⁻¹, locally calibrated for other bird species using fossil records (Shields and Wilson 1987, Randi 1996). Standard errors for the distance estimates were calculated by creating 100 matrices through bootstrapping of the original matrix used for branch-lengths estimation. Matrices were created using SEQBOOT in PHYLIP, version 3.6 (Felsenstein 2005); ML branch lengths were estimated based on each one of the 100 bootstrapped matrices using PAUP*.

To further explore the data and better understand the relative times of speciation, we analyzed the ML tree obtained in the analysis of the combined matrix (cytochrome *b* and control region) using the semiparametric, penalized likelihood (PL) method (Sanderson 2002). This method makes no assumption of clock-like sequence evolution and allows for some degree of rate heterogeneity among branches (Sanderson 2002). The PL method used the Truncated-Newton (TN) algorithm and was implemented in the program r8s, version 1.7 (Sanderson 2003), with a smoothing factor of 1, as determined by the cross-validation analysis. Because there is no calibration for the *Pyrrhura* phylogeny, this analysis was performed assigning an arbitrary age of "10" to the most basal node inside the genus (the place where the outgroup attaches to the ingroup). Relative ages and standard errors were then estimated for all other nodes, and these values could be compared in a relative framework to understand the temporal patterns of diversification inside the genus.

RESULTS

Sequence characteristics and informative sites.—The complete cytochrome-*b* gene (1,140 base pairs [bp]) was sequenced for all studied individuals. Translation of the nucleotide sequences

found no unexpected stop codons. The complete control region in *P. leucotis* (individual 3921) had 1,514 bp. There was some length variation in other taxa. A 10-bp deletion was found at position 1161 in some individuals of *P. roseifrons*, *P. picta*, *P. pfrimeri*, and *P. cruentata*.

Polymerase chain reaction amplifications yielded single products of the expected sizes. The sequences obtained aligned easily with those of other parrots, and the base composition found was that expected for avian mtDNA. A duplication of the control region of the mtDNA has been described in some Neotropical short-tailed parrot genera (*Amazona* and *Pionus*; Eberhard et al. 2001); whereas some Neotropical long-tailed parrot genera do not have this duplication and have the same gene order as in Jungle Fowl (*Gallus gallus*) (E. S. Tavares pers. comm.). Amplification using the primers Lthr (Eberhard et al. 2001) and ND6H16522 (E. S. Tavares pers. comm.) resulted in a product of ~500 bp, the expected size when there is not a copy of the control region between the genes for Threonine tRNA (Thr) and ND6 (see Eberhard et al. 2001). Also, the region between the cytochrome-*b* gene and the 12S rDNA was sequenced for several long-tailed taxa, and it was confirmed that *Pyrrhura* does not have the duplication of the control region (E. S. Tavares pers. comm.). These results indicate that control-region sequences analyzed are homologous and of mitochondrial origins.

After alignment of all *Pyrrhura* sequences, a control-region matrix containing 1,430 bp was obtained, ranging from position 54 to position 1484. After alignment with the outgroup sequence, a matrix of 1,457 bp was obtained. From this matrix, 58 characters were removed because of indels that caused uncertainty about homology in the alignment. The final aligned control-region matrix had 1,399 characters. The combined matrix had 2,539 bp for 79 taxa (78 from *Pyrrhura* and the outgroup).

Base composition of the *Pyrrhura* sequences (Table 2) was typical of mitochondrial sequences of birds. No base composition bias was detected in the data set, even when only variable sites were analyzed ($P = 0.99$). Variation in cytochrome-*b* sequences was concentrated on the third position. The control region had higher net proportion of variable and informative sites, but the percentage in cytochrome-*b* third positions was higher than in control-region sequences (Table 2). The cytochrome-*b* protein putative sequence had 380 amino acids, 38 and 23 of which were variable among all species of *Pyrrhura* and within the *picta-leucotis* complex, respectively. Cytochrome-*b* third positions showed very low proportions of guanine (Table 2). The data matrices were not saturated (data not shown). The partition homogeneity test showed no incongruence between the two data sets (cytochrome *b* and control region; $P = 0.725$).

TABLE 2. Percentage of variable sites, percentage of informative sites, nucleotide composition, and Ts:Tv ratios of the mitochondrial regions.

	Cytochrome <i>b</i>	Control region
Number of base pairs	1,140	1,399
<i>Picta-leucotis</i> complex (54 terminals)		
Variable (%)	5.7% (1st), 2.6% (2nd), 17.6% (3rd)	14.4%
Informative (%)	2.4% (1st), 0.8% (2nd), 13.4% (3rd)	11.3%
A (%)	27.6 (1st), 20.8 (2nd), 36.3 (3rd)	25.3
C (%)	29.2 (1st), 27.6 (2nd), 48.6 (3rd)	26.1
G (%)	19.9 (1st), 12.6 (2nd), 4.8 (3rd)	15.4
T (%)	23.3 (1st), 39.0 (2nd), 10.3 (3rd)	33.3
Ts:Tv ratio	9.8	9.5
All <i>Pyrrhura</i> (78 terminals)		
Number variable (%)	10.8% (1st), 4.5% (2nd), 38.4% (3rd)	24.4%
Number informative (%)	8.7% (1st), 2.4% (2nd), 33.4% (3rd)	20.7%
A (%)	27.6 (1st), 20.7 (2nd), 36.1 (3rd)	24.9
C (%)	29.2 (1st), 27.6 (2nd), 49.2 (3rd)	25.8
G (%)	19.9 (1st), 12.7 (2nd), 5.0 (3rd)	15.9
T (%)	23.3 (1st), 39.0 (2nd), 9.7 (3rd)	33.4
Ts:Tv ratio	10.8	8.2

FIG. 2. Strict consensus of the four most parsimonious trees obtained based on 2,539 bp of cytochrome *b* and control region. Numbers on branches correspond to bootstrap support (1,000 replicates) and decay indices. Asterisk indicates bootstrap value <50%. Thicker branches indicate well-supported, morphologically distinct lineages.

FIG. 3. Single most-likely tree obtained with ML analysis, including representatives of all analyzed taxa, based on 2,539 bp of cytochrome *b* and control region. Bootstrap values $\geq 50\%$ are shown above branches; posterior probabilities $\geq 80\%$ are shown below branches.

FIG. 4. Single most-likely tree obtained with ML analysis, including all individuals belonging to the *picta*–*leucotis* complex, based on 2,539 bp of cytochrome *b* and control region. Bootstrap values $\geq 50\%$ are shown above branches; posterior probabilities $\geq 80\%$ are shown below branches.

TABLE 3. Average *p*-distance within groups of individuals belonging to the same clade (*n* = number of individuals in each clade).

	<i>n</i>	Cytochrome <i>b</i>	Control region
<i>P. snethlageae</i> – <i>P. amazonum</i>	13	0.003	0.011
<i>P. griseipectus</i> – <i>P. leucotis</i>	8	0.005	0.010
<i>P. pfrimeri</i>	5	0.001	0.002
<i>P. eisenmanni</i>	2	0.000	0.001
<i>P. emma</i>	5	0.002	0.003
<i>P. picta</i>	7	0.002	0.004
<i>P. peruviana</i> – <i>P. roseifrons</i>	14	0.005	0.012
<i>P. orcesi</i>	2	0.001	0.005
<i>P. rhodocephala</i>	2	0.003	0.004
<i>P. albipectus</i> – <i>P. melanura</i>	7	0.007	0.008
<i>P. molinae</i>	2	0.002	0.013
<i>P. frontalis</i>	1	–	–
<i>P. lepida</i>	2	0.001	0.002
<i>P. perlata</i>	2	0.004	0.005
<i>P. hoffmanni</i>	2	0.000	0.001
<i>P. rupicola</i>	2	0.016	0.018
<i>P. cruentata</i>	2	0.000	0.004

Divergence times.—The likelihood ratio test could not reject rate constancy only when the outgroup and *P. cruentata* sequences were removed from the cytochrome-*b* matrix. In this way, a matrix containing 20 individuals, representing the 20 recognized species of *Pyrrhura* studied here, excluding *P. cruentata*, was used for molecular clock analyses. This matrix passed the LRT (*P* > 0.05) and, thus, the branch lengths of the resulting linearized ML tree were converted to units of time using the rates of 1.6% and 2.0% sequence divergence per million years.

We estimate that diversification within the *picta*–*leucotis* complex started at 1.59 ± 0.22 Ma, if the rate of 1.6% Ma⁻¹ is used; or at 1.27 ± 0.17 Ma, if 2.0% Ma⁻¹ is used. Diversification among all other *Pyrrhura*, except *P. cruentata*, started at 2.36 ± 0.34 Ma with the rate of 1.6% Ma⁻¹ and at 1.89 ± 0.27 Ma with 2.0% Ma⁻¹. These results indicate that these two clades diversified relatively recently, and that diversification within the *picta*–*leucotis* complex occurred still later. Divergence between the two clades occurred at 4.64 ± 0.71 Ma (1.6% Ma⁻¹) or at 3.71 ± 0.57 Ma (2.0% Ma⁻¹).

When the basal split inside *Pyrrhura* (the node in which the outgroup attaches to the ingroup in the tree of Fig. 4) was assigned an arbitrary value of 10 in the PL analysis, the diversification of the *picta*–*leucotis* clade was estimated to have started at 3.78 (3.24–4.41) and continued until 1.63

(1.26–2.06), whereas diversification of the other clade was estimated to have occurred from 4.81 (4.00–5.66) until 1.47 (1.02–2.10). This confirms the results that the two main clades diversified in similar periods of time, and that the *picta*–*leucotis* clade is a little younger. These results also show that diversification in both clades was concentrated in a relatively short period (intervals of 2.15 and 3.34 units in a total of 10). The origin of *P. cruentata* lineage is almost as old (9.27) as the basal node of the tree, indicating an old isolation of this lineage. It is also interesting to note that diversification of the two clades mentioned above started at ~5 in the scale that puts the basal node of the tree at 10, which indicates that a long time elapsed after the split of the two lineages and before diversification of the two extant clades.

DISCUSSION

Diversification patterns in Pyrrhura.—*Pyrrhura* is phylogenetically divided into three core lineages: one comprising the *picta*–*leucotis* complex; one comprising only *P. cruentata*, from the Atlantic Forest; and one comprising all other species sampled. The MP results indicate an initial separation between the ancestor of *P. cruentata* and the ancestor of all other *Pyrrhura*. Later diversification among these species occurred by way of a further split into two main clades during the Pliocene. *Pyrrhura cruentata*

TABLE 4. The *p*-distances among *Pyrrhura* clades, with cytochrome *b* below and control region above diagonal. Lines define the three main lineages found: the *picta-leucotis* complex, most of the other *Pyrrhura* species, and *P. cruentata*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 <i>P. snethlageae</i> - <i>P. amazonum</i>	0.031	0.027	0.037	0.040	0.036	0.034	0.087	0.085	0.078	0.086	0.088	0.088	0.084	0.081	0.079	0.080	0.095
2 <i>P. griseipectus</i> - <i>P. leucotis</i>	0.012	0.040	0.049	0.054	0.045	0.038	0.085	0.085	0.078	0.083	0.083	0.083	0.083	0.082	0.076	0.080	0.088
3 <i>P. pflimeri</i>	0.020	0.021	-	0.033	0.041	0.032	0.035	0.081	0.079	0.075	0.081	0.082	0.078	0.076	0.071	0.080	0.094
4 <i>P. eisenmanni</i>	0.015	0.019	0.021	-	0.039	0.035	0.045	0.085	0.082	0.077	0.085	0.087	0.084	0.080	0.076	0.080	0.094
5 <i>P. enma</i>	0.018	0.021	0.022	0.015	-	0.032	0.041	0.087	0.088	0.082	0.088	0.089	0.089	0.083	0.082	0.084	0.095
6 <i>P. picta</i>	0.018	0.019	0.022	0.016	0.011	-	0.038	0.073	0.070	0.066	0.072	0.072	0.071	0.066	0.065	0.072	0.089
7 <i>P. peruviana</i> - <i>P. roseifrons</i>	0.018	0.019	0.022	0.018	0.017	0.017	-	0.082	0.082	0.073	0.081	0.081	0.079	0.078	0.075	0.078	0.089
8 <i>P. orcesi</i>	0.050	0.048	0.050	0.047	0.046	0.048	0.045	-	0.034	0.037	0.038	0.032	0.033	0.035	0.031	0.040	0.080
9 <i>P. rhodocephala</i>	0.050	0.050	0.051	0.048	0.047	0.049	0.048	0.024	-	0.038	0.036	0.037	0.034	0.034	0.030	0.040	0.086
10 <i>P. albipectus</i> - <i>P. melanura</i>	0.055	0.053	0.056	0.054	0.052	0.053	0.050	0.024	0.026	-	0.036	0.038	0.038	0.036	0.031	0.030	0.077
11 <i>P. molinae</i>	0.056	0.055	0.058	0.052	0.048	0.048	0.050	0.033	0.027	0.034	-	0.027	0.025	0.024	0.029	0.034	0.082
12 <i>P. frontalis</i>	0.053	0.051	0.052	0.050	0.047	0.047	0.046	0.029	0.026	0.032	0.023	-	0.022	0.026	0.029	0.040	0.077
13 <i>P. lepida</i>	0.055	0.053	0.057	0.050	0.047	0.044	0.048	0.034	0.029	0.034	0.023	0.023	-	0.013	0.031	0.036	0.079
14 <i>P. perlata</i>	0.053	0.053	0.055	0.051	0.048	0.047	0.050	0.030	0.028	0.032	0.020	0.023	0.015	-	0.031	0.032	0.076
15 <i>P. hoffmanni</i>	0.052	0.052	0.054	0.048	0.046	0.047	0.045	0.021	0.020	0.025	0.026	0.027	0.027	0.024	-	0.033	0.078
16 <i>P. rupicola</i>	0.055	0.055	0.058	0.049	0.051	0.054	0.054	0.029	0.030	0.033	0.036	0.038	0.036	0.035	0.032	-	0.075
17 <i>P. cruentata</i>	0.051	0.053	0.056	0.049	0.050	0.053	0.051	0.051	0.060	0.060	0.060	0.054	0.056	0.054	0.054	0.066	-

diverged from the ancestor of those two clades considerably earlier than that, judging from the PL analysis, but non-clock-like evolution in its sequences precludes an estimation of timing of that divergence. A further key finding is that these two clades both radiated and evolved their present mtDNA diversity in two short periods in the Plio-Pleistocene, substantially later than the split that separated the two clades themselves. Today, members of both clades occur in all three blocks of humid Neotropical forests (Central American, Amazonian, and Atlantic forests) as well as in drier peripheral areas. Species from these two clades are often sympatric, but sympatry is almost absent among species of the same clade.

The lack of resolution in the relationships among taxa inside the two main clades may be related to rapid diversification. The *p*-distances observed among taxa corroborate this, because distances within each of the two main clades have a very low variance (9.5×10^{-6} and 2.9×10^{-5} for cytochrome *b*, 4.0×10^{-5} and 3.2×10^{-5} for control region), without any evident hierarchical structure (Table 4). Short branch lengths and internodes, and the results of the PL analysis, are similarly corroborative of rapid radiation. Also, divergences are low inside both clades, agreeing with recent diversification dates.

Faunal relationships between Amazonia and the Atlantic Forest of southeastern Brazil have been examined by many authors. Increasingly, two interrelated trends are apparent. First, the Atlantic forest fauna appears to be a composite, with some old endemics and some primarily Amazonian taxa (Costa 2003, da Silva et al. 2004). This can be seen in *Pyrrhura*, where Atlantic forest taxa are represented in its three main evolutionary lineages and in two main temporal periods of diversification: *P. cruentata*, which we have found to be an old lineage in *Pyrrhura*, is endemic to the Atlantic Forest; *P. leucotis* and *P. griseipectus*, which together are southern and northern elements of Atlantic Forest fauna, respectively (see Andrade-Lima 1982), are related to southern Amazonian taxa with low genetic distances (1.2% in cytochrome *b* and 3.1% in control region); and the Atlantic forest *P. frontalis* is sister to *P. perlata*, *P. lepida*, and *P. molinae*, from southern and western Amazonia, also with low genetic distances (2.3% in cytochrome *b* and 2.2 to 2.7 % in control region). A second trend is the spatial and temporal

complexity of biogeographical connections between the two forests. That is, other than the Atlantic Forest being a biogeographical composite, the diversity and incongruence of biogeographical connections between the two areas in various taxa (e.g., Costa 2003) suggests that connections between them have varied in location as well as in the times at which they have occurred. The uncertain position of *P. pfrimeri* of central Brazil in our study contributes to this complexity. Perhaps a factor in obscuring these relationships is relatively short-term, millennial-scale movement of rainforest boundaries described for southern and central Amazonia (e.g., Mayle et al. 2000) that may well underlie some of this spatial and temporal complexity.

The relationships of the northern South American taxa, *P. picta*, *P. emma*, and *P. eisenmanni* were not resolved. There was posterior probability support for them being united on the topology of Figure 3, but high posterior probabilities support a different arrangement in Figure 4. Posterior probabilities are known to overestimate support for groups with very low branch lengths (Lewis et al. 2005, Yang and Rannala 2005), and this may be the case here. Notably, though, no analysis suggested that Central American *P. eisenmanni* is basal within the *picta-leucotis* clade, and no analysis placed it close to *P. griseipectus*, with which it shares, presumably as a homoplasy or symplesiomorphy, broad pale barring on feathers of its chest. It is also important to note that our analyses are robust in not uniting *P. picta*, which occurs in northern and easternmost Amazonia and the Guianas, with Amazonian taxa with which it has so often been linked taxonomically, such as *P. roseifrons* and *P. amazonum*.

Only five species of *Pyrrhura* outside the *picta-leucotis* complex were excluded from this analysis. One of them is *P. devillei*, which forms a superspecies with *P. frontalis* (Forshaw 1989, Collar 1997) and, thus, we predict that this species would be included in the clade uniting *P. molinae*, *P. perlata*, *P. lepida*, and *P. frontalis*. These species show an interesting pattern of distribution, replacing one another in a geographical sequence from the Atlantic Forest, and then to southern and western Amazonia. The only other well-supported association of species outside the *picta-leucotis* clade unites *P. melanura* and *P. albipectus*.

The pattern of diversification found in *Pyrrhura*, with radiations concentrated in two short and partially superimposed periods, suggests that similar changes at similar periods affected habitats in all of the Neotropical forests where these birds occur. The dates estimated for the radiations indicate that they occurred during the end of the Pliocene and in the Pleistocene. Among the proposed mechanisms that may have generated Neotropical diversity, the ones that can be associated with the pattern found here are sea-level changes (Klammer 1984, Räsänen 1995, Marroig and Cerqueira 1997, Nore 1999) and climate changes related to climatic cycles that seem to have been significant during the Pleistocene (Haffer 1969, 1993, 1997; Bush 1994). Other mechanisms, such as tectonic movements and river formation (Salo et al. 1986), probably had little influence on *Pyrrhura* diversification, because during this period the Amazon river system was already developed, and the Andes had almost attained their present height (Clapperton 1993). We note, however, that ecological interactions between members of the two main clades of *Pyrrhura* could be involved in explaining otherwise odd but apparently real gaps in distribution in Amazonia (see Joseph 2002).

Species limits and taxonomic recommendations.—The present study allows some conclusions about the evolutionary units that exist in *Pyrrhura*. Further, it is the first phylogenetic hypothesis for *Pyrrhura* that delimits groups for more detailed studies in the future.

Here, we discuss what our phylogenetic analyses of mtDNA can offer to the clarification of species limits. Reciprocal monophyly for neutral characters between sister lineages is one consequence of the process of speciation and is the most suitable characteristic for establishing species limits in mtDNA-based phylogenies (Moritz 1994). However, if mtDNA data are to be used to investigate species limits, they must be interpreted together with other kinds of data, such as diagnostic morphological characters, nuclear DNA, ecology, and behavior.

The first systematic conclusion of the present study is that *P. picta* and *P. leucotis*, *sensu* Peters (1937) are non-monophyletic assemblages, as Joseph (2000) argued. Conversely, the taxa included in the *picta-leucotis* complex constitute a strongly supported monophyletic group within which there are more than two species.

Pyrrhura leucotis and *Pyrrhura griseipectus*.—The taxa *leucotis* and *griseipectus* were treated as two of five subspecies of *P. leucotis* by Peters (1937). On the basis of diagnostic differences in plumage and size, Joseph (2000) suggested that they and all the other then recognized subspecies of *P. leucotis* could be considered different species. In the present phylogenetic analysis, *P. leucotis* and *P. griseipectus* are in a well-supported clade. *Pyrrhura griseipectus* is monophyletic within this clade, but *P. leucotis* is paraphyletic with respect to it. This pattern, associated with the low genetic distances (0.5% in cytochrome *b* and 1.0% in control region among *leucotis* and *griseipectus* individuals), suggests recent divergence of these two taxa from their most recent common ancestor. Specific status is suggested by the plumage diagnosis, but there are only two diagnostic characters for *P. griseipectus* in the molecular data. *Pyrrhura griseipectus* occurs in an isolated patch of Atlantic Forest that is surrounded by drier vegetation (Andrade-Lima 1982). Its geographical isolation from its closest relative, *P. leucotis*, may have been related to recent climate alterations that, in turn, may have caused the evolution of the “caatinga” vegetation in northeastern Brazil that effectively isolated Atlantic and Amazonian forests (Costa 2003, Auler et al. 2004).

Pyrrhura roseifrons and *Pyrrhura peruviana*.—*Pyrrhura roseifrons* has long been considered a subspecies of *P. picta* (Peters 1937). Recently, however, it has been restored to species rank and recognized to occur in two disjunct populations (Joseph 2000, 2002). A further species, *P. peruviana*, has also been diagnosed by combination of its heavily barred underparts, brown crown, and lack of any of the other diagnostic markings of *P. roseifrons*. *Pyrrhura peruviana* is notable for a lack of any bright red or yellow in its plumage, such traits being prominent in several characters of *P. roseifrons* (Joseph 2002). The molecular phylogeny included *P. roseifrons* individuals from three different localities, one on the range of the “southern” population (Madre de Dios, Peru) and two in the “northern” population (Contamana and Pucallpa, Peru; Table 1 and Figs. 1, 2, and 4; see Joseph 2002). The Pucallpa individuals formed a clade, but the Madre de Dios individual was not separated from the others, showing no differentiation between the two proposed populations.

The Contamana individuals were paraphyletic within the well-supported clade that includes all *P. roseifrons* and *P. peruviana* individuals. *Ptychocheilus peruviana* was monophyletic and appeared as sister to one of the Contamana *P. roseifrons* individuals (number 12; see Figs. 2 and 4). Thus, *P. roseifrons* is paraphyletic with respect to *P. peruviana*. There are only three diagnostic characters for *P. peruviana*; whereas there are eight characters uniting the two *P. peruviana* individuals with *P. roseifrons* individual number 12. Genetic distances are very low within the *peruviana*–*roseifrons* clade (0.5% in cytochrome *b* and 1.2% in control region; Table 3), and there are intraspecific distances within *P. roseifrons* that are higher than distances between *P. roseifrons* and *P. peruviana*.

Two alternative hypotheses emerge from these results. One is that *P. roseifrons* and *P. peruviana*, which are highly differentiated from each other in plumage (Joseph 2002), are recently evolved species whose mtDNA is incompletely sorted. The alternative is that they are geographical variants of a single species. Discriminating between these alternatives and explaining the history of their leapfrog distribution pattern will require more thorough sampling of *P. peruviana*, especially its other, disjunct southern population, as well as more directed sampling of areas from where *P. peruviana* and *P. roseifrons* approach each other geographically. Also involved and possibly critical to resolution of this issue is the phylogenetic position and taxonomic status of a further morphologically distinct population that Joseph (2002) recognized as occurring between northern populations of *P. peruviana* and *P. roseifrons* (Group 6, *sensu* Joseph 2000) that could not be sampled for molecular characters.

Ptychocheilus amazonum and *Ptychocheilus snethlageae*.—Individuals identified as *P. snethlageae* and *P. amazonum* are united in a well-supported clade in all analyses, but relationships among individuals within the clade are not well resolved. Only two groups appear with moderate to good support inside the clade, and these are all *P. snethlageae* (see below): (1) one with four individuals: 24 and 25 from Santa Cruz, Bolivia; 26 from Porto Velho, Brazil; and 27 from Cachoeiro de Nazare, Brazil; and (2) another with five individuals: 28 and 29 from Cachoeiro de Nazare; and 30, 31, and 32 from Alta Floresta. There is no support for uniting

these two groups, and there is no support for the position of the remaining four individuals (33 from Jacareacanga, and 34, 35, and 36 from Altamira). Although the localities of individuals 30, 31, and 32 (Alta Floresta) were assigned by Joseph (2002) to *P. amazonum*, this was an error, and the voucher specimens accompanying these birds as well as individual 33 from Jacareacanga clearly show them to be *P. snethlageae*. Even *P. amazonum* individuals collected in Altamira (34, 35, and 36), a locality that is far from the distribution described for *P. snethlageae*, have intermediate phenotypes, which certainly suggests that there is morphological intergradation between *P. amazonum* and *P. snethlageae*. There is generally low mtDNA divergence between these taxa (the highest distance among all *snethlageae*–*amazonum* individuals was 1.3%, between individuals 26 and 31); and individuals 33, 34, 35, and 36 could not be reliably associated with any other *amazonum*–*snethlageae* individuals within this clade. These results show that the mtDNA evolution in this group is not indicative of differentiation between the two proposed species. Thus, the morphological diagnostic characters that Joseph (2002) used to define these species may be a consequence of very recent isolation that is still not reflected in the mtDNA data, or they may represent morphological polymorphism inside a single species. More studies, including nuclear DNA analyses and finer-scale sampling, are necessary to discriminate between these hypotheses. The present taxonomy provides a more meaningful framework within which to conduct that testing.

Ptychocheilus albipectus and *Ptychocheilus melanura*.—*Ptychocheilus albipectus* has a very restricted range in southeastern Ecuador. Possible hybrids with *P. melanura berlepschii* have been described by Robbins et al. (1987). These authors also mentioned the possibility that *P. albipectus* and *P. melanura* are separated by altitude when breeding but that *P. albipectus* descends to lower elevations when not breeding. Thus, in the nonbreeding season, the ranges of *P. albipectus* and *P. melanura* would overlap. This has led some to propose that *P. albipectus* may, in fact, be a subspecies of *P. melanura*, which occurs from southwestern Colombia to northern Peru and in which five subspecies are recognized. In our analyses, the two *P. albipectus* individuals were sister to *P. melanura melanura* individuals

68, 69, and 70, from southwestern Venezuela. The other two individuals, identified as *P. melanura souancei*, from Ecuador, were, in turn, sister to the above clade, thus rendering *P. melanura* paraphyletic (see Fig. 2). These results indicate that closer study of relationships in the *melanura-albipectus* complex is clearly necessary.

Conclusions.—Our results clarify the confused systematics of *Pyrrhura*, suggest that evolution of most taxa has occurred in relatively short periods of radiation, and provide a basis for understanding part of what appears to be the complex biogeographical history of the group. The value of data from nuclear DNA markers, and of carefully planned field work to sample populations in key areas such as zones of contact between taxa, is evident from our work.

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Anexo IX

The assembly of montane biotas: linking Andean tectonics and climatic oscillations to independent regimes of diversification in *Pionus* parrots

Camila C. Ribas^{1,2,*}, Robert G. Moyle³, Cristina Y. Miyaki² and Joel Cracraft¹

¹Department of Ornithology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA

²Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, 05508-090, São Paulo, Brazil

³Department of Ecology and Evolutionary Biology and Natural History Museum and Biodiversity Research Center, University of Kansas, Lawrence, KS 66045-7561, USA

The mechanisms underlying the taxonomic assembly of montane biotas are still poorly understood. Most hypotheses have assumed that the diversification of montane biotas is loosely coupled to Earth history and have emphasized instead the importance of multiple long-distance dispersal events and biotic interactions, particularly competition, for structuring the taxonomic composition and distribution of montane biotic elements. Here we use phylogenetic and biogeographic analyses of species in the parrot genus *Pionus* to demonstrate that standing diversity within montane lineages is directly attributable to events of Earth history. Phylogenetic relationships confirm three independent biogeographic disjunctions between montane lineages, on one hand, and lowland dry-forest/wet-forest lineages on the other. Temporal estimates of lineage diversification are consistent with the interpretation that the three lineages were transported passively to high elevations by mountain building, and that subsequent diversification within the Andes was driven primarily by Pleistocene climatic oscillations and their large-scale effects on habitat change. These results support a mechanistic link between diversification and Earth history and have general implications for explaining high altitudinal disjuncts and the origin of montane biotas.

Keywords: montane biotas; historical biogeography; speciation; diversification; Andes; Psittacidae

1. INTRODUCTION

Formed behind an eastward-dipping ocean–continental subduction zone, the Andes stretch more than 7000 km from Colombia to Chile and have extensive land area above 4000 m, particularly from Peru through northern Chile and Argentina. This topographic complexity, and the fact that the Andes border the megadiverse lowland Amazonian biota, has contributed to their harbouring the world's most diverse montane avifauna in terms of numbers of species as well as endemics (Fjeldså & Krabbe 1990; Stotz *et al.* 1996).

The origin of the montane Andean flora and fauna, and specifically that of birds, is poorly understood. Two general hypotheses have been proposed. The most widely invoked is that montane elements have been largely derived from lowland relatives via long-distance dispersal (Descimon 1986; Monasterio & Vuilleumier 1986; van der Hammen & Cleef 1986) or by colonization as populations expanded their ranges upslope, became isolated due to climatically induced habitat change, and then became differentiated (Brumfield & Edwards 2007). An alternative hypothesis is that ancestral populations were distributed across both lowland and pre-Andean landscapes, with tectonic uplift passively transporting some to increasingly higher elevations where they

differentiated within a variety of montane habitats (Lynch 1986; Reig 1986; van der Hammen & Cleef 1986; Hall 2005).

Neither hypothesis would be expected to be applicable across all groups, and it might not always be possible to distinguish between them in specific cases. What has generally been lacking in earlier studies, however, is a corroborated phylogenetic hypothesis, which, along with inferences about the spatial and temporal history of the group, could be used to discriminate between these two alternatives. Following vicariance biogeographic principles (Nelson & Platnick 1981), Lynch (1986) outlined such a test, applied it to several small clades of frogs in the genus *Eleutherodactylus*, and proposed that lowland/montane disjunctions were the result of vicariance. Later, Bates & Zink (1994) implied altitudinal vicariance for four species of tyrannid flycatchers (*Leptopogon*). Using a general mitochondrial clock calibration of 2% divergence per million years (Myr), they estimated a lowland/montane age of vicariance at 9–6 Myr (ago). Recently, Hall (2005) constructed a phylogeny for 11 species of butterflies (*Ithomiola*), and inferred a vicariance origin for the montane taxa, but did not provide a temporal framework.

Here we describe the phylogenetic and biogeographic history of species within a single monophyletic genus of parrots (*Pionus*) and infer three independent instances of lowland/high montane vicariance and subsequent diversification. We use phylogenetic and biogeographic patterns,

* Author for correspondence (ribas@amnh.org).

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as well as multiple estimates of divergence times based on molecular sequence data, to propose a causal link between diversification and Earth history, including mountain building and climatic oscillations, as understood from tectonic, palaeogeographic and palaeobotanical data. We discuss the implications these results have for investigating the origins of montane biotas and for understanding mechanisms of biological diversification.

2. MATERIAL AND METHODS

(a) Taxonomic diversity and phylogenetic species

Discovery of all smallest diagnosable taxonomic units, or phylogenetic species, is critical for describing how a group's taxonomic diversity originated across space and time (Cracraft 1997). Our starting point was to test whether previously described subspecies of *Pionus* are diagnosably distinct (phylogenetic species); therefore, we scored 18 external morphological characters for all subspecies (Forshaw 1989; Juniper & Parr 1998) using a large series of museum study skins (498 individuals; see table S1 and 'Taxonomic diversity and distributions', both in the electronic supplementary material).

(b) DNA sequence acquisition

We collected different sets of molecular data. For reconstructing species-level phylogenetic relationships, we obtained 2181 base pairs (bp) of sequence for the complete cytochrome *b* (*cyt b*, GenBank accession numbers EF517602–EF517636) and NADH dehydrogenase 2 (*ND2*, GenBank accession numbers EF517637–EF517671) mitochondrial genes from 35 individuals representing all 19 presumptive phylogenetic species within *Pionus* (table S2 in the electronic supplementary material). Monophyly of *Pionus* was confirmed by undertaking preliminary analyses using a diversity of South American parrots with presumed close and distant relationships (Tavares *et al.* 2004, 2006; Ribas *et al.* 2005; for further details see 'methods' in the electronic supplementary material). From these analyses, close relatives of *Pionus*, including *Gypopsitta barrabandi*, *Amazona farinosa*, *Amazona xanthops* and *Graydidascalus brachyurus*, were chosen as outgroups.

For the molecular clock analyses, sequences from the nuclear recombination activating genes, *RAG-1* and *RAG-2*, were obtained for several genera of parrots (table S3 in the electronic supplementary material). Most *RAG-1* sequences were obtained from GenBank from a recent study (Tavares *et al.* 2006) that focused on Neotropical genera. *RAG-2* sequences were obtained in our laboratory for selected genera including all main lineages of parrots, all main Neotropical lineages, and five *Pionus* species (table S3).

(c) DNA extraction, sequencing and phylogenetic data analysis

DNA extraction, amplification, sequencing and alignment were performed as described (Ribas *et al.* 2005, 2006; for further details see 'methods' in the electronic supplementary material). All primers used are shown in table S4 (in the electronic supplementary material).

Tests were performed to assess incongruence of phylogenetic signal between the two mitochondrial genes and among all three codon positions (ILD test; Farris *et al.* 1995). We further determined whether there was evidence of saturation due to multiple substitutions and evaluated the uniformity of base composition (see 'methods' in the electronic supplementary material).

Phylogenetic hypotheses were constructed through heuristic maximum-parsimony (MP) and maximum-likelihood (ML) searches in PAUP* v. 4.0b10 (Swofford 2002), as well as with Bayesian analysis using MRBAYES v. 3.1 (Huelsenbeck & Ronquist 2001). For MP analyses of the combined morphological and molecular data, the 18 morphological characters were added to a molecular matrix containing only one representative of each taxon. Support for nodes in MP and ML was assessed using the non-parametric bootstrap. Bayesian analyses were conducted with a mixed-model approach. Three independent analyses of 10 million generations each were performed (for further details on MP, ML and Bayesian analyses see 'methods' in the electronic supplementary material).

(d) Biogeographic analyses

Because montane versus lowland elevational distributions are discrete, they were scored as a binary character and each species was coded as being either lowland or montane (distributional data are provided in 'taxonomic diversity and distributions' in the electronic supplementary material). Historical biogeographic change in elevational distribution was assessed using ancestral character-state reconstruction with delayed optimization (Maddison & Maddison 1999).

(e) Molecular dating of divergence times

Estimating ages of divergence across lineages with precision is subject to many caveats (Arbogast *et al.* 2002; Lovette 2004), thus our goal was to seek an internally consistent estimate of divergence times. Because no fossil calibration exists for *Pionus*, three different strategies were implemented to test for concision in age estimates.

Our first approach employed rates of molecular evolution in *cyt b* to bracket divergence time estimates in *Pionus*. The most commonly used rates for *cyt b* have been 1.6% (Fleischer *et al.* 1998) and 2% (Brown *et al.* 1979; Randi 1996) sequence divergence per million years (Myr). It is not known how applicable these rates are across birds as a whole, but the congruence of rates estimated from different studies lends some credibility to using them to bracket divergence times in groups for which no fossil record is available. As *Pionus cyt b* sequences evolve in a clock-like manner ($p > 0.1$, likelihood ratio test), we adopted both 1.6 and 2% rates to convert ML distances to times of divergence within *Pionus*.

For the remaining two strategies, relaxed molecular clock methods were employed. Penalized likelihood (PL) allows for different rates of evolution across the tree, but applies a non-parametric roughness penalty (rate smoothing) that costs the model more if rates change too quickly between branches (Sanderson 2002). The parametric method proposed by Thorne *et al.* (1998) uses a probabilistic model based on priors to describe the change in evolutionary rates over time and uses the Markov chain Monte Carlo procedure to derive the posterior distribution of rates and times.

Previous phylogenetic studies have shown that the endemic New Zealand parrot genera, *Nestor* and *Strigops*, are basal to all other parrots (de Kloet & de Kloet 2005; Tavares *et al.* 2006). This provides a framework for calibrating this basal split using the date of geological separation of New Zealand from West Antarctica (e.g. Barker *et al.* 2004; Tavares *et al.* 2006), estimated to be 85–82 Myr ago (Yan & Kroenke 1993). Because saturation of nucleotide substitutions places limits on the use of mtDNA sequences for dating deep phylogenetic nodes (Jansa *et al.* 2006; Roelants *et al.* 2007), we employed

Figure 1. Topologies generated by the combined analysis of *cyt b* and ND2 (2181 bp). Numbers in parenthesis indicate the number of specimens sampled. Boxes highlight the tree montane clades. (a) ML topology using the GTR + Γ model. Numbers along branches indicate ML bootstrap values greater than 50%/Bayesian posterior probabilities greater than 0.9. Solid circles indicate a bootstrap value greater than 95% and a posterior probability of 1.0. (b) MP topology. Numbers along branches are MP bootstrap values greater than 50% without/with the morphological characters included in the analysis. Solid circles indicate a bootstrap value greater than 95%.

nuclear sequences to estimate divergence dates across a generic tree for parrots using the New Zealand geological calibration. We then adopted these dates to calibrate the mtDNA tree for *Pionus* (see Wiens *et al.* 2007). Thus, we built a tree for *RAG-2* sequences (1155 bp, GenBank accession numbers EF517676–EF517702) for 22 genera of parrots, including 5 *Pionus* species (table S3) and analysed their temporal diversification with both r8s (PL, Sanderson 2003) and MULTIDIVTIME (Thorne & Kishino 2002), fixing the age of the split between *Nestor* and all other genera at 85 Myr ago. Results from this analysis were then used to calibrate the mtDNA *Pionus* phylogeny (for details on the settings used for both r8s and MULTIDIVTIME analyses, see 'methods' in the electronic supplementary material).

Our final approach was designed to examine the precision of the ages determined by *RAG-2*. We obtained nuclear *RAG-1* (2703 bp) sequences from GenBank (Tavares *et al.* 2006) for 30 parrot genera. As this pre-existing dataset was composed mostly of Neotropical genera, we added *RAG-1* sequences from *Nestor notabilis*, *Agapornis personata*, *Micropsitta bruijnii* and *Psittacus erithacus* (table S3; GenBank accession numbers EF517672–EF517675), so that the same geological calibration could be applied. The final *RAG-1* matrix had 34 terminals, including 14 genera in common with the *RAG-2* dataset.

3. RESULTS

(a) Basal taxa (phylogenetic species)

Morphological and genetic data support the recognition of 19 phylogenetic species, although additional sampling will

be required to corroborate the status of several of these taxa (for details, see 'Taxonomic diversity and distributions' in the electronic supplementary material).

(b) Phylogenetic analyses

No insertions or deletions were found, and start and stop codons were in the expected positions. Patterns of base composition were as expected for avian mtDNA. No base composition bias was detected ($p=0.08$ for *cyt b* and $p=0.95$ for ND2). The incongruence length difference test (ILD; Farris *et al.* 1995) rejected incongruence between the two genes ($p>0.05$) and among the three codon positions (*cyt b* $p>0.15$ and ND2 $p>0.20$). Plots of pairwise divergences (Ts and Tv versus p -distance) did not show evidence of saturation. The GTR + Γ model was implemented for *cyt b* ($\alpha=0.155$) and combined ($\alpha=0.195$) mitochondrial data, while TrN + Γ was applied to the ND2 ($\alpha=0.238$) data.

The trees found by ML and MP analyses are shown in figure 1. Bayesian analysis yielded the same topology as the ML tree. Five clades (species groups) were recovered, four of which (*menstruus*, *chalcopterus*, *tumultuosus* and *maximiliani*) have strong support. The *sordidus* species group was less well supported but was monophyletic in all analyses, especially when morphological characters were added to the molecular data (figure 1b). There was consistently high support for a relationship between the *menstruus*, *chalcopterus* and *tumultuosus* species groups, on one hand, and the *maximiliani* and *sordidus* groups on the other, but two phylogenetic uncertainties were also

Figure 2. Distribution and altitudinal ranges of the *menstruus*, *chalcopterus* and *tumultuosus* species groups. Phylogenetic relationships are based on ML analysis (figure 1a). See electronic supplementary material for detailed discussion about distributions and altitudinal ranges. Plates courtesy of William T. Cooper (*Parrots of the world*, 1st edn, 1973).

revealed. First, both ML and Bayesian analyses united the *menstruus* and *chalcopterus* groups with low support (figure 1a), whereas MP clustered the *chalcopterus* and *tumultuosus* groups. This latter clade had low MP support with molecular data alone but had moderate support when morphological characters were included (figure 1b). Second, *Pionus fuscus* is an old lineage, and its association with the *maximiliani* + *sordidus* clade appeared in ML and Bayesian analyses with high support. In contrast, the MP analysis placed *P. fuscus* basal to all other *Pionus* taxa, but this arrangement had very low support (figure 1b).

(c) *Biogeographic patterns within Pionus*

Phylogenetic results revealed that the three Andean clades are not jointly monophyletic but instead have independent relationships to lowland taxa (figure 1). *Pionus chalcopterus* and *Pionus cyanescens* are mid-montane sister taxa of the northern Andes (figure 2). Together they are related to *P. senilis*, a lowland species of Middle America and Mexico. On the ML tree, these three species are related to the lowland *menstruus* species group, which exhibits classic *cis*-, *trans*-Andean and Brazilian Serra do Mar tropical forest distributions. The sister group of these two species groups, as determined by ML, is the *tumultuosus* species group. The latter contains two high montane species, *Pionus tumultuosus* and *Pionus seniloides*. The MP solution (figure 1b) united the *chalcopterus* and *tumultuosus* species groups, and thus on the MP tree montane origins are optimized ambiguously: either there was a single origin for the *chalcopterus* and *tumultuosus* species groups, with a reversal back to lowlands in *Pionus senilis*, or these

species groups became montane independently. Divergence time estimates, discussed below, suggest *P. senilis* separated from *P. cyanescens*/*P. chalcopterus* 2.2–1.2 Myr ago (table 1, node 5), after the emplacement of continuous Panamanian Isthmus lowlands (Duque-Caro 1990). This finding, and the fact that the current distribution of *P. senilis* is centred in the lowlands, strongly implies a primitively lowland distribution for this species. Both trees of figure 1 thus point to three independent montane origins.

Nearly all species of *Pionus*, particularly the montane ones, have their distributions in one or more long-recognized areas of endemism (Cracraft 1985a). Shared patterns of endemism are most parsimoniously explained in terms of a common vicariance history (Nelson & Platnick 1981; Cracraft 1992, 1994), and indeed sister species within montane *Pionus* are allopatric, so that their distributions are consistent with vicariance. Species ranges within *Pionus* are limited by many of the same barriers that affect other montane groups, primarily strong topographical lows having disparate ecologies (e.g. hotter lowland forest or drier intermontane shrubland) when compared with adjacent montane forests.

Examining distributions of Andean species groups phylogenetically reveals shared montane ecological histories within, but disparity among, these clades (figures 2 and 3). Of the three montane clades, the two species of the *tumultuosus* species group occur at the highest elevations in the high temperate zone and both range between 2000 and 3000 m (figure 2). Intermediate in altitude are the highland species within the *chalcopterus* species group (figure 2), with both species being found in mid-montane cloud forests. Finally, taxa of the *sordidus*

Table 1. Estimates of divergence time (Myr ago) within *Pionus*. Nodes are numbered as in figure 4. Fixed calibration times marked with asterisks are derived from a *RAG-2* analysis as described in §2 (see also electronic supplementary material, table S5).

node	cytochrome <i>b</i>		cytochrome <i>b</i> + <i>ND2</i>		cytochrome <i>b</i> + <i>ND2</i>	
	1.6%	2.0%	penalized likelihood (r8s)		Bayesian (MULTIDIVTIME)	
			mean	95% interval	mean	95% interval
1	—	—	9.21*	—	13.30*	—
2	5.83	4.66	4.28*	—	6.90*	—
3	4.38	3.50	3.36	2.08–4.71	5.58	4.46–6.69
4	0.43	0.34	0.37	0.15–0.63	0.76	0.33–1.41
5	1.93	1.54	1.22	0.78–1.74	2.18	1.27–3.30
6	0.25	0.20	0.19	0.12–0.45	0.45	0.12–0.92
7	1.50	1.20	1.29	0.75–1.91	2.34	1.41–3.50
8	5.00	4.00	3.81	2.04–5.14	6.31	5.30–6.91
9	2.38	1.90	1.68	1.26–2.16	2.98	1.98–4.18
10	1.15	0.92	0.64	0.38–0.98	1.18	0.53–2.06
11	0.06	0.05	0.04	0.00–0.15	0.15	0.01–0.45
12	0.04	0.03	0.02	0.00–0.11	0.12	0.01–0.35
13	1.94	1.55	1.35	0.95–1.83	2.41	1.52–3.50
14	0.66	0.53	0.23	0.005–0.49	0.34	0.02–0.91
15	1.66	1.33	1.14	0.84–1.54	2.01	1.18–3.02
16	0.25	0.20	0.11	0.00–0.27	0.32	0.06–0.73

species group have the lowest altitudinal distributions of the three montane clades and occupy low- to mid-montane forests and may range to lower elevations, especially in northern Venezuela (figure 3; for further details about distributions, see ‘taxonomic diversity and distributions’ in the electronic supplementary material).

(d) *Temporal pattern of diversification.*

Based on age estimates using rates of 2.0 and 1.6% sequence divergence per Myr, the basal split within *Pionus* (figure 4, node 2) took place between *ca* 4.7 Myr ago and a maximum of *ca* 5.8 Myr ago (table 1). Importantly, the estimated dates bracket the origins of the three montane lineages as having taken place between *ca* 4.4 and 1.5 Myr ago (table 1, nodes 3, 5 and 9) and also suggest that the radiation of extant species was almost entirely within the Pleistocene.

MP and ML analyses of the *RAG-2* dataset resulted in a topology that agrees with previously inferred phylogenetic relationships among parrot genera (de Kloet & de Kloet 2005; Tavares *et al.* 2006), with the species of *Pionus* forming a well-supported clade, and the New Zealand genus *Nestor* resolved as the sister group of all other parrots (figure S1 in the electronic supplementary material). A PL analysis of the *RAG-2* ML tree, using the external geological calibration, estimated the split between *Pionus* and its sister genera, *Graydidascalus* and *Amazona*, at *ca* 9.2 Myr ago (95% CI: 3.9–18.5 Myr ago), whereas the most basal split within *Pionus* took place *ca* 4.3 Myr ago (95% CI: 1.2–12.4 Myr ago; nodes 8 and 9; table S5 in the electronic supplementary material). The Bayesian analysis of the same *RAG-2* data estimated these two splits at *ca* 13.3 Myr ago (95% CI: 5.2–26 Myr ago and 6.9 Myr ago (95% CI: 1.7–16 Myr ago), respectively (nodes 8 and 9, table S5).

Splits inside *Pionus* were estimated using the mtDNA dataset and applying the *RAG-2* dates as calibration points. Excluding the most basal (fixed-age) node, divergence dates within *Pionus* ranged from 3.81 to

Figure 3. Distribution and altitudinal ranges of the *sordidus* and *maximiliani* species groups and *P. fuscus*. Phylogenetic relationships are based on ML analysis (figure 1a). See electronic supplementary material for detailed discussion about distributions and altitudinal ranges. Plates courtesy of William T. Cooper (*Parrots of the world*, 1st edn. 1973).

0.02 Myr ago using PL and from 6.31 to 0.12 Myr ago in the Bayesian analysis. The dates and confidence intervals are given in table 1, and the chronogram derived from the Bayesian analysis is shown in figure 4.

PL and Bayesian analyses of the *RAG-1* dataset using the same settings and calibration point as that for *RAG-2* resulted in the dates and confidence intervals shown in table S5.

Figure 4. Bayesian MULTIDIVTIME chronogram based on the combined *ND2* and *cyt b* datasets. Nodes 1 and 2 are fixed calibration points derived from a Bayesian MULTIDIVTIME analysis of *RAG-2* data. Time scale is in millions of years. Numbered nodes correspond to those of table 1. Montane clades are shown in boxes. Shaded area shows estimated window of time during which montane/lowland vicariance occurred.

Despite disparate taxon sampling between the *RAG-1* and *RAG-2* datasets, estimated ages for the nodes common to both analyses were similar (figures S1 and S2, and table S5), strengthening our calibration of the mtDNA *Pionus* tree.

We found slight biases related to the different datasets and method of analysis. *RAG-1* age estimates are slightly older than *RAG-2* using the same geological calibration point and same methods, and Bayesian estimates are older than those for PL, given the same calibration and data. As a result of this, the mtDNA age estimates within the genus are slightly younger using PL (table 1). Owing to the lack of multiple calibration points, confidence and credibility intervals are large and overlap substantially.

Despite the variance just noted, multiple approaches to dating diversification within *Pionus* yielded a consistent interpretation: (i) major lineages within the genus arose in the Late Miocene or Pliocene and (ii) most of the species arose in the Pleistocene (figure 4).

4. DISCUSSION

(a) Vicariance origin of montane *Pionus* and Earth history

Taken as a whole, several lines of evidence are consistent with a vicariance hypothesis for the origin of the three montane clades within *Pionus*. First, distributions of these clades are strongly allopatric relative to their lowland sister

clades, and in each case the putative barrier is intervening low montane forest or lowland moist or dry habitats created as the Andes uplifted. These highland/lowland disjunctions are therefore better explained by vicariance rather than three independent long-distance dispersal events. Second, within each montane clade, species are distributed allopatrically in well-known areas of endemism that are shared by many other montane clades of birds. Such congruent patterns of endemism imply a vicariant history in generating species diversity within those clades (Cracraft 1994). Finally, the reconstructed temporal history of diversification (figure 4) is correlative with the uplift history of the Andes, thus establishing a plausible causal linkage to the process of vicariance.

The Central Andean Altiplano and Eastern Cordillera of Peru and northern Bolivia had attained approximately 40–50% of its current elevation by 10 Myr ago, and 2300–3500 m additional uplift has taken place since then (Gregory-Wodzicki 2000). Palaeofloras (Gregory-Wodzicki *et al.* 1998; Gregory-Wodzicki 2000, 2002; Graham *et al.* 2001) and geological analyses (Barke & Lamb 2006; Ghosh *et al.* 2006) for the Eastern Cordillera of the Central Andes indicate Miocene–Pliocene (*ca* 6–5 Myr ago) palaeoaltitudes of approximately 1000–2000 m, which is much lower than present-day elevations (3500 m or greater). At that time, our data suggest that several lineages of *Pionus* were diversifying in this region (figure 4). A 1000–2000 m

palaeoaltitude is at the lower part of the current altitudinal range of one of these lineages, the *P. tumultuosus* species group, which is also the oldest montane clade. The habitat at 1000–1500 m would have been near the cloud forest/tropical forest interface (Graham et al. 2001). We estimate that between 5.58 and 2.34 Myr ago (nodes 3 and 7, table 1 and figure 4) the ancestor of *P. tumultuosus*/*P. seniloides* would have been uplifted approximately 650–1000 m (at 0.2–0.3 mm/yr assuming continuous uplift; Gregory-Wodzicki 2000) and an additional 450–700 m since then, thereby transporting the lineage and its habitat from a subtropical/tropical zone to a temperate one.

In the northern Andes of Ecuador and Colombia, in contrast, the *chalcopterus* species group arose later in time (2.18 Myr ago; node 5, figure 4) and is distributed at lower elevations (figure 2), which may reflect differences in tectonic history. The Ecuadorian Cordillera began developing in the Middle Miocene, with uplift of approximately 6100 m taking place over Plio-Pleistocene times (Steinmann et al. 1999; Coltorti & Ollier 2000). The Eastern Cordillera of Colombia was a continuous range by 11.8 Myr ago (Hoorn et al. 1995; Flynn et al. 1997; Guerrero 1997), but at an elevation of approximately 700 m it had lowland, or perhaps lower montane, forest depending on location (Gregory-Wodzicki 2000). The Eastern Cordillera of Colombia and the Merida Andes of Venezuela experienced rapid Plio-Pleistocene uplift (Gregory-Wodzicki 2000; Chacin et al. 2005), and montane forest environments were established via 1500–1800 m of uplift within the past 4 Myr (Gregory-Wodzicki 2000). This time frame of uplift is consistent with the origins of the *P. chalcopterus/cyanescens* clade (node 5, figure 4) and the *P. sordidus* species group (node 9, figure 4). Within the later, the first split (node 13, figure 4) isolated northern Colombian/Venezuelan taxa (*saturatus*, *antilius*, *sordidus* and *ponsi*) from the Central and Northern Andean taxa (*corallinus* and *mindoensis*) that occur today at higher elevations. This split is dated at ca 2.4 Myr ago, a time when the Northern Andes were undergoing their most recent significant burst of uplift. The northern Colombian/Venezuelan clade was isolated in the Mérida, Perijá and Santa Marta ranges and diversified during a period of uplift (Cediel et al. 2003), with the Santa Marta endemic (*Pionus saturatus*) being the first to be isolated at ca 2 Myr ago (node 15, figure 4). Finally, our temporal estimates (figure 4; table 1) suggest that *P. senilis* separated from *chalcopterus/cyanescens* ca 2.2 Myr ago, well after the Panamanian Isthmus was formed (Duque-Caro 1990). What isolated *senilis* and why it is not found in southern Panama are unknown.

(b) Speciation and Pleistocene climate change

Except for *P. tumultuosus*, *P. seniloides* and *P. saturatus*, all montane species apparently originated within the last 0.5 Myr (table 1). Distributional patterns and relationships of the latter are consistent with vicariance driven by cyclic climatic changes that shifted montane environments to lower elevations during cooler/drier (glacial) times and back to higher elevations during warmer/wetter (interglacial) periods (Haffer 1974). The elevation of the upper forest line has been estimated to have oscillated between 2000 and 3400 m in the Colombian Andes (Hooghiemstra & van der Hammen 2004; Weng et al. 2007). During interglacial conditions montane populations of *Pionus* would have been

isolated by intermontane valleys, tropical forest habitats or both. This mechanism has also been suggested to have promoted diversification in several other groups of Andean birds (Roy et al. 1997; Garcia-Moreno et al. 1999a,b; Chesser 2000).

(c) Vicariance, origin and evolution of montane avifaunas

Although some phylogenetic studies of Andean birds have suggested that montane clades originated prior to the Pleistocene (Bates & Zink 1994; Bleiweiss 1998; Garcia-Moreno et al. 1998, 2001; Burns & Naoki 2004; Chesser 2004; Pérez-Emán 2005), the origins of those clades were not explicitly attributed to vicariance via Andean uplift (Bates & Zink (1994) and Bleiweiss (1998) implied a role for Andean uplift in establishing montane groups). Many authors, in contrast, have proposed that subsequent diversification within these groups was strongly influenced by tectonic activity and climate change (Garcia-Moreno et al. 1998, 2001; Pérez-Emán 2005; Cadena et al. in press; Chaves et al. 2007; among others). Thus, Andean orogenesis has been invoked more frequently as being important for generating diversity within the Andes rather than as a mechanism underlying the origin of the montane avifauna.

We propose that highland-lowland vicariance has been underappreciated for several reasons: (i) the relevant phylogenetic-biogeographic patterns have been overlooked, (ii) avian divergences are generally thought to be too young relative to the time scale of mountain building, (iii) it has long been assumed that ecological (biotic) factors structure altitudinal distributions, (iv) a vicariance split between disjunct montane and tropical lowland clades has been seen as improbable, and (v) some biogeographic methods, especially those that estimate ancestral nodal distributions across a tree using either a parsimony or model-based framework, can themselves create a bias for accepting a dispersalist interpretation. In contrast to Bates & Zink (1994) and this paper, other researchers have identified biogeographic disjunctions between highland and lowland sister groups but did not link their divergence to vicariance via mountain building, for example in *Henicorhina* wood-wrens (Dingle et al. 2006) and *Thamnophilus* antshrikes (Brumfield & Edwards 2007). The latter authors propose a 'colonization' model for some highland taxa, which they proposed post-dated Andean uplift, in contrast to the history suggested here for *Pionus*. Moreover, they applied their model to taxa that have large overlaps in altitudinal distribution, which further suggests it is not applicable to biogeographic patterns such as discussed here.

These observations lead to the conclusion that more attention should be paid to deciphering the phylogenetic and biogeographic histories of montane biotas. In addition to the Andes, mountain ranges in Eurasia, New Guinea and New Zealand underwent rapid elevational change in the Late Neogene; thus, it can be predicted that patterns of lowland/montane vicariance are likely to be more widespread than previously realized. This prediction is testable by biogeographic analysis of montane clades and their lowland sister groups. Many biogeographic studies of montane taxa do not include sampling of lowland relatives, thus lowland/highland vicariance has been probably overlooked. The approach discussed here can

also be used to test the hypothesis that mountain biotas are a source for tropical lowland diversity (Fjeldså 1994). Support for this hypothesis has lacked rigorous phylogenetic and historical biogeographic testing (but see Weir 2006), and our results provide a falsifying instance.

Finally, the observation that allopatric or parapatric distributions of congeners along an altitudinal gradient can be explained as a function of biotic interactions (Terborgh 1971; Diamond 1973) needs critical re-examination. Without discounting possible secondary effects of biotic interactions on distributions, our results and those of others (Arctander & Fjeldså 1994; Bates & Zink 1994; Garcia-Moreno *et al.* 1998, 1999a,b; Cadena 2007) demonstrate that altitudinally parapatric congeners are often not sister taxa, thus providing falsifying instances of the hypothesis of parapatric speciation across altitudinal zones. Increasingly, these studies suggest that allopatric speciation driven by Earth history can explain north-south and altitudinal distributional patterns and is likely to be the primary mechanism underlying the taxonomic assembly of montane communities.

(d) Drivers of diversification

Historical biogeographic analysis interpreted within a framework of Earth history (Nelson & Platnick 1981; Cracraft 1985b, 1992, 1994) is fundamental for developing a mechanistic understanding of biological diversification. Because speciation in plants and animals appears to be largely allopatric (Coyne & Orr 2004), historical biogeographic methods can be used to test the hypothesis (Cracraft 1992, 1994) that allopatry is predominately driven by vicariance due to geological/environmental change rather than by long-distance dispersal. This approach is essential for testing alternative hypotheses about the rate control of speciation, which, along with extinction, is the first-order determinant of patterns of diversity across space and time. The results of this study provide evidence that spatio-temporal diversification in *Pionus* is causally linked to Andean tectonic and palaeoclimate change through vicariance. Because many other species in the Andes share similar spatial patterns, it is probable that these physical controls on diversification extend to them as well.

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Anexo X

ORIGINAL
ARTICLE

Phylogenetic relationships, diversification and biogeography in Neotropical *Brotogeris* parakeets

Camila C. Ribas^{1,2*}, Cristina Y. Miyaki¹ and Joel Cracraft²

¹Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil and ²Department of Ornithology, American Museum of Natural History, New York, NY, USA

ABSTRACT

Aim We present a molecular phylogenetic analysis of *Brotogeris* (Psittacidae) using several distinct and complementary approaches: we test the monophyly of the genus, delineate the basal taxa within it, uncover their phylogenetic relationships, and finally, based on these results, we perform temporal and spatial comparative analyses to help elucidate the historical biogeography of the Neotropical region.

Location Neotropical lowlands, including dry and humid forests.

Methods Phylogenetic relationships within *Brotogeris* were investigated using the complete sequences of the mitochondrial genes *cyt b* and ND2, and partial sequences of the nuclear intron 7 of the gene for Beta Fibrinogen for all eight species and 12 of the 17 taxa recognized within the genus (total of 63 individuals). In order to delineate the basal taxa within the genus we used both molecular and plumage variation, the latter being based on the examination of 597 skin specimens. Dates of divergence and confidence intervals were estimated using penalized likelihood. Spatial and temporal comparative analyses were performed including several closely related parrot genera.

Results *Brotogeris* was found to be a monophyletic genus, sister to *Myiopsitta*. The phylogenetic analyses recovered eight well-supported clades representing the recognized biological species. Although some described subspecies are diagnosably distinct based on morphology, there was generally little intraspecific mtDNA variation. The Amazonian species had different phylogenetic affinities and did not group in a monophyletic clade. *Brotogeris* diversification took place during the last 6 Myr, the same time-frame as previously found for *Pionus* and *Pyrilia*.

Main conclusions The biogeographical history of *Brotogeris* implies a dynamic history for South American biomes since the Pliocene. It corroborates the idea that the geological evolution of Amazonia has been important in shaping its biodiversity, argues against the idea that the region has been environmentally stable during the Quaternary, and suggests dynamic interactions between wet and dry forest habitats in South America, with representatives of the Amazonian biota having several independent close relationships with taxa endemic to other biomes.

Keywords

Amazonia, biogeography, birds, *Brotogeris*, diversification, molecular clock, molecular phylogeny, Neotropics, Psittacidae, South America.

*Correspondence: Camila C. Ribas, Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, R. do Matão, tr. 14, 05508-900, São Paulo, SP, Brazil. E-mail: ribas@amnh.org; ribas@usp.br

INTRODUCTION

Neotropical diversity patterns and their origins have been the focus of much recent research and debate (Haffer, 1969, 1974; Campbell, 1990; Colinvaux, 1993; Bush, 1994; Gascon *et al.*, 2000; Moritz *et al.*, 2000). This interest has been stimulated by the high number of species and their complex distribution patterns, by the diversity of habitats, and by the high threat to this diversity during the last few decades (Mittermeier *et al.*, 2003; Lees & Peres, 2006). Birds are among the most diverse groups of Neotropical vertebrates (Stotz *et al.*, 1996) and have also been among the most studied (Haffer, 1967, 1969, 1974, 1985; Bates, 2001; Aleixo & Rossetti, 2007). Despite these efforts, even patterns of distribution and taxonomy of Neotropical birds are still incompletely understood, and recent studies frequently challenge the traditional taxonomy, demonstrating how much more work is needed before we have a good picture of species limits, distributions, and evolutionary relationships (Bates & Demos, 2001; Aleixo, 2002; Funk & Omland, 2003; Ribas & Miyaki, 2004; Ribas *et al.*, 2005, 2006, 2007a,b; Tobias *et al.*, 2008). As a consequence, well-supported generalizations about patterns of diversification will depend on having additional high-quality taxonomic and distributional data as well as phylogenetic analyses using broad character and taxon sampling.

Neotropical parrots have been the subject of several recent molecular phylogenetic and biogeographical analyses (Ribas & Miyaki, 2004; Russello & Amato, 2004; Tavares *et al.*, 2004, 2006; Ribas *et al.*, 2005, 2006, 2007a,b). These studies have established well-supported genus-level relationships within Neotropical parrots (tribe Arini) and have provided biogeographical and temporal hypotheses of species-level diversification for several genera. Tavares *et al.* (2006) found that Neotropical parrots can be subdivided into three major clades: 'parrotlets'; 'macaws, conures and relatives'; and 'amazons and allies', and that the third group includes the genera *Amazona*, *Pionus*, *Graydidascalus*, *Pionopsitta*, *Triclaria*, *Myiopsitta* and *Brotogeris*. Ribas *et al.* (2005) discovered that *Pionopsitta* is not a monophyletic assemblage, and separated the genus into *Pionopsitta* (comprising only *P. pileata*) and *Gypopsitta* [including all other species; the generic name *Gypopsitta* has subsequently been replaced by the older name *Pyrilia* (Banks *et al.*, 2008)]. Ribas *et al.* (2005) also showed that the Andean genus *Hapalopsittaca* is closely related to *Pyrilia*, and thus is clustered within the 'amazons and allies' clade. Within this major clade, there are only two genera for which species-level relationships have not yet been studied: *Brotogeris* and *Hapalopsittaca*.

The genus *Brotogeris* includes eight recognized biological species and 17 subspecies (Forshaw, 1989; Collar, 1997; Juniper & Parr, 1998). Morphological variability within the genus (Cory, 1918; Miranda-Ribeiro, 1920; Sick, 1997) and differences in karyotype (Lucca *et al.*, 1991) have led some authors to doubt the monophyly of *Brotogeris*. The eight species occur in the Neotropical lowlands (below 1500 m), both east (Atlantic forest, Amazonia and Cerrado) and west (Chocó

and Tumbesian centres, Central American lowlands) of the Andes. Four of these species, with as many as 10 subspecies, have distinct distributions within the Amazon Basin, whereas only one species occurs in the Atlantic forest of south-eastern Brazil. Owing to the diversity of habitats occupied by the different taxa, an understanding of the phylogenetic and temporal pattern of diversification within *Brotogeris* will add to an understanding of several aspects of Neotropical lowland biogeography, especially the biogeographical affinities of Amazonian taxa. Furthermore, inasmuch as diversification patterns of most of the closely related genera have already been described, a phylogenetic and biogeographical analysis of *Brotogeris* will facilitate comparisons across a large group of related genera.

Here we present a molecular phylogenetic analysis of *Brotogeris* using several distinct and complementary approaches: first we test the monophyly of the genus by conducting preliminary phylogenetic analyses including representatives of all other genera within the 'Amazon and allies' clade (hereafter called the 'Amazon' clade) that was recovered by Tavares *et al.* (2006); then we focus on delineating the basal taxa within the genus and uncovering their phylogenetic and biogeographical relationships; and finally, based on these results, we discuss spatial and temporal patterns of diversification both within *Brotogeris* and within the entire Amazon clade.

MATERIALS AND METHODS

Plumage variation and diagnosability

It is widely appreciated that gene trees may not represent species trees, but what is often less emphasized in phylogenetic and phylogeographic studies is that any *a priori* hypothesis of taxonomic limits sets important constraints on interpreting those gene trees. Thus, delimiting basal taxa – having a hypothesis about those units – is essential for understanding patterns of variation, both morphological and genetic, as well as patterns of spatial history. Mitochondrial DNA (mtDNA) sequences have the potential to provide positive character evidence for taxonomic limits when the time to coalescence has been sufficient for character fixation. On the other hand, tree topologies from mtDNA sequences may also be positively misleading about the boundaries of taxa if coalescence has not been achieved within currently independent evolutionary lineages. Many of the pitfalls of misinterpreting gene trees vs. species trees can be avoided if phenotypic evidence is incorporated into the formulation of hypotheses about taxonomic units. We therefore examined large series of specimens housed in the Department of Ornithology, American Museum of Natural History (AMNH), and in the Museu de Zoologia, Universidade de São Paulo (MZUSP) (see Appendix S1 in Supporting Information). We also consulted previous studies on the diagnosability of currently described taxa within *Brotogeris* (Todd, 1917; Neumann, 1931; Gyldestolpe, 1941; Bond & de Schauensee, 1943; Traylor, 1958; Forshaw, 1989; Collar, 1997; Juniper & Parr, 1998; Dickinson, 2003).

Sampling for molecular analysis

All *Brotogeris* species, including 12 of the 17 recognized taxa (species + subspecies) identified in the most recent reviews of the family (Forshaw, 1989; Collar, 1997; Juniper & Parr, 1998), were sampled, with several individuals being sampled per taxon (Table 1). Most vouchers of tissues included in the molecular analysis were examined to confirm identification (except for a few that were not prepared as skins). In order to test *Brotogeris* monophyly, we conducted preliminary analyses that sampled all genera within the Amazon clade of Tavares *et al.* (2006), including *Myiopsitta*, *Pionus*, *Pionopsitta*, *Pyrilia*, *Amazona*, *Alipiopsitta*, *Graydidascalus*, *Triclaria* and *Hapalopsittaca*, plus representatives of the other two clades of Neotropical parrots: *Bolborhynchus*, *Nannopsittaca*, *Touit*, *Ara*, *Aratinga* and *Pyrrhura*. The African genus *Melopsittacus* was used as an outgroup for these preliminary analyses (Tavares *et al.*, 2004, 2006; Ribas *et al.*, 2007a). All sequences of genera other than *Brotogeris* were obtained from GenBank (Table 1).

Data collection

Whole mitochondrial genes encoding NADH dehydrogenase subunit 2 (ND2) and cytochrome *b* (cyt *b*) were sequenced for all 63 *Brotogeris* samples. The nuclear intron 7 of the gene for Beta Fibrinogen (BFib7) was sequenced for a subset of 16 samples (Table 1).

DNA extraction was performed with the DNeasy Tissue Kit (Qiagen, Valencia, CA). Amplifications were conducted using the external primers N5L14750 and HB20 (Ribas *et al.*, 2005) for cyt *b*; LMet and H6313 (Sorenson *et al.*, 1999; Ribas *et al.*, 2005) for ND2; and FIB-BI7U and FIB-BI7L (Prychitko & Moore, 1997) for BFib7. Amplification of the whole gene fragments was conducted through denaturation at 94°C for 5 mins, followed by 40 cycles of denaturation/annealing/extension, with annealing temperature starting at 57°C (first 10 cycles), and dropping to 54°C (last 30 cycles). The amplification of large gene fragments and the touchdown procedure were adopted to minimize the possibility of amplifying non-homologous regions of the genome.

Amplification products were visualized in agarose gels, purified with Perfectprep PCR cleanup kits (Eppendorf, Hamburg, Germany), and used as templates on sequencing reactions with BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA) using the external primers described above plus the internal primers CBL15298 (Cheng *et al.*, 1994) and CBH15764 (Miyaki *et al.*, 1998) for cyt *b*; and H5764 and L5602 (Ribas *et al.*, 2005) for ND2. Cycle sequencing products were run out on an ABI Prism 3100 automated DNA Sequencer (Applied Biosystems). SEQUENCHER 4.1 (Genecodes Corporation, Ann Arbor, MI) was used to assemble chromatograms of complementary fragments, and to produce preliminary alignments, which were later manually corrected using MACCLADE (Maddison & Maddison, 1999).

Phylogenetic analysis

The incongruence length difference (ILD) test (Farris *et al.*, 1994, 1995) implemented in PAUP*4.0b10 (Swofford, 2001) was employed to check for congruence of phylogenetic signal between the two mitochondrial genes and between nuclear and mitochondrial regions. Two datasets were analysed: the first contained mitochondrial sequences for all 51 distinctive haplotypes found among the *Brotogeris* individuals analysed, plus outgroups; and the second was composed of mitochondrial (cyt *b* and ND2) and nuclear (BFib7) sequences for a subset of 16 *Brotogeris* individuals, plus outgroups. Nuclear sequences were also analysed independently to evaluate their phylogenetic signal. Saturation of substitutions in the mtDNA was evaluated through plots of corrected maximum likelihood distances against absolute number of transitions for each pairwise comparison in each codon position.

Maximum parsimony (MP) and maximum likelihood (ML) analyses were conducted in PAUP* 4.0b10 through heuristic searches, and tree bisection–reconnection branch swapping with the starting tree obtained by stepwise addition with 10 random additions of taxa. MP analyses were conducted with all characters equally weighted. An evolutionary model for ML analyses was selected by MODELTEST 3.7 (Posada & Crandall, 1998) using the Akaike information criterion (AIC). Support for inferred clades was obtained through the non-parametric bootstrap (1000 replicates for MP, 500 for ML).

Bayesian analyses were conducted in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). We employed a mixed-model approach to account for potential differences in evolutionary model parameters for subsets of the data (Ronquist & Huelsenbeck, 2003; Nylander *et al.*, 2004). Bayes factors were used to choose the appropriate partitioning scheme and avoid over-parameterization of the data (Kass & Raftery, 1995; Brandley *et al.*, 2005; Brown & Lemmon, 2007). Alternatives tested were: all data in a single partition, partition by gene, partition by codon position, and partition by gene and codon position. Bayes factors were calculated using the harmonic mean from the *sump* command in MRBAYES. The appropriate number of model parameters was determined by running MrMODELTEST 2.2 (Nylander, 2004) on each data partition. After the partition scheme was selected, four runs with four chains each were performed for 10 million generations each. Burn-in was estimated by visual inspection of plots of generation $\times$ likelihood for each run, to determine when likelihood values stabilized. Burn-in results were discarded and the remaining trees from the four runs were combined in order to estimate the majority rule consensus tree.

Patterns of intrataxon genetic variation

Within *Brotogeris*, the *chrysopterus/cyanoptera* complex has the largest taxonomic complexity, with eight subspecies recognized, and is distributed across most of the Amazon Basin (Fig. 1). Because of this, sampling for this complex was

Table 1 Identification, locality code, voucher number, institution of origin, collection locality, and GenBank accession numbers for *Brotogeris* and outgroup samples used.

Taxon	Code	Voucher	Inst.*	Locality	Cyt <i>b</i>	ND2	BFib7
<i>B. tirica</i>	T1	5366	USP	Campus USP, São Paulo, Brazil	FJ652848	FJ652911	FJ667196
	T2	399117	FMNH	Ibateguara, Alagoas, Brazil	FJ652849	FJ652912	–
<i>B. versicolurus</i>	V1	1592	USP	Macapá, Amapá, Brazil	FJ652850	FJ652913	–
	V2	1595	USP	Ilha Caviana, Pará, Brazil	FJ652851	FJ652914	FJ667189
	V2	1596	USP	Ilha Caviana, Pará, Brazil	FJ652852	FJ652915	–
	V3	A08493	MPEG	Ilha do Marajó, Chaves, Pará, Brazil	FJ652853	FJ652916	FJ667192
	V4	B 7252	LSUMNH	Isla Pasto, Rio Amazonas, Loreto, Peru	FJ652854	FJ652917	–
<i>B. chiriri</i>	C1	5486	USP	Urú, São Paulo, Brazil	FJ652857	FJ652920	FJ667190
	C2	3631	KUMNH	Itapua, San Rafael Natl Park, Paraguay	FJ652858	FJ652921	–
	C3	B 37971	LSUMNH	Santa Fé, Santa Cruz, Bolivia	FJ652855	FJ652918	–
	C4	B 37539	LSUMNH	Mina Don Mano, Santa Cruz, Bolivia	FJ652856	FJ652919	–
	C5	71475	UWBM	Samaipata, Santa Cruz, Bolivia	FJ652859	FJ652922	FJ667191
<i>B.s. sanctithomae</i>	S1	2060	USP	Fazenda Apuí, Rio Iaco, Acre, Brazil	FJ652895	FJ652958	FJ667193
	S2	58358	MPEG	Serra do Divisor, Tabocal, Acre, Brazil	FJ652896	FJ652959	FJ667195
	S2	58359	MPEG	Serra do Divisor, Tabocal, Acre, Brazil	FJ652897	FJ652960	–
	S3	58828	MPEG	Assis Brasil, Acre, Brazil	FJ652898	FJ652961	–
	S4	B 7287	LSUMNH	Isla Pasto, Rio Amazonas, Loreto, Peru	FJ652899	FJ652962	–
<i>B.s. takatsukasae</i>	S5	58037	MPEG	Ilha do Marajó, Chaves, Pará, Brazil	FJ652900	FJ652963	FJ667194
	S5	58038	MPEG	Ilha do Marajó, Chaves, Pará, Brazil	FJ652901	FJ652964	–
	S6	20588	MPEG	Omnia Minérios, Juruti, Pará, Brazil	FJ652902	FJ652965	–
<i>B. pyrrhopterus</i>	P1	1730	ANSP	Celica, Rio Catamayo, Ecuador	FJ652860	FJ652923	–
	P1	1733	ANSP	Celica, Rio Catamayo, Ecuador	FJ652861	FJ652924	–
	P1	1782	ANSP	Celica, Rio Catamayo, Ecuador	FJ652862	FJ652925	–
	P1	1809	ANSP	Celica, Rio Catamayo, Ecuador	FJ652863	FJ652926	–
	P2	3493	ANSP	Manta Real, Ecuador	FJ652864	FJ652927	–
<i>B. jugularis</i>	P2	3547	ANSP	Manta Real, Ecuador	FJ652865	FJ652928	–
	J1	B 1543	NMNH	Chiriqui, Panama	FJ652903	FJ652966	FJ667199
	J2	B 28768	LSU	Achiote Road, Colon, Panama	FJ652906	FJ652969	–
	J2	B 28767	LSU	Achiote Road, Colon, Panama	FJ652907	FJ652970	–
	J3	B 46751	LSU	Isla Coiba, Veraguas, Panama	FJ652908	FJ652971	–
	J3	B 46752	LSU	Isla Coiba, Veraguas, Panama	FJ652909	FJ652972	–
	J3	B 46753	LSU	Isla Coiba, Veraguas, Panama	FJ652910	FJ652973	–
	J4	69042	UWBM	Las Nubes, Masaya, Nicaragua	FJ652904	FJ652967	FJ667197
<i>B. cyanoptera</i>	J5	70139	UWBM	Managua, Nicaragua	FJ652905	FJ652968	FJ667198
	Y1	2064	USP	FLONA Maracua, Acre, Brazil	FJ652866	FJ652929	–
	Y2	ESEC 28	MPEG	Assis Brasil, Acre, Brazil	FJ652871	FJ652934	FJ667202
	Y2	ESEC 109	MPEG	Assis Brasil, Acre, Brazil	FJ652872	FJ652935	–
	Y3	B 9534	LSUMNH	Nicolas Suarez, Pando, Bolivia	FJ652875	FJ652938	–
	Y4	B 11167	LSUMNH	Tahuayo, Pucallpa, Ucayali, Peru	FJ652873	FJ652936	–
	Y5	B 39744	LSUMNH	Juanjui, Rio Pauya, Loreto, Peru	FJ652874	FJ652937	–
	Y6	1490	ANSP	Santiago, Ecuador	FJ652867	FJ652930	–
<i>B.c. chrysopterus</i>	Y6	1640	ANSP	Santiago, Ecuador	FJ652868	FJ652931	–
	Y7	2647	ANSP	Taisha, Ecuador	FJ652869	FJ652932	–
	Y8	4873	ANSP	Pasohurco, Ecuador	FJ652870	FJ652933	–
	R1	7778	ANSP	Iwokrama Reserve, Guyana	FJ652876	FJ652939	–
	R1	7791	ANSP	Iwokrama Reserve, Guyana	FJ652878	FJ652941	–
	R1	1262	KUMNH	Iwokrama Reserve, Guyana	FJ652879	FJ652942	FJ667204
	R1	7788	ANSP	Iwokrama Reserve, Guyana	FJ652877	FJ652940	–
	R2	B 11598	NMNH	Upper Essequibo River, Guyana	FJ652882	FJ652945	–
	R2	B 11427	NMNH	Upper Essequibo River, Guyana	FJ652880	FJ652943	–
	R3	B 9732	NMNH	Barima-Waini, Baramita, Guyana	FJ652883	FJ652946	–
<i>B.c. chrysosema</i>	R4	B 12727	NMNH	Parabara Savanna, Guyana	FJ652881	FJ652944	–
	R4	B 12730	NMNH	Parabara Savanna, Guyana	FJ652884	FJ652947	–
	R5	389684	FMNH	Cachoeira Nazaré, Rondônia, Brazil	FJ652885	FJ652948	–
	R5	389685	FMNH	Cachoeira Nazaré, Rondônia, Brazil	FJ652886	FJ652949	–
	R5	389686	FMNH	Cachoeira Nazaré, Rondônia, Brazil	FJ652887	FJ652950	FJ667200

Table 1 Continued.

Taxon	Code	Voucher	Inst.*	Locality	Cyt <i>b</i>	ND2	BFib7
<i>B.c. tuipara</i>	R6	56993	MPEG	Rio Urucu, Amazonas, Brazil	FJ652894	FJ652957	–
	R7	2948	USP	Jacareacanga, Pará, Brazil	FJ652888	FJ652951	FJ667201
	R8	B 7095	NMNH	Altamira, Pará, Brazil	FJ652889	FJ652952	–
	R8	B 7096	NMNH	Altamira, Pará, Brazil	FJ652890	FJ652953	–
	R8	B 7002	NMNH	Altamira, Pará, Brazil	FJ652891	FJ652954	–
	R8	B 7029	NMNH	Altamira, Pará, Brazil	FJ652892	FJ652955	–
	R9	58248	MPEG	Juruti, Pará, Brazil	FJ652893	FJ652956	FJ667203
Outgroups					Cyt <i>b</i>	ND2	Fib7
<i>Myiopsitta monachus</i>		2821	USP	Captive	AF370778	DQ143318	FJ667205
<i>Pionus menstruus</i>		2087	USP	Basiléia, Acre, Brazil	EF517605	EF517640	–
<i>Pionopsitta pileata</i>		494	USP	Captive	AY669436	AY669480	–
<i>Pyrrhura barrabandi</i>		B9596	LSUMNH	Nicolas Suarez, Pando, Bolivia	AY669420	AY669464	–
<i>Amazona farinosa</i>		3683	USP	Captive	DQ143282	AY194461	–
<i>Alipopsitta xanthops</i>		1876	USP	Captive	DQ143293	DQ143316	–
<i>Graydidascalus brachiurus</i>		1623	USP	Ilha Caviana, Pará, Brazil	AY669439	AY669483	–
<i>Triclaria malachitacea</i>		415	USP	Captive	AY669442	AY669486	–
<i>Hapalopsittaca amazonina</i>		B32099	LSUMNH	Cajamarca, Peru	AY669443	AY669487	–
<i>Ara ararauna</i>		13	USP	Captive	DQ150994	DQ143315	–
<i>Aratinga weddelli</i>		2085	USP	FLONA Maracá, Acre, Brazil	AY669401	AY669445	–
<i>Pyrrhura picta</i>		B09287	NMNH	Baramita, Guyana	AY669400	AY669444	–

*USP, Laboratório de Genética e Evolução Molecular de Aves, Universidade de São Paulo; FMNH, Field Museum of Natural History; MPEG, Museu Paraense Emílio Goeldi; LSUMNH, Louisiana State University Museum of Natural History; UWBM, University of Washington Burke Museum; ANSP, Academy of Natural Sciences of Philadelphia; KUMNH, Kansas University Museum of Natural History; NMNH, National Museum of Natural History.

increased so that finer detail in its spatial and demographic history might be revealed from analyses of genetic variation. To visualize genealogical relationships among individuals, haplotype networks were constructed in *rcs* (Clement *et al.*, 2000). In addition, we characterized within-taxon diversity by calculating nucleotide diversity (Nei, 1987) and haplotype diversity. We also undertook statistical tests for detecting past population growth [Tajima's (1989) *D*, Fu's (1997) *F_s*, and Ramos-Onsins & Rozas's (2002) *R₂*] using the program DNASP 4.10.2 (Rozas *et al.*, 2003). Significant values of the various test statistics imply either that population size has not been constant through time, or that these mtDNA genes have been under selection. As there has been recent concern about mtDNA being selectively neutral in birds (Zink, 2005), MK tests (McDonald & Kreitman, 1991), also implemented in DNASP, were used to test for neutrality of *cyt b* and ND2 variation.

Molecular-clock dating

We employed a reduced mtDNA matrix containing two individuals from each of the main clades found in the phylogenetic analyses to estimate divergence dates among taxa. This matrix was analysed by ML in *PAUP** 4.0b10, as described above, and the resulting topology and branch lengths were used for inferring diversification times using the method of penalized likelihood (PL; Sanderson, 2002)

implemented in the program *r8s* (Sanderson, 2003). PL is a semi-parametric smoothing method that combines rate variation among branches with a roughness penalty that discourages rates from varying too much across a phylogeny (Sanderson, 2003). The value of smoothing most appropriate for the data was selected through a cross-validation analysis, also implemented in *r8s*. Empirical 95% confidence intervals for time estimates were obtained as follows: 100 bootstrapped matrices were constructed using the program *seqboot* from the *PHYLIP* package (Felsenstein, 2008); branch lengths were estimated in *PAUP** 4.0b10 for each bootstrapped dataset on the ML topology with likelihood parameters estimated for the original matrix; finally, these trees with their branch lengths were analysed in *r8s* and the standard deviation for each node was determined using the *profile* command. Although saturation of substitutions was observed within the *Brotoogeris* mtDNA dataset, especially in the third positions (see Results), the PL method employs branch lengths obtained through a ML analysis using an evolutionary model that corrects for saturation and variation in rates of substitutions among sites (Sanderson, 2002).

The calibration adopted was based on a molecular dating analysis of nuclear RAG1 and RAG2 genes for several genera of parrots (Ribas *et al.*, 2007a), which was calibrated for the basal split of parrots separating the New Zealand taxa (genera *Nestor* and *Strigops*) from all other parrots. This split was assumed to have occurred between 82 and 85 million years ago (Ma),

Figure 1 Maps showing the distribution of *Brotogeris* species and the Neotropical collection localities for samples used. (a) *B. versicolurus* (V, red); *B. sanctithomae* (S, blue; the blue bar marks the approximate division between the taxa *B. s. sanctithomae* to the west and *B. s. takatsukasae* to the east; see text for details); *B. chiriri* (C, green); and *B. tirica* (T, blue). (b) *B. jugularis* (J, crimson); *B. pyrropterus* (P, green); *B. cyanoptera* (Y, blue); and *B. chrysopterus* (R, red; the specimen localities for diagnosable subspecies are shown in red for *chrysopterus*, magenta for *chrysosoma*, and yellow for *tuipara*; see text for details). Distributions from Ridgely *et al.* (2003); note that one sample (R6) is outside the published distribution area. This individual has been checked for morphological placement within *B. chrysopterus* (Whittaker *et al.*, 2008).

which is the estimated time when New Zealand became isolated from Gondwana and Australia (Yan & Kroenke, 1993). According to the PL results, the genera *Brotogeris* and *Myiopsitta* last shared a common ancestor 16.3 Ma (RAG 2) or 19.1 Ma (RAG 1) (Ribas *et al.*, 2007a). Adopting these results, two PL analyses of the mtDNA dataset were run, one fixing the node corresponding to the split between *Brotogeris* and *Myiopsitta* at 16.3 Ma and the other fixing this node at 19.1 Ma.

Biogeographical patterns

In order to compare spatial and temporal patterns of diversification in *Brotogeris* with patterns found in other closely related Neotropical parrot genera, a matrix of *cyt b* and ND2 sequences was constructed, including one representative of each species of the genera *Brotogeris*, *Pyrilia* and *Pionus* (Ribas *et al.*, 2005, 2007a). To complete the sampling of all genera in the Amazon clade (Tavares *et al.*, 2006), we also included representatives of the monotypic genera *Pionopsitta*, *Graydidascalus*, *Alipiopsitta* and *Triclaria*, and of one species of *Myiopsitta* [*M. monachus*; the genus *Myiopsitta* has recently been shown to comprise two species (Russello *et al.*, 2008)]. *Cyt b* and ND2 sequences for most species of *Hapalopsittaca* and *Amazona* are not yet available, and therefore only one species of *Hapalopsittaca* and four of *Amazona* were included. *Pyrrhura picta* was chosen as the outgroup. This matrix, with 46 taxa and 2181 bp, was analysed through ML using the evolutionary model selected by MODELTEST. The resulting tree with branch lengths was used to estimate divergence dates through PL in r8s. For the r8s analysis, calibration points used were derived from the PL RAG2 analysis of Ribas *et al.* (2007a), and were set as follows: 16.3 Ma for the divergence between *Brotogeris* and *Myiopsitta*; 16.8 Ma for the crown group containing *Pionopsitta*, *Graydidascalus*, *Alipiopsitta*, *Pionus*, *Amazona*, *Pyrilia*, *Hapalopsittaca* and *Triclaria*; and 31.5 Ma for the crown Amazon group. A cross-validation procedure was applied to determine the best smoothing parameter for this analysis. Confidence intervals were obtained through the bootstrap approach as described above.

RESULTS

Sequence characteristics and phylogenetic relationships

Sequences for the whole mitochondrial genes *cyt b* (1140 bp) and ND2 (1041 bp) were obtained for 63 samples of *Brotogeris*, representing 51 distinct haplotypes. A fragment of the BFib7 nuclear intron (880 bp) was sequenced for a subset of 16 individuals. All sequences of this sample aligned unambiguously, as well as with homologous sequences from other Neotropical parrot genera. The partition homogeneity test did not indicate conflict of phylogenetic signal between the two mitochondrial genes (*cyt b* and ND2, $P = 0.55$), nor among the three codon positions within these genes ($P = 0.94$). The test also did not indicate conflict between mitochondrial and nuclear BFib7 data ($P = 0.08$). Saturation of transition substitutions was detected in the three codon positions and was particularly well marked in third positions (Fig. 2).

Our preliminary analysis showed that *Brotogeris* is monophyletic and confirmed that it is sister to *Myiopsitta* (Tavares *et al.*, 2006; Ribas *et al.*, 2007a; Wright *et al.*, 2008). All subsequent analyses were conducted including only *Brotogeris* sequences (51 distinct haplotypes), with *Myiopsitta* and *Pionus*

Figure 2 Plots used to evaluate the presence of saturation in the mitochondrial dataset. (a) Absolute number of transitions relative to maximum likelihood (ML) distance for all pairwise comparisons within *Brotogeris* at first (black diamonds), second (grey squares) and third (dark grey triangles) positions. (b) *p*-distance vs. ML distance for all pairwise comparisons within *Brotogeris*. For reference, the dashed line represents equal values.

as outgroups. Analyses of the mtDNA dataset (53 terminals, 2181 bp) by ML, MP and Bayesian inference resulted in very similar topologies. The MP analysis yielded 29,622 trees with a length of 915 steps. The large number of trees resulted from the variation in the relationships among haplotypes within each major lineage, but relationships among major lineages were the same in all topologies. The ML analysis was performed using the Tamura–Nei model of nucleotide evolution with gamma correction ($\alpha = 0.163$) and resulted in a tree with an ML score of 7487.45 (Fig. 3). The best partition for the BI analysis, as selected by the Bayes factors procedure, was by gene and codon position (six partitions). This partition scheme had a 2ln Bayes-factor value of 240.06 when compared with partition by codon, of 417.12 when compared with partition by gene, and of 439.22 when compared with no partition [2ln Bayes-factor > 10 indicates strong evidence against the alternative hypothesis with fewer partitions (Kass & Raftery, 1995; Brandley *et al.*, 2005; Brown & Lemmon, 2007)]. The consensus of the

best trees found in the BI analysis (after burn-in) corresponds to a topology identical to the ML topology, with the posterior probabilities shown in Figure 3.

All analyses identified eight major lineages within the genus that correspond to traditional biological species (see comments on taxonomic limits below), divided into two well-supported groups: Clade 1, composed of *B. tirica*, *B. versicolurus*, *B. chiriri* and *B. sanctithomae*; and Clade 2, composed of *B. pyrrhopterus*, *B. jugularis*, and the *B. cyanoptera* and *B. chrysopterus* complexes (Fig. 3). The only difference between the MP tree and the ML tree shown in Figure 3 is the position of *B. tirica*: in the ML tree this taxon is basal within Clade 1 with little support, whereas in the MP tree it is sister to *B. versicolurus* + *B. chiriri* with a bootstrap support of 81%, and *B. sanctithomae* is sister to these three species.

A fragment of the BFib7 nuclear intron (880 bp) was sequenced for 16 *Brotogeris* samples (Table 1). No representative of *B. pyrrhopterus* was sequenced, as it was not possible to amplify BFib7 for any of the six tissue samples available (possibly as a result of degraded DNA). Within *Brotogeris* BFib7 sequences we found 27 variable characters, with 10 being parsimony-informative. MP analysis of BFib7 data alone using *Myiopsitta* as the outgroup resulted in 72 trees having 14 steps and recovered only a single clade – ((*chiriri*, *versicolurus*) *sanctithomae*) – with all other taxa appearing in a basal polytomy (including *B. tirica*). Bootstrap support (1000 replicates) was 62% for *chiriri* + *versicolurus* and 88% for ((*chiriri*, *versicolurus*) *sanctithomae*). There was a 12-bp insertion common to sequences of *chrysopterus* and *cyanoptera*, and when the MP analysis was run with gaps being considered as a '5th state', the *chrysopterus* + *cyanoptera* clade was recovered with 100% support (1000 bootstrap replicates).

Analyses of the combined mitochondrial and nuclear matrix (cyt *b*, ND2 and BFib7; 3061 bp) included 16 *Brotogeris* sequences and *Myiopsitta* as the outgroup. MP, ML and BI analyses recovered trees with topologies identical to the ones found in the MP, ML and BI analyses of the mitochondrial data alone. In the ML analysis, bootstrap support for Clade 1 increased from 65% to 78% with the combined data. Support for the relationship ((*B. versicolurus*, *B. chiriri*) *B. sanctithomae*) increased to 88% bootstrap in ML, and to a posterior probability of 1 in the Bayesian analysis, strongly supporting the basal position of *B. tirica* within Clade 1. In contrast, support for the position of *B. tirica* as sister to (*B. versicolurus*, *B. chiriri*) dropped from 88% to 64% in the MP analysis.

Patterns of morphological and genetic variation

As shown above, phylogenetic analysis identified well-supported mitochondrial clades that correspond to the eight biological species recognized within *Brotogeris* by recent compilations (Collar, 1997; Dickinson, 2003). Genetic distances within these biological species are extremely shallow overall. In contrast, subspecies have been described within five of them, and our analysis of morphological variation confirmed diagnosability for some of these. Here we comment on

the systematic status of these taxa, integrating morphological and genetic variability.

1. *Brotogeris versicolurus* and *B. chiriri* are sometimes united into a single species (Forshaw, 1989), but most recent authors (Collar, 1997; Dickinson, 2003) treat them as distinct taxa. Evidence from morphology supports the latter (see also Forshaw, 1978): the overall plumage of *B. versicolurus*, especially of the upperparts, is darker than that of *B. chiriri*, which is a lighter, brighter green. Molecular evidence agrees and recovers these two taxa in well-supported, reciprocally monophyletic clades. Another taxon allied to *B. chiriri* is *B. c. behni*, from central southern Bolivia. Neumann (1931) described *B. c. behni* as being larger than *B. c. chiriri*, with the underparts characterized as blue-green rather than yellow-green. Although Bolivian specimens assigned to *B. c. behni* tend to be larger than those of *B. c. chiriri*, there is considerable variation, and moreover we could find no consistent difference in tone either of the underparts or of the underside of the tail (Forshaw, 1978: p. 482). Currently, there is no evidence that *behni* and *chiriri* are isolated, and variation between them may be clinal. The lack of variability

and structure among mtDNA sequences within *B. chiriri* (Fig. 3) is consistent with these observations.

2. The biological species *B. sanctithomae* contains two sub-species: *B. s. sanctithomae* of western Amazonia and *B. s. takatsukasae* of eastern Amazonia along the Amazon River (Fig. 1). These two taxa are diagnosably distinct in morphology, although not strongly so (*takatsukasae* has a yellow streak of variable size behind the eye, whereas *sanctithomae* does not). Published distribution maps show them to be allopatric, with the slight gap in their distributions being located between the Tapajós and Madeira rivers (Forshaw, 1989), but examination of specimens at MZUSP (see Appendix S1) showed that *takatsukasae* occurs as far west as the Madeira River, where there is overlap between the two phenotypes in one locality (Rio Madeira, Nova Olinda). Individuals assigned to these two subspecies were not distinct in the analysis of mtDNA (Figs 1 and 3; Table 1).

3. The two taxa included in the biological species *B. jugularis*, namely *B. j. jugularis* of north-western South America and Middle America, and *B. j. exsul* of northern Venezuela and adjacent Colombia, appear to be diagnostic (based on three

specimens, the lower flanks and thighs of *exsul* are green, not bluish: Todd, 1917; Forshaw, 1989). A comprehensive study is needed to confirm this difference and to understand better their distribution in northern South America. It is not clear, for example, whether the subspecies are allopatric, and if so, what the barrier between them might be. We had no tissues from *B. j. exsul*, precluding comment on mtDNA variation.

4. The most taxonomically complicated biological species of *Brotogeris* is *B. chrysopterus*, with five described subspecies, three of which (*chrysopterus*, *chrysosema* and *tuipara*) were found to be diagnosably distinct based on the specimens analysed (see Appendix S1). The other two subspecies, *solimoiensis* and *tenuifrons*, need further investigation as we had access to only a few specimens. *Brotogeris c. tuipara* and *B. c. chrysosema*, which occur south of the Amazon River, have reddish orange frontal bands and chin spots, whereas *B. c. chrysopterus*, which occurs in the northern portion of the Basin, has the frontal band greatly reduced, with the band and the chin spot both being brownish/blackish. However, some specimens collected north of the Amazon River have orange chin spots and some also have light brown foreheads (A. Aleixo, personal communication), indicating some introgression between *B. c. chrysopterus* and *B. c. tuipara*. *Brotogeris c. chrysosema* is characterized by yellow primary coverts, which are orange in the other taxa. Although the Tapajós is reported in the literature to limit the distributions of *tuipara* and *chrysosema*, we have examined 13 specimens (MZUSP10636; MZUSP10637; MZUSP10638; MZUSP46423; MZUSP58242; MZUSP58243; MZUSP58244; MZUSP58255; MZUSP58256; MZUSP60483; MZUSP63033; MZUSP64817; MZUSP64818) from the northern portion of the Madeira–Tapajós interfluvium that show *tuipara* phenotypes, and this is confirmed by the genetic data. There is a partially structured genetic pattern within this species-group. The haplotype network (Fig. 4a) shows two main groups separated by four mutational steps. One is composed exclusively of *chrysopterus* haplotypes that occur north of the Amazon River at localities R1–4 (Fig. 1). The second group contains haplotypes of two diagnosably distinct taxa south of the Amazon River (*chrysosema* at localities R5 and R6; and *tuipara* at localities R7–9), but this group also includes two individuals of *chrysopterus* phenotype collected north of the Amazon River at localities R2 and R4 (Fig. 4a).

5. The biological species *B. cyanopectera* comprises three named subspecies. All three appear to be diagnosably distinct (Gyldenstolpe, 1941: p. 9; Forshaw, 1978: p. 488). According to the literature, *B. c. cyanopectera*, lacks yellow along the bend of the wing and the carpals, although we have examined several individuals that have a very small amount of yellow in this region. In *B. c. gustavi*, the edge of the wing, carpals and anteriormost underwing coverts are yellow, whereas the edge of the wing, carpals and primary coverts of *B. c. beniensis* are yellow, but the anteriormost underwing coverts are not. Hybrids are known between *cyanopectera* and *gustavi* (Traylor, 1958) and between *cyanopectera* and *beniensis* (Bond & de Schauensee, 1943). Because *beniensis* and *gustavi* are allopatric,

Figure 4 Haplotype networks of mtDNA gene sequences (cyt *b* and ND2) in *Brotogeris*. Circle size is proportional to the number of individuals that share one haplotype (shown in brackets when more than one sample from the same locality has the same haplotype). (a) *Brotogeris chrysopterus*. R1–R4: *chrysopterus*; R5–R6: *chrysosema*; R7–R9: *tuipara*. White circles correspond to samples collected south of the Amazon River, grey circles to samples collected north of the Amazon River. (b) *Brotogeris c. cyanopectera*. White circles correspond to samples from Ecuador.

it is doubtful that any of this variation is clinal, but certainly much additional analysis should be done to clarify the distribution of the morphological variation. Sampling for the mtDNA analysis included one bird from northern Bolivia (Y3, Fig. 1b), near the locality from where *beniensis* was described, and one from the Huallaga valley region, in north-central Peru (Y5), near where *gustavi* was described. Both individuals, however, have very little yellow in the bend of wing and carpals, and are phenotypically closer to *cyanopectera*. As a consequence, we lacked tissue samples from ‘true’ *beniensis* and *gustavi*. Among the *cyanopectera* samples sequenced, two distinct mtDNA clades are identified that are separated by 10 mutational steps (Fig. 4b), one of them includes only Ecuadorian individuals (Y6–8), whereas the other includes samples from northern Bolivia, western Brazil and eastern Peru (Fig. 1b). No phenotypic variation was detected corresponding to these two clades.

Corrected ML distances among biological species varied from 0.55% (*B. chiriri* and *B. versicolorus*) to 5.77% (*B. jugularis* and *B. sanctithomae*). ML distances within those species ranged from 0.05% (within *B. chiriri*) to 1.11% (within *B. cyanopectera*). Nucleotide diversity within species was generally low (Table 2). Tajima’s *D*, Fu’s *F_s*, and Ramos-Onsins and Rozas’ *R₂* test statistics were mostly non-significant ($P > 0.05$). The only significant results were Fu’s *F_s* and *R₂* for *B. jugularis* ($P = 0.01$, Table 2), rejecting the null hypothesis of demographic stability for this species. The MK tests showed that

Table 2 Number of individuals sampled from each taxon of *Brotogeris* (*N*), number of haplotypes (*H*), nucleotide diversity (*Pi*) and summary statistics (Tajima's *D*, Fu's *F_s*, and *R₂*). *Significant values of *P*.

	<i>N</i>	<i>H</i>	<i>Pi</i> (%)	Tajima's <i>D</i> (<i>P</i> -value)	Fu's <i>F_s</i> (<i>P</i> -value)	<i>R₂</i> (<i>P</i> -value)
<i>B. tirica</i>	2	2	0.092 (0.046)	–	–	–
<i>B. versicolurus</i>	5	4	0.170 (0.057)	–	–	–
<i>B. chiriri</i>	5	3	0.038 (0.014)	–	–	–
<i>B. sanctithomae</i>	8	6	0.163 (0.021)	–0.060 (0.52)	–1.301 (0.22)	0.1561 (0.23)
<i>B. pyrrhopterus</i>	6	4	0.055 (0.017)	–0.447 (0.44)	–1.454 (0.17)	0.1805 (0.05)
<i>B. jugularis</i>	8	8	0.253 (0.132)	–1.168 (0.15)	–3.872 (0.01)*	0.0736 (0.01)*
<i>B. cyanoptera</i>	10	10	0.532 (0.200)	–0.019 (0.51)	–3.147 (0.05)	0.1357 (0.26)
<i>B. chrysopterus</i>	19	14	0.332 (0.025)	–0.615 (0.29)	–3.561 (0.07)	0.0991 (0.13)

neutrality could not be rejected for any lineage within *Brotogeris*.

Temporal and spatial patterns of diversification

The calibrated divergence between *Brotogeris* and *Myiopsitta* is estimated to be between 16.3 and 19.1 Ma (Ribas *et al.*, 2007a). Based on these dates, the crown-clade diversification of *Brotogeris* was estimated to have begun between 5.0 and 6.0 Ma, with the split between the two main clades (Fig. 5, node 2; Table 3). Further splits within both clades took place within the last 3 Myr (Fig. 5). Two sister-species, however, are much younger: *B. versicolurus* and *B. chiriri* split around 380 and 440 thousand years ago (Fig. 5, node 5; Table 3), and *B. cyanoptera* and *B. chrysopterus* diverged around 0.93 and 1.09 Ma (Fig. 5, node 8; Table 3).

The mean substitution rates for mtDNA were estimated by r8s to be 0.009 and 0.011 substitutions per site per lineage per million years, employing calibrations derived from RAG1 and RAG2 analyses, respectively (Table 3). These values are close to the commonly used rate of 2% per million years, which corresponds to 0.01 substitutions per site per lineage per million years. The analysis including all the genera within the

Amazon clade (Tavares *et al.*, 2006) found that most of the diversification within the genera occurred during the last 8 Myr (Fig. 6). Moreover, we infer that *Brotogeris* diversified within a time-frame similar to those seen in *Pionus* and *Pyrilia*.

DISCUSSION

Brotogeris is monophyletic with high support and consists of an old lineage, separated from its sister-genus, *Myiopsitta*, by large sequence divergence values (c. 14% uncorrected *p*-distance; c. 30% corrected ML distance). *Brotogeris* monophyly shows that tail morphology, which is rounded and short in some taxa (*B. cyanoptera*, *B. chrysopterus*, and *B. sanctithomae*) and cunneated and long in the others, does not map to phylogenetic relationships. This adds to previous molecular evidence (Tavares *et al.*, 2006) that concluded that this character does not reflect major phylogenetic breaks within Neotropical parrots, as had been proposed previously by Sick (1997). Karyotype diversity within the genus, reflected in the quite distinct karyotype described for *B. sanctithomae* and *B. versicolurus* (Lucca *et al.*, 1991), is also proved not to be indicative of large phylogenetic distance.

Patterns of molecular and plumage variation

Brotogeris parakeets occupy a diverse array of habitats, but, as with most Neotropical parrots, their distribution is associated with the presence of large trees that are used for nesting (Forshaw, 1989). Most species occur in forested habitats but do occupy forest borders and adjacent open areas, and several are reported to be quite tolerant to habitat change (Forshaw, 1989). This broad habitat tolerance is probably one of the reasons for the large distributions and our finding of relatively little intraspecific geographical genetic structure.

All biological species of *Brotogeris* strongly covary in their morphological and genetic distinctness, but within biological species, patterns of genetic and morphological variation do not always agree. There are instances in which variation covaries but not strongly. Thus, the ML analysis of mtDNA (Fig. 3) recovers the diagnosable taxa *tuipara* and *chrysosema* within the biological species *B. chrysopterus*, but these clades are not strongly supported. On the other hand, lack of covariation is seen within *B. sanctithomae*: allopatric or near-allopatric populations are diagnosable in morphological characters

Figure 5 Chronogram derived from a penalized likelihood analysis using the calibration of 16.3 Ma for the split between *Brotogeris* and *Myiopsitta*. Confidence intervals are shown as grey bars. Dates and confidence intervals for numbered nodes are shown in Table 3.

Method	PL	PL	ML distance (mtDNA)
Calibration of basal node	RAG2	RAG1	
Node 1 (fixed basal node)	16.3	19.1	
Node 2	5.08 (4.01–6.75)	5.95 (4.69–7.91)	11.67% (1.10)
Node 3	3.05 (2.26–5.63)	3.58 (2.65–6.65)	5.48% (1.02)
Node 4	2.89 (1.90–5.63)	3.39 (2.22–6.60)	5.77% (0.15)
Node 5	0.38 (0.09–0.67)	0.44 (0.11–0.70)	0.55% (0.19)
Node 6	2.92 (2.18–4.78)	3.43 (2.56–5.60)	7.34% (0.72)
Node 7	2.01 (1.32–3.03)	2.36 (1.54–3.87)	5.63% (0.19)
Node 8	0.93 (0.51–1.43)	1.09 (0.60–1.73)	1.82% (0.10)
Mean substitution rate*	0.011 (7.8×10^{-06})	0.009 (9.2×10^{-06})	

Table 3 Divergence dates (confidence intervals) in Ma and maximum likelihood distances (standard deviation) obtained from the mtDNA dataset among *Brotogetis* taxa and corresponding to the nodes of the tree shown in Figure 5. For the penalized likelihood (PL) dating, calibrations based on previous analyses of the nuclear genes RAG1 and RAG2 were employed. *Substitutions per site per lineage per Myr.

Figure 6 Chronogram derived from a penalized likelihood analysis of the ‘Amazons and allies’ clade of Tavares *et al.* (2006). Numbers on nodes correspond to common area relationships among different genera: 1, split between *cis*- and *trans*-Andean taxa; 2, origin of Atlantic forest endemic taxa; 3, split between eastern and western Amazonian taxa; 4, split between Central American and *cis*-Andean taxa. Confidence intervals are shown as grey bars. Distribution codes: ATL, Atlantic forest; AMZ, Amazon forest; CSA, dry forests of central South America; CAM, Central America; AND, Andes; NSA, northern South America; CHO, Chocó; SSA, southern South America; TMB, Tumbesian centre of endemism.

(*sanctithomae* and *takatsukasae*), but they are not reciprocally monophyletic in mtDNA (Fig. 3, Table 1). And interestingly, the opposite situation is seen within *B. cyanopectera*: genetic data

provide strong evidence for a diagnosably distinct population in eastern Ecuador (Fig. 4b) but that distinctness is not paralleled by morphological differences.

The most general explanation for these observations is that there are temporal discrepancies between the mtDNA of isolated populations becoming reciprocally monophyletic, on the one hand, and plumage characters, under the control of nuclear loci, becoming fixed, on the other. The manifestations of these processes may or may not be synchronized in populations that have recently diverged, as our data suggest. At the same time, these patterns may be influenced by gene flow, through introgression, as apparently is the case among the diagnosable taxa (subspecies) of *B. cyanopectera* and *B. chrysopterus* (see below). In any case, these results stress the importance of the correct individuation of taxonomic units, through the use of both molecular and morphological markers.

Temporal and spatial patterns of diversification

An important biogeographical pattern that emerges from the analysis of relationships within *Brotogeris* is that the Amazonian lineages do not constitute a monophyletic clade. This characteristic of the Amazonian avifauna has been found in several other studies of groups that are restricted to forested habitats, including *Schiffornis turdina* (Nyári, 2007), *Glyphorhynchus spirurus* (Marks *et al.*, 2002) and *Pyrrhura* (Ribas *et al.*, 2006). These close relationships of Amazonian taxa to groups that occur in other forests corroborate the ideas that the Amazonian biota, taken as a whole, has not been isolated for long, and that connections to other currently isolated forest blocks have existed at different points in time.

Brotogeris is divided into two clades. Clade 1 (Fig. 3) includes Amazonian *B. versicolor* and *B. sanctithomae*, which occur mostly in seasonally inundated forest (várzea), river islands and forest edges; the Atlantic forest endemic *B. tirica*; and *B. chiriri*, which occurs in open woodlands, cerrado gallery forests, and in the dry Caatinga vegetation of north-eastern Brazil. Clade 2 (Fig. 3) includes Amazonian *B. cyanopectera* and *B. chrysopterus*, associated with 'terra firme' forest; the Tumbesian endemic, *B. pyrrhopterus*; and *B. jugularis*, which occurs mostly in 'lightly wooded areas' of northern South America (east and west of the Andes) and Central America (Forshaw, 1989). All *Brotogeris* species distributed in lowland forests are reported also to occupy adjacent open areas as long as there are suitable large trees for nesting (Forshaw, 1989). Only two species (*B. chiriri* and *B. jugularis*) are primarily associated with open formations (although *B. chiriri* is reported to occupy gallery forests within the cerrado, and *B. jugularis* can be found in the canopy of humid forest; Ridgely, 1981; Forshaw, 1989).

The basal position of the Atlantic forest endemic *B. tirica* in Clade 1 is supported by the combined nuclear and mitochondrial analysis. Among the genera closely related to *Brotogeris*, there are examples of old Atlantic forest endemics, such as *Pionopsitta pileata* and *Triclaria malachitacea* (Fig. 6), and examples of Atlantic forest endemics recently split from Amazonian and Central American taxa, such as *Pionus reichenowi* (Fig. 6). *Brotogeris tirica* seems to have split from

its Amazonian/Central Brazilian sister clade at about 3.0–3.6 Ma, at the end of the Pliocene. Since then, palaeoclimatic changes have affected the distribution of Neotropical forests, and there is clear evidence that wet events occurred in presently semi-arid north-eastern Brazil throughout much of the Pleistocene, with current dry conditions having been established recently. This suggests that Atlantic and Amazonian forests may have been in contact several times during the Pleistocene (Auler *et al.*, 2004). The relatively old age of the Atlantic forest endemic *B. tirica*, when compared with *Pionus reichenowi*, for example, shows that these contacts between the Atlantic and Amazonian forests affected closely related genera in different ways, isolating Atlantic forest endemic taxa at different times.

Soon after the first diversification event that isolated the Atlantic forest endemic *B. tirica*, the Amazonian lineage split into *B. sanctithomae*, on the one hand, and the ancestor of *B. versicolor* + *B. chiriri*, on the other, with both descendants probably still associated with floodplain forests. Currently, the two largely sympatric floodplain forest species differ slightly in their habitats. *Brotogeris versicolor* occurs in seasonally inundated forests and river islands, whereas *B. sanctithomae* occupies primarily the margins of seasonally flooded forests along the larger rivers, and also ranges out into clearings close to watercourses (Ridgely, 1981). This suggests that, although the vicariance event may have been related to fragmentation of the currently continuous floodplain forest at about 3.0 Ma, ecological specialization within the várzea habitat may also have played a role in originating and maintaining the initial isolation between these two taxa. Both species lack phylogeographic structure, as individuals collected at the extremes of their rather long and narrow distributions – localities on Marajó Island (V1–V3 and S5) and in northern Peru (V4, S4), more than 2500 km apart – show very little genetic differentiation (Figs 1 and 3).

A similar pattern of little phylogeographic structure has been described for other várzea-specialist birds, such as *Xiphorhynchus kienerii* and *X. obsoletus* (Aleixo, 2006), as well as for floodplain-specialist rodents (Patton *et al.*, 1994; Matocq *et al.* 2000). The pattern seen in these floodplain *Brotogeris* taxa, though, is not different from a general pattern of little intraspecific phylogeographic structure found in most species within the genus (Fig. 3). Although signs of recent population expansion have been detected only within *B. jugularis* (Table 2), the current sampling does not rule out this possibility for the remaining taxa. The other possible explanation for the relative lack of phylogeographic structure is that these taxa have high dispersal rates, with a possible exception being the Ecuadorian populations of *B. cyanopectera*, and recent restrictions to gene flow among *B. chrysopterus* populations.

The recent divergence between *B. versicolor* and *B. chiriri* (< 0.5 Ma) represents a disjunction between continuous floodplain forest habitats and gallery forests embedded within open areas. *Brotogeris chiriri* occupies open vegetation enclaves within Amazonia (Pinto & Camargo, 1957), and has recently been reported from a patch of savanna in southern Amazonia

(Aleixo & Poletto, 2007), expanding its reported range, although the two taxa have never been recorded in sympatry (Aleixo & Poletto, 2007). Divergence between them is likely to have been affected by drying events during the Pleistocene, followed by the contraction and fragmentation of the flood-plain forest, which may have isolated the ancestor of *B. chiriri* in gallery forests.

Within Clade 2, taxa that occur in Central America and in northern South America (*B. jugularis* and *B. pyrrhopterus*) split from Amazonian taxa around 3.0 Ma (Table 3, node 6 in Fig. 5), more recently than the split between *cis*- and *trans*-Andean taxa observed in *Pyrilia*, which was dated at around 8.0 Ma (Fig. 6). One of the two *trans*-Andean species (*B. jugularis*) occupies open and dry areas both east and west of the Andes (*B. j. exsul* and *B. j. jugularis*, respectively), including the Llanos, indicating that the Andean mountains probably have not been a strong barrier for these taxa, as there is a possible connection through the Caribbean lowlands around the northern end of the mountains, at least in periods of low sea-level stands (Haffer, 1967; Brumfield & Capparella, 1996). This interpretation is favoured because the split between Amazonian and northern South American/west Andean taxa within *Brotogeris* occurred at a time (c. 3.0 Ma) when the Andes had already developed to high elevations (Gregory-Wodzicki, 2000), although tectonic activity was still occurring in the region. Thus, in contrast to the situation in *Pyrilia* (Fig. 6), the split in *Brotogeris* cannot be strongly related to Andean uplift. Instead, the vicariance event between *B. jugularis* + *B. pyrrhopterus* and *B. chrysopterus* + *B. cyanoptera* may be another example within the genus in which speciation might have been related to habitat shift from primarily humid forest habitats to more open and dry formations, following the development of a drier and cooler climate in northern South America during the Pliocene (Hooghiemstra & van der Hammen, 1998).

Brotogeris pyrrhopterus is endemic to the dry forests of the Tumbesian centre of endemism in western Ecuador and north-western Peru (Cracraft, 1985; Best & Kessler, 1995; Fig. 1). This region contains one of the highest concentrations of restricted-range bird species in South America (Terborgh & Winter, 1983). Of the 35 Tumbesian endemic bird species whose presumed closest relatives are known, 15 have their closest relatives in other arid regions, whereas the remaining species are sister to taxa that occur in humid habitats, including Andean and lowland forests (Best & Kessler, 1995). The Tumbesian dry forests are isolated from other lowland dry forest habitats by the humid forests of the Chocó region. Avifaunal connections between the Tumbesian region, the arid Marañón valley, and the dry forests of Central America have been described (Chapman, 1926), suggesting the existence of a dry-forest corridor in dry periods of the Pleistocene. Such a corridor would have allowed connections between dry-forest biotas (Best & Kessler, 1995). As both *B. pyrrhopterus* and its sister species, *B. jugularis*, occur mostly in more open and dry habitats, it is possible that the Chocó humid lowland forest has been the barrier separating these two taxa. Their split is

estimated at about 2 Ma, at the Pliocene/Pleistocene transition, pointing to an older, pre-Pleistocene, isolation of the Tumbesian dry forests, and corroborating the notion that these forests have been a relatively stable habitat throughout the Pleistocene. *Pyrhura orcesi*, another endemic Tumbesian psittacid, also seems to be related to the Central American and western Venezuelan species *P. rhodocephala* and *P. hoffmanni* (although with little bootstrap support) and differs from these two taxa by c. 2.0% mtDNA genetic distance (Ribas *et al.*, 2006).

Despite being locally common, *B. pyrrhopterus* is still under threat as a result of the pressures of habitat loss and the pet trade (Best & Kessler, 1995). The restricted range of this species, its low intra-specific diversity (Table 2), and the large genetic distance (c. 5.6%) to its sister-group, *B. jugularis*, are all factors that indicate that *B. pyrrhopterus* is a unique and vulnerable lineage.

***Brotogeris chrysopterus* and *Brotogeris cyanoptera*: diversification patterns within Amazonia**

Brotogeris chrysopterus and *B. cyanoptera* are found in eastern and western Amazonia, respectively. The precise limits of their distribution are still incompletely known (Fig. 1b). The two taxa have been reported in sympatry in central Amazonia (Rio Urucu, Amazonas, Brazil; Whittaker *et al.*, 2008), although hybrids have not been reported. Both taxa are reciprocally monophyletic with high support, and their split was dated to around 1.0 Ma (Figs 3 and 5). A similar east/west break within the Amazon Basin is also seen in *Pyrilia*, but at an earlier time (around 4.0–5.0 Ma; Fig. 6).

As discussed above, there are morphologically diagnosable taxa within *B. chrysopterus*, with *chrysopterus* being distributed north, and *tuipara* and *chrysosema* south of the Amazon River. Although there is some molecular differentiation between *B. chrysopterus* populations north and south of the Amazon River, the haplotypes of two individuals collected north of the river at localities R2 and R4 are resolved on the haplotype tree as being linked to southern Amazonian individuals (most closely to an individual collected at locality R7; Fig. 4a). The voucher of one of the individuals at locality R4 confirms it as having the *chrysopterus* phenotype. Moreover, other samples from localities R2 and R4 sort with *chrysopterus* on the haplotype network. This pattern may be the result of remnant ancestral polymorphism, or gene flow. Sampling along the north bank of the Amazon is insufficient to prefer one explanation over the other, but the finding of some 'intermediate' phenotypes between *chrysopterus* and *tuipara* in this region suggests ongoing gene flow. These results indicate that the lower portion of the Amazon River is not a strong barrier for this group, contrasting with patterns seen in several other Amazonian groups that usually do not show close relationships between taxa or populations from the Guiana and Brazilian shields (reviewed in Aleixo & Rossetti, 2007). The fact that *tuipara* individuals occur west of the Tapajós River, being recorded in the northern portion of the Madeira–Tapajós

interfluvium, also suggests an apparent weakness of large rivers as barriers for *Brotogeris*.

Although a north/south split coinciding with the position of the Amazon River is a clear biogeographical break for Amazonian taxa, the patterns seen in *Pyrilia* and *Brotogeris* show that east/west breaks have played an important role as well, although the possible barriers associated with these breaks are not so evident. Vicariance separating eastern and western taxa within Amazonia could be related to periods of drying in central Amazonia, or to historical differences in the evolution of eastern and western portions of the Amazon Basin (Rossetti *et al.*, 2005; Campbell *et al.*, 2006; Aleixo & Rossetti, 2007). The drying of Amazonia and consequent formation of forest refugia (Haffer, 1969) has become a contentious idea. Although climate modelling reconstructions indicate extensive replacement of humid forest by drier vegetation in central Amazonia (Anhuf *et al.*, 2006), especially south of the Amazon River, other lines of evidence suggest there was not an appreciable decrease in rainfall in the Southern Hemisphere during Northern Hemisphere glacial periods (Cruz *et al.*, 2005). At the same time, there has been a growing amount of evidence pointing to the prevalence of braided fluvial systems and wetlands in western Amazonia during the Quaternary, prior to the development of the current drainage system (Rossetti *et al.*, 2005; Campbell *et al.*, 2006). The effects that this recent Amazonian wetland system might have had on the diversification of the biota are still largely unexplored. Bates (2001) and Aleixo & Rossetti (2007) proposed that 'terra firme' taxa found in western Amazonia should be relatively young, since this region would only recently have been available to species that occur in non-inundated forest. Another outcome of this scenario would be the isolation of 'terra firme' forest species in Andean foreland forests and in eastern Amazonia, with their later expansion after the drainage of the western Amazonian lowlands. The pattern seen in *Brotogeris* adds to this discussion in an interesting way, as large rivers apparently do not constitute strong barriers to dispersal in this group when compared with many other Amazonian clades. Importantly, our results point to a recent split between *B. chrysopterus* and *B. cyanoptera*, which supports the notion that at c. 1.0 Ma unsuitable habitat for these taxa developed in central Amazonia (and a similar pattern in *Pyrilia* suggests that such a vicariance event operated at an earlier time as well).

Comparisons with other Amazonian parrot genera

Several Amazonian *Brotogeris* species occur in sympatry. They occupy forest edges, floodplain forest, riverbanks and river islands and are thus adapted to 'open' areas within the forest. Within *Pyrilia*, there are no species that occur in open formations, and most species are allopatric within Amazonia, with the exception of the pair *P. aurantiocephala*/*P. vulturina* (Gaban-Lima *et al.*, 2002; Ribas *et al.*, 2005). The two genera diversified over a similar period of time (Fig. 6) and have the same number of Amazonian taxa, although in *Pyrilia*

Amazonian species form a monophyletic clade. The Amazonian species of *Pionus* (*P. fuscus* and *P. menstruus*) are broadly sympatric, and have larger distributions than species of *Pyrilia*. In a study of activity patterns in parrots within the Peruvian Amazon, Gilardi & Munn (1998) compared flight height relative to canopy among several genera of parrots and showed that *Pyrilia barrabandi* flies within the canopy whereas *B. sanctithomae*, *B. cyanoptera* and *Pionus menstruus* fly 8–16 m above the canopy. This is only an indication of how different the habits of these closely related parrot genera are, and provides one possible explanation for why *Pyrilia* may be more limited by habitat changes or large river channels within the forest than are *Brotogeris* and *Pionus*.

The biogeographical sequence of diversification events is very similar within *Pyrilia* and within Clade 2 of *Brotogeris*: a first split separating Central American/west Andean taxa from those in Amazonia, followed by a split within each new clade separating east/west Amazonia and Central America/west Andes (Fig. 6). Despite this correspondence, the timings of the vicariance events are different, as are the habitat preferences of the two genera. This illustrates the importance of incorporating more information, such as time and habitat preference, in biogeographical analyses, rather than relying solely on the topology of area cladograms.

CONCLUSIONS

Because of the broad habitat tolerances of species in this genus, the challenge when studying speciation in *Brotogeris* is to identify the barriers, either physical or ecological, that were responsible for generating the diversity we see today. The biogeographical history of *Brotogeris* implies a dynamic history for South American biomes since the Pliocene, especially in Amazonia. These results agree with the idea that the geological evolution of Amazonia has been important in shaping its biodiversity, and at the same time argue against the idea that the region has been environmentally stable during the Quaternary. The biogeographical pattern of *Brotogeris* also suggests dynamic interactions between wet and dry forest habitats within South America, with representatives of the Amazonian biota having several independent close relationships with taxa endemic to other biomes. To understand current biogeographical patterns we need to integrate knowledge across taxa found in different habitats and ecosystems, considering how these habitats have changed through time, both spatially and ecologically, and how the species are consequently influenced by those environmental changes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Information on the specimens of *Brotogeris* analysed.

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BIOSKETCHES

Camila C. Ribas currently has a postdoctoral position at the Zoology Department, Universidade de São Paulo, Brazil, and is a research associate of the Department of Ornithology, American Museum of Natural History. Her research interests include historical biogeography, molecular systematics, phylogeography and patterns of diversification of Neotropical birds.

Cristina Y. Miyaki is Associate Professor at the Departamento de Genética e Biologia Evolutiva at the Universidade de São Paulo. Her research focuses on the molecular systematics, historical biogeography, phylogeography and conservation genetics of birds.

Joel Cracraft is Lamont Curator of Birds and Curator-in-Charge, Department of Ornithology, American Museum of Natural History in New York. His research interests include avian systematics, historical biogeography and biological diversification.

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Anexo XI

Molecular markers for population genetic analyses in the family Psittacidae (Psittaciformes, Aves)

Patrícia J. Faria and Cristina Y. Miyaki

Universidade de São Paulo, Instituto de Biociências, Departamento de Genética e Biologia Evolutiva, São Paulo, SP, Brazil.

Abstract

The selection of molecular markers for population studies is an important tool for biodiversity conservation. The family Psittacidae contains many endangered and vulnerable species and we tested three kinds of molecular markers for their potential use in population studies of five psittacid species: 43 hyacinth macaws (*Anodorhynchus hyacinthinus*), 42 blue-and-yellow macaws (*Ara ararauna*), 23 red-and-green macaws (*Ara chloroptera*), 19 red-spectacled amazons (*Amazona pretrei*); and 18 red-tailed amazons (*Amazona brasiliensis*). We tested 21 clones from a genomic library of golden conure (*Guarouba guarouba*) minisatellites and 12 pairs of microsatellite primers developed for the domestic chicken (*Gallus gallus*) and *A. hyacinthinus*. We also tested seven tetranucleotide repeat primers for their ability to amplify regions between microsatellite loci (inter simple sequence repeats, ISSRs). We were able to select seven markers that were variable in different degrees for three species (*A. hyacinthinus*, *A. chloroptera* and *A. ararauna*). The mini and microsatellites produced more polymorphic patterns than the ISSRs. The genetic variability of the species studied seems to be correlated with their endangered status.

Key words: microsatellites, minisatellites, molecular markers, parrots, population studies.

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Introduction

Population or species viability depends on stochastic and deterministic demographic, environmental and genetic factors. Estimations of genetic diversity can be very important in programs for the conservation of biodiversity but such data need to be used with caution. In some situations, such as when the habitat is being rapidly destroyed, it is futile to be concerned with long-term goals such as genetic variability analysis (Haig, 1998) and it would be better to concentrate resources on the effective protection of the environment instead of conducting genetic studies. However, genetic diversity is important for the population to be able to face future environmental changes and to ensure a long-term response to selection (see Gilpin and Soulé, 1986; Hunter, 1996; Frankham *et al.*, 2002; Fernandez *et al.*, 2004).

Concern over the conservation of many threatened species has highlighted the importance of genetic data and the maintenance of genetic diversity is the major goal for some biodiversity conservation programs (Frankham *et al.*,

2002; Fernandez *et al.*, 2004). Neutral genetic markers are assumed to reflect adaptive genetic variation that is important to the evolutionary potential of the species (Hunter, 1996; Frankham *et al.*, 2002) and consequently, the selection of useful molecular markers is necessary to conduct these studies.

Among the molecular techniques available, DNA fingerprinting, developed by Jeffreys *et al.* (1985), has been widely utilized in studies of various groups of animals including threatened species of birds (Miyaki *et al.*, 1993; Craveiro and Miyaki, 2000; Caparroz *et al.*, 2001). This technique is based on the detection of multilocus minisatellites (*i.e.* variable number of tandem repeats (VNTRs); Jeffreys *et al.*, 1985). These minisatellite loci can also be isolated from genomic libraries and analyzed separately. The use of single locus minisatellites presents some advantages over the use of multilocus minisatellites, including the possibility of comparing the band patterns from many specimens on different gels (Burke *et al.*, 1996; Wetton and Parkin, 1997). The cloned single locus minisatellites are usually species-specific, a disadvantage of using this kind of marker (Burke *et al.*, 1996), but some studies have shown that many cloned loci can be used in other species of the same genus or family (Hanotte *et al.*, 1992; Wetton and

Send correspondence to Cristina Yumi Miyaki. Universidade de São Paulo, Instituto de Biociências, Departamento de Genética e Biologia Evolutiva, Rua do Matão 277, 05508-090 São Paulo, SP, Brazil. E-mail: cymiyaki@ib.usp.br.

Parkin, 1997). Single locus minisatellites have also been widely used in different animals including mammals (Amos *et al.*, 1993; Sherwin *et al.*, 1993), fish (Taggart and Ferguson, 1990) and birds (Hanotte *et al.*, 1992; Dixon *et al.*, 1994; Wetton *et al.*, 1995).

Microsatellites are also VNTRs, but are less frequent in the avian genome than in other organisms (Primmer *et al.*, 1997). This kind of marker has the advantage of being able to be amplified by PCR (and thus, it does not require large amounts of DNA), is usually highly polymorphic in the number of repeat units and shows a single locus pattern which allows the comparison of populations based on their allele frequencies (Bruford *et al.*, 1996). However, the development of microsatellite markers also requires a large amount of work, but, fortunately, many primers developed for one species can be used in related species (Moore *et al.*, 1991; Crooijmans *et al.*, 1993; Hanotte *et al.*, 1994; Bruford *et al.*, 1996; Primmer *et al.*, 1996). Microsatellites have been useful in many animal conservation studies, including Komodo dragons (Ciofi and Bruford, 1999), turtles (FitzSimmons *et al.*, 1997), whales (Buchanan *et al.*, 1996), wolves (Roy *et al.*, 1994), snakes (Prosser *et al.*, 1999), bears (Paetkau *et al.*, 1998), butterflies (Keyghobadi *et al.*, 1999) and birds (Hansson *et al.*, 2000; Nesje *et al.*, 2000; Johnson *et al.*, 2003).

Another approach for estimating genetic variability is to use primers of short repeated sequences (inter simple sequence repeats, ISSRs) to amplify anonymous genomic regions between two microsatellite loci, and it has been demonstrated that using this technique (called the single primer amplification reaction, SPAR) it is possible to obtain molecular markers in plants and animals (Gupta *et al.*, 1994; Wolfe *et al.*, 1998; Esselman *et al.*, 1999; Ge and Sun, 1999; Fernandes-Matioli *et al.*, 2000). The principal advantage of this kind of approach is that it is not necessary to construct genomic libraries.

One of the most important uses of molecular markers is to determine the genetic variability in species and populations that suffer from human disturbances, such as birds of the family Psittacidae (Aves: Psittaciformes). About 30% of the species from this family are endangered or vulnerable. Their ability to imitate human voice, their high intelligence and colorful plumage make them attractive to humans. Illegal trading, habitat destruction and competition for suitable nest sites are the major factor leading to the reduction in population size of these birds (Collar, 1997). In Brazil there are 70 psittacid species, 14 of which are considered endangered or vulnerable (Collar *et al.*, 1992; Collar, 1997; Sick, 1997; BirdLife International, 2000). One example is *Cyanopsitta spixii* (Spix's macaw) that has been extinct in the wild since 2000 and of which there are only about 60 birds in captivity. Another critically endangered species is *Anodorhynchus leari* (Lear's macaw), of which there are only about 500 specimens remaining.

In the study described in this paper our aim was to select suitable molecular markers for population analysis of psittacid species and to estimate the genetic variability of five species from the family Psittacidae (*Anodorhynchus hyacinthinus*, *Amazona brasiliensis*, *Amazonia pretrei*, *Ara ararauna* and *Ara chloroptera*).

Materials and Methods

Psittacid specimens and DNA extraction

We analyzed 145 captive or wild psittacids: 43 hyacinth macaws (*Anodorhynchus hyacinthinus*), 42 blue-and-yellow macaws (*Ara ararauna*), 23 red-and-green macaws (*Ara chloroptera*), 19 red-spectacled amazons (*Amazona pretrei*) and 18 red-tailed amazons (*Amazona brasiliensis*). Golden conure (*Guarouba guarouba*) DNA was used as a positive control for the hybridizations using single-locus minisatellite probes. Blood samples (0.1 mL) were taken from the brachial vein in the wing, mixed with 0.5 mL of absolute ethanol and stored at room temperature. Total DNA was extracted using the method of Bruford *et al.* (1992).

Single locus minisatellite markers

Single locus minisatellites were detected using probes (Table 1) from a Golden Conure (*Guarouba guarouba*) genomic library (CY Miyaki, unpublished). The banding patterns were initially analyzed in three to five specimens from each species using *G. guarouba* DNA as the positive control. The loci deemed to be suitable as markers (GguA₄, GguA₆, GguA₁₂, and GguB₁₀) were then used to investigate all the specimens of *A. hyacinthinus* and the two *Ara* species. During the course of this study, the banding patterns obtained with the GguA₄ probe could not be reproduced and it was abandoned. We used the following methodology for both an initial screening and a more detailed investigation. For each specimen tested, 6 µg of genomic DNA was digested with the *Mbo* I restriction enzyme and the fragments separated by electrophoresis on a 25 cm long horizontal agarose (1% w/v) gel. The fractionated DNA fragments were transferred onto nylon membranes by standard capillary Southern blotting (Sambrook *et al.*, 1989) and hybridized with the probes selected from a *G. guarouba* genomic library. The probes were labeled with α P³² dCTP by random priming and hybridization was carried out at 65 °C for 24 h in a solution containing 0.263 M Na₂HPO₄, 1 mM EDTA, 7% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) bovine serum albumin (BSA) and 10 µg mL⁻¹ chicken or salmon competitor DNA. After hybridization the membranes were washed at 65 °C with a solution of 40 mM Na₂HPO₄ containing 1% SDS (w/v) for 10 min and then in 0.1 X saline-sodium citrate (SSC) buffer containing 0.1% SDS (w/v) for 10 to 25 min. These membranes were placed on intensifying screens in contact with Kodak RX X-ray film for 2-7 days at -70 °C.

Table 1 - Type of banding patterns obtained with the 21 *Guarouba guarouba* probes tested in three to five specimens of *G. guarouba* (positive control), *Anodorhynchus hyacinthinus*, *Ara chloroptera* and *Ara ararauna*.

Probe	Type of banding patterns			
	<i>G. guarouba</i>	<i>A. hyacinthinus</i>	<i>A. chloroptera</i>	<i>A. ararauna</i>
GguA ₂	ML	ML	ML	ML
GguA ₄ and GguA ₇	SL	SL	SL	SL
GguA ₆	ML	SL	SL	ML
GguA ₈	ML	ML	ML	ML
GguA ₉ (small)	MO	MO	MO	MO
GguA ₉ (large)	MO	MO	MO	MO
GguA ₁₂	MO	MO	SL	MO
GguB ₁ (small)	SA	SA	SA	SA
GguB ₁ (large)	SL	SA	SA	SA
GguB ₇ , GguB ₈ , GguB ₉ , GguB ₁₀ , GguC ₁₀ , GguD ₂ and GguD ₃	SL	SL	SL	SL
GguG ₁	SA	SA	SA	SA
GguG ₂	SA	SA	SA	SA
GguG ₇	SA	SA	SA	SA
GguH ₅	SA	SA	SA	SA

ML = multilocus; SL = single locus polymorphic; MO = monomorphic; SA = satellite. The words 'small' and 'large' indicate the insert sizes of the clone. The probes GguA₄ and GguA₇ gave equal banding patterns for the same specimens (indicating that they detected the same loci), as did the probes GguB₇, GguB₈, GguB₉, GguB₁₀, GguC₁₀, GguD₂ and GguD₃.

Microsatellite markers

We also tested 12 pairs of microsatellite primers (Table 2), ten of which were obtained from a chicken (*Gallus gallus*) genomic library (Gibbs *et al.*, 1997) and two from an *A. hyacinthinus* genomic library (Scott K. Davis, personal communication), using three to five specimens from each species. After screening we used the HYA 1172 and MAC 436 primers in all the specimens of *A. hyacinthinus* and of the two *Ara* species. Polymerase chain reactions (PCRs) were performed in 10 µL of 1X amplification buffer (Pharmacia) containing 0.2 mM of each dNTP, 0.75 units of *Taq* DNA polymerase (Pharmacia), 10 pmol of each primer and 25 ng of psitacid genomic DNA using 35 cycles of 95 °C for 60 s, 48-55 °C for 30 s and 72 °C for 40 s. In some cases the conditions (annealing temperatures and magnesium concentrations) were varied to try to get better results from specific primers. The products were separated on 6.5% (w/v) acrylamide gels and the fragments visualized using two methods: incorporation of α P³² dCTP during the PCR and then placing the gels onto intensifying screens in contact with Kodak RX X-ray film which was exposed for 1-2 days at -70 °C; and silver staining of the acrylamide gel followed by photographing the films using general photographic techniques.

Inter simple sequence repeat markers

To investigate inter simple sequence repeats (ISSRs) we tested seven tetranucleotide repeat primers ((GGAT)₄, (CACT)₄, (GGGT)₄, (GGAC)₄, (GACA)₄, (AAGC)₄ and (TAGG)₄) using three to five specimens from each species. Then we used the (GACA)₄ and (AAGC)₄ primers to con-

Table 2 - Microsatellite primers tested, ten from the domestic chicken (*Gallus gallus*) and two from the hyacinth macaw (*Anodorhynchus hyacinthinus*).

Locus	Primer sequences (5'-3')
45A7 ¹	GGATGAAGTGCCACCATCAGG ATGCGTGCTTAGAGGCCAGTG
41F4 ¹	TGAAACATGTATGGAGTCTCAGCA GACAGCTAAATGCAGTTTCATGG
50D5 ¹	ACATTTCCACAGCTCCCTGC AAGCTGACATTCACCACCAGGA
53B10 ¹	GATTCACCTTGATGGTCGAGG GATCTAATGTGGAATGCCTGATAC
73B11 ¹	TCCTTCACTGGGCGTGCTC CAAGTGTGAAGCCCATAGTGCA
70A3 ¹	GCGGAGAGCAATTAGTCTGCAA CGGCTCGGGAACAATCAC
75C3.2 ¹	GATCCAGGTGGTGGCTCTAACACGA TTAGTCTCTGCTTGCTACTGCA
66E7 ¹	ATCTCATTATCCTTGTGTGAAACTG ATCATGCATTGTATTGCGC
40H10.2 ¹	ACATTTCTCAGTGGAGCTGGGC CCGCGCACTAAGTCATCCG
72E2 ¹	GACGAAAGGCCGCTGTCTGT GCGTGAATAGGAGCGCGG
MAC436 ²	GCACCAAACACAACATCTTATTC TTGGGACACCAATGTAATTTG
HYA1172 ²	GATCCTTGTCTTAAGACAGATGTC GAGTGAAATACACATTCAGCTTCTG

¹From *G. gallus*, Gibbs *et al.*, 1997; ²From *A. hyacinthinus*, Scott K. Davis (unpublished).

duct a more thorough investigation using all the specimens of all five psitacid species. The PCRs were performed in 10 µL of 1X amplification buffer (Pharmacia) containing

0.2 mM of each dNTP, 0.75 units of *Taq* DNA polymerase (Pharmacia), 10 pmol of primer and 25 ng of psitacid genomic DNA using 35 cycles of 94 °C for 45 s, 48-60 °C for 60 s and 72 °C for 60 s. In some cases the conditions (annealing temperature, amount and quality of target DNA and primer concentration) were varied to try to get better results from specific primers. The products were then separated by electrophoresis on 2% (w/v) agarose gels and photographed. The reactions were repeated several times for each species.

Genetic variability analyses

These analyses were carried out by comparing the banding patterns obtained (mono or polymorphic), the level of variation observed in these patterns, the number of alleles (N) and the heterozygosity (H). For minisatellites and microsatellites, the variability was estimated by calculating the number of alleles detected and the heterozygosity. For ISSR this was done by determining the number of haplotypes and the genetic diversity in each species. The Arlequin program version 2.0 (Schneider *et al.*, 2000) was used to calculate the mean observed and expected heterozygosities and the genetic diversity.

Results

Single locus minisatellite markers

The initial screening hybridizations with only three to five specimens per species revealed that the 21 probes from

the *G. guarouba* genomic library did not hybridize with the DNA of the two *Amazona* species (*A. brasiliensis* and *A. pretrei*) but did result in characteristic satellite or mini-satellite (multilocus and single-locus monomorphic and polymorphic) patterns in the two *Ara* species (*A. chloroptera* and *A. ararauna*) and *Anodorhynchus hyacinthinus* (Table 1).

The probes GguB₇, GguB₈, GguB₉, GguB₁₀, GguC₁₀, GguD₂ and GguD₃ were initially considered as different clones but showed equal banding patterns for the same specimens, indicating that they detected the same loci. This was also observed for the probes GguA₄ and GguA₇ that detect another locus. Consequently, just one of each of these probes was tested for its polymorphism in all specimens.

The probes that showed polymorphic single locus pattern (GguA₄, GguA₆, GguA₁₂ and GguB₁₀) were selected for the analysis of a broader sampling. As the banding patterns obtained with the GguA₄ probe could not be reproduced, it was abandoned. The number, size and frequency of the alleles obtained with each probe are presented in Table 3.

Microsatellite markers

Only one of the ten *Gallus gallus* microsatellite primers tested (50D5) was able to amplify the DNA of the five psitacid species tested but it was monomorphic. Both of the *A. hyacinthinus* microsatellite primers (HYA 1172 and MAC 436) produced amplification products from samples of three species of macaws (*Anodorhynchus hyacinthinus*

Table 3 - Results for all specimens of the macaw species *Anodorhynchus hyacinthinus*, *Ara chloroptera* and *Ara ararauna* using the single locus minisatellite probes GguA₆, GguA₁₂ and GguB₁₀. N = number of alleles; S = band size in kilobase pairs; F = relative allele frequencies; and H = heterozygosity. A dash (-) indicates no data available.

Probe	<i>A. hyacinthinus</i>				<i>Ara chloroptera</i>				<i>Ara ararauna</i>			
	N	S	F	H	N	S	F	H	N	S	F	H
GguA ₆	6	5.7	0.061	0.68	8	9.8	0.042	0.84	-	-	-	-
		6.0	0.076			10.5	0.062					
		6.6	0.015			10.7	0.062					
		8.2	0.424			11.6	0.104					
		8.5	0.409			12.2	0.458					
		9.2	0.015			13.2	0.146					
						14.6	0.104					
GguA ₁₂	2	1.7	0.50	-	2	2.9	0.317	0.84	1	3.4	-	-
		1.9	0.50			3.2	0.683					
GguB ₁₀	4	1.7	0.025	0.43	8	1.70	0.061	0.82	12	2.3	0.10	0.92
		1.8	0.025			1.80	0.183			2.4	0.16	
		1.9	0.710			1.86	0.211			2.6	0.04	
		2.0	0.240			1.90	0.055			2.7	0.04	
						2.00	0.217			2.8	0.12	
						2.20	0.228			2.9	0.16	
						2.40	0.017			3.0	0.04	
						3.10	0.028			3.1	0.02	
										3.2	0.08	
										3.3	0.04	
										3.5	0.16	
										3.6	0.04	

and the two *Ara* species) and generated polymorphic patterns. The number, size and frequency of the alleles obtained in each species being shown in Table 4. Even with various annealing temperatures and magnesium concentrations the HYA 1172 primer pair did not produce an analyzable pattern for the *Amazona* specimens. The sequences of the alleles from some *Amazona* specimens showed that there was a nucleotide addition in the middle of the repeat block and a substitution of an AC repeat by a GC in some specimens (data not shown). Also, the MAC436 primer pair could not be used in the *Amazona* specimens because it did not amplify. It thus appears that these two loci are not suitable molecular markers for the genus *Amazona*.

Inter simple sequence repeats

We tested seven primers over a broad range of annealing temperatures in the five species of Psittacidae. The primers (GGAT)₄, (CACT)₄, and (GGGT)₄ did not amplify the DNA of any of the species tested. Even though the (GGAC)₄ primer revealed polymorphic patterns between

different genera, it was not suitable as a population marker because it produced monomorphic patterns in different species of the same genus. Despite being polymorphic between different species the (TAGG)₄ primer was also unsuitable because the banding patterns were extremely weak (even after many tests in which the annealing temperature, amount and quality of target DNA and primer concentration were varied), which made it impossible to identify the haplotypes. The (GACA)₄ primer at 56 °C and the (AAGC)₄ primer at 55 °C produced banding patterns that were exclusive to each species but variable between specimens of *Ara ararauna* and of *Ara chloroptera*, while only monomorphic patterns were produced in *Anodorhynchus hyacinthinus*, in *Amazona brasiliensis* and in *Amazona pretrei*. The number of haplotypes, their band sizes and frequencies are shown in Table 5.

Genetic variability

Given the few available markers for the two *Amazona* species we only compared the genetic variability data be-

Table 4 - Results for microsatellite primers HYA1172 and MAC436 in all specimens of *Anodorhynchus hyacinthinus*, *Ara chloroptera* and *Ara ararauna*. N = number of alleles; S = band size in base pairs; F = relative allele frequencies; and H = heterozygosity. A dash (-) indicates no data available.

Primer	<i>A. hyacinthinus</i>				<i>Ara chloroptera</i>				<i>Ara ararauna</i>			
	N	S	F	H	N	S	F	H	N	S	F	H
HYA1172	2	134	0.821	0.28	2	134	0.969	0.12	2	134	0.568	0.41
		136	0.179			136	0.031			136	0.432	
MAC436	4	131	0.696	0.32	9	123	0.050	0.80	4	148	0.107	0.44
		135	0.050			141	0.125			150	0.387	
		145	0.246			145	0.033			152	0.470	
		151	0.008			147	0.067			154	0.036	
						149	0.325					
						151	0.191					
						153	0.158					
						185	0.033					
						195	0.016					

Table 5 - Number of haplotypes (N), size range of the bands in base pairs (S), relative haplotype frequencies (F) and genetic diversity (H) obtained with the inter simple sequence repeat (ISSR) primers (GACA)₄ and (AAGC)₄.

Species	ISSR primer							
	(GACA) ₄				(AAGC) ₄			
	N	S	F	H	N	S	F	H
<i>Anodorhynchus hyacinthinus</i>	1	900	1.0	-	1	800-1000	1.0	-
<i>Ara chloroptera</i>	4	850-1100	0.133 0.646 0.158 0.063	0.58	2	800-1000	0.317 0.683	0.47
<i>Ara ararauna</i>	3	850-1100	0.137 0.244 0.619	0.45	4	700-1000	0.286 0.250 0.024 0.110	0.46
<i>Amazona brasiliensis</i>	1	700-800	1.0	-	1	800-1000	1.0	-
<i>Amazona pretrei</i>	1	700-950	1.0	-	1	800-1000	1.0	-

tween the three macaw species. Considering all the molecular markers analyzed, the values of genetic diversity or heterozygosity (Tables 3 to 5) were lowest in *Anodorhynchus hyacinthinus* (0.43 and 0.68; 0.28 and 0.32 for mini and microsatellites, respectively), intermediate in *Ara ararauna* (0.92; 0.41 and 0.44; 0.45 and 0.46 for minisatellites, microsatellites and ISSRs, respectively) and highest in *Ara chloroptera* (0.82–0.84; 0.12 and 0.80; 0.47 and 0.58 for minisatellites, microsatellites and ISSRs, respectively).

Discussion

The development of suitable molecular markers for population analysis usually incurs the construction of a genomic library of the species followed by the isolation of specific loci such as mini and microsatellites. However, the isolation of mini and microsatellites is a laborious and expensive procedure and some loci do not produce good results when applied to other taxa. In some cases, heterologous probes (minisatellites) or primers (microsatellites) detect only monomorphic loci, while in other cases, the level of polymorphism is so high that is difficult to make reliable comparisons. These results are possibly related to the evolutionary distance between the taxa from which the markers were developed and those under study.

An alternative approach is to test more universal markers such as inter simple sequence repeats (ISSRs). In this study we tested both VNTR (mini and microsatellites) and ISSR markers and found that, in general (and as expected), the more specific markers (mini and microsatellite) could only be applied to closely related taxa. The mini and microsatellite loci were isolated from two Psittacidae species (*G. guarouba* and *A. hyacinthinus*) belonging to a psittacid group with long tail that is distantly related to the short tailed psittacid group that includes the genus *Amazona* (Miyaki *et al.*, 1998; Tavares *et al.*, 2004). These two psittacid groups have been estimated to have diverged around 50 million years ago (Miyaki *et al.*, 1998) so VNTR markers developed for taxa from one of these groups would not be expected to be useful for studies in taxa from the other group, although the less specific ISSR marker primers should be able to amplify DNA from a wider range of species, which was indeed the case in our study.

Minisatellites

In this study we found that it was possible to use minisatellite probes cloned from one psittacid species (*Guarouba guarouba*) in some taxa (*Anodorhynchus hyacinthinus*, *Ara chloroptera* and *Ara ararauna*) from other genera of the same family. However, the probes did not hybridize with DNA from the two species of the genus *Amazona* tested (*A. brasiliensis* and *A. pretrei*).

We observed that each minisatellite probe recognized different numbers of bands in distinct species. For example,

the GguA₆ probe detected various bands (multilocus pattern) in *G. guarouba* and *A. ararauna* while in the other species it produced single-locus banding. Whilst the banding pattern of the GguA₁₂ probe was monomorphic in *Anodorhynchus hyacinthinus* and *Ara ararauna* but polymorphic in *Ara chloroptera*. The fact that different results were obtained even for species from the same genus reinforces the importance of conducting broad surveys on a species by species basis. Similar results were obtained by Hanotte *et al.* (1992) using minisatellite probes from the passerine *Passer domesticus* in other species of the family Passeridae. Wetton and Parkin (1997) also found similar results when they tested minisatellite probes from genomic libraries of *Falco peregrinus* and *Falco columbarius* in other species of the genus *Falco*.

The high number of alleles generated by some of the probes used in our study produced unique banding patterns for each specimen, which meant that population analysis with small sample sizes was not viable because of the high variability. An example of this was the GguB₁₀ probe, which detected 12 alleles in the *Ara ararauna* population ($n = 43$). Such high variability also made it more difficult to identify alleles due to the fact that many alleles were of similar size, a factor which was even more critical when it was necessary to compare various specimens from the same species on different hybridization membranes.

Even though the methodology to detect minisatellites is more laborious than the one used to amplify microsatellites, minisatellites can be more informative in addressing some biological questions. In a study of the endangered kakapo (*Strigops habroptilus*) Miller *et al.* (2003) observed no variation in seven microsatellite loci, but when minisatellite profiles were produced, two different lineages were identified and the parentage of individual specimens was determined.

Microsatellites

With the exception of the monomorphic 50D5 loci, the *G. gallus* microsatellites were unable to amplify the DNA from the five psittacid species investigated, probably as a result of the wide taxonomic distance between the species tested and species from which the genomic libraries were constructed. Both *Anodorhynchus hyacinthinus* microsatellite primers (HYA 1172 and MAC 436) amplified DNA from *A. hyacinthinus* and from the two *Ara* species but only the HYA 1172 primer was able to amplify DNA from the genus *Amazona*. Once again, different results were obtained with different loci and species, demonstrating the importance of a wide survey. Such differences were probably also related to the different taxonomic distances between the species from which the marker was isolated and the taxa tested.

The analysis of microsatellites presents some difficulties associated with the kind of repeat unit that is amplified. The HYA 1172 and MAC 436 amplified micro-

satellites with dinucleotide repeats and many stutter bands were produced, such non-specific products probably being due to DNA slippage during amplification (Schlotterer, 1998). This, together with problems of gel distortion, meant that we had to use two DNA visualization techniques (PCR with one radioactive nucleotide and silver staining) to allow the correct identification of alleles and genotypes. Another alternative, that we did not investigate, is the use of fluorescent primers and analysis in an automated sequencer (Schlotterer, 1998).

In the genus *Amazona*, the alleles obtained using the HYA 1172 primer differed in size by only one nucleotide. Thus, the analysis was more difficult since it was necessary to sequence some specimens to verify the exact number of repeats and to try to establish their genotypes. In doing so we discovered that in the genus *Amazona* there is an addition of a nucleotide in the middle of the repeat block and a substitution of an AC repeat by a GC in some specimens, although in *Anodorhynchus hyacinthinus* neither the addition nor the substitution was present. The HYA 1172 locus could be amplified in the genus *Amazona* because the mutation is inside the microsatellite region and not where the primers hybridize. The fact that the MAC 436 primer did not amplify the DNA of the taxa from the genus *Amazona* is possibly due to the occurrence of mutations in the flanking regions where the primers hybridize or because this locus is absent in the specimens studied and possibly in the species analyzed.

Similar results have been obtained for *Amazona guildingii* (Russello *et al.*, 2001) and *Ara ararauna* (Caparroz *et al.*, 2003). Microsatellites primers were developed for these species and were tested for cross amplification in other psittacid species. Nine microsatellite loci were developed for *A. guildingii* and only two resulted in successful amplification in *Ara chloroptera* and five in *A. ararauna* (Russello *et al.*, 2001). While six primers were developed for *A. ararauna* and three failed to amplify any product from samples of species from the genus *Amazona* (Caparroz *et al.*, 2003).

Inter simple sequence repeats

The use of universal primers, such as those used to amplify sequences between microsatellites loci (*i.e.* ISSRs) by simple sequence repeat primer amplification reactions (SPAR), have some advantages over the other techniques used in this study because it is unnecessary to construct genomic libraries and such primers can be applied to various organisms (Gupta *et al.*, 1994). There are, however, some disadvantages of the SPAR technique, including high sensibility to PCR conditions such as the concentration and purity of DNA (Williams *et al.*, 1990) and weak intensity of banding patterns. The low resolution of agarose gels is another problem, because small size differences (less than 100 bp) cannot be visualized. In order to discard technical artifacts we repeated the amplification reactions many

times for each of the five species tested and the same results were obtained each time, indicating the repeatability of this methodology.

However, as explained in the results, monomorphic patterns between specimens from the same species were produced in *Anodorhynchus hyacinthinus* and the two species of the genus *Amazona* (*brasiliensis* and *pretrei*), so it appears that the application of these markers to population studies of psittacids is limited.

Genetic variability

The genetic variation as estimated by neutral molecular markers may not be related to the variability (*i.e.* fitness-related traits) needed by the organism to respond to environmental changes (Amos and Balmford, 2001; Fernandez *et al.*, 2004). However, if a study applies a large number of molecular markers that cover the entire genome and that are probably linked to some of the selective genes these markers will be reflecting the adaptive genetic variation, which is responsible for the ability of the population to evolve (Frankham *et al.*, 2002; Fernandez *et al.*, 2004).

Despite the fact that genetic variation is usually measured by heterozygosity (Nei, 1973), some authors suggest that allelic diversity is the most relevant criterion for measuring diversity because high levels of allelic diversity should be a source of single-locus variation for important qualitative traits. Also, allelic diversity tends to be more sensitive to population bottlenecks than genetic diversity because rare alleles are lost in a higher proportion than more common alleles, while most of the heterozygosity is retained (Bataillon *et al.*, 1996; Petit *et al.*, 1998; Amos and Balmford, 2001).

The use of molecular markers in the analysis of the genetic variability and vulnerability of species is a very important tool in biological conservation and the results obtained in our study seem to be coherent with the status of each of the psittacid species studied. The hyacinth macaw (*Anodorhynchus hyacinthinus*) is an endangered species which is highly specialized in regard to diet and species of nesting-tree and is restricted to few areas in Brazil (Guedes and Harper, 1995). The other two macaw species investigated (*Ara ararauna* and *Ara chloroptera*) are not considered endangered, have broader geographic distributions and are less specialized than *A. hyacinthinus*. This former species presented the lowest values of genetic variability (heterozygosity and allele diversity) with all the markers used in this study. *A. ararauna* presented intermediate genetic variability and *A. chloroptera* high genetic variability.

The results obtained in our study agree with those obtained using DNA fingerprinting on samples from the same populations that were investigated in the present study. The indexes of variability based on band sharing coefficients obtained in the studies by Miyaki (unpublished) and Caparroz *et al.* (2001) were considered low and similar to those found in endangered species for *Anodorhynchus*

hyacinthinus (65.60%) and *Ara ararauna* 68.50%, while for *Ara chloroptera* (76.90%) the variability index was similar to that observed in non-endangered species.

Concluding remarks

In general, all the species of macaws and parrots face similar threats, such as habitat destruction, illegal trade and loss of nest cavities. For example, *A. ararauna* is one of the most frequently illegally traded species in Brazil (Diário Oficial, 1998) and its natural habitat has been largely destroyed, mainly for soy culture, while *A. chloroptera* populations breeding in the Brazilian Pantanal face strong competition for nesting sites (Guedes and Harper, 1995). All these threats can decrease the effective population size of a species, and in small populations genetic factors such as inbreeding depression and genetic drift will be more accentuated than in larger populations and can drive the species into an extinction vortex (Gilpin and Soulé, 1986; Saccheri *et al.*, 1998).

In the study presented in this paper we selected molecular markers (mini and microsatellites and ISSR) that showed specific and polymorphic patterns in five neotropical parrot species and also described a comparative genetic variability analyses of three macaw species. We hope that these markers will be useful for future population studies that will eventually help plan conservation programs.

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Anexo XII

PRIMER NOTE

Characterization of microsatellite loci in the Blue-and-gold Macaw, *Ara ararauna* (Psittaciformes: Aves)

R. CAPARROZ*, C. Y. MIYAKI* and A. J. BAKER†

*Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, 277, Cidade Universitária, São Paulo, SP 05508-090, Brazil, †Centre for Biodiversity and Conservation Biology, Royal Ontario Museum, Toronto, Ontario, Canada

Abstract

Microsatellite loci were isolated from the Blue-and-gold Macaw (*Ara ararauna*), a Neotropical parrot, from a GT_n and CT_n enriched genomic library. Six loci were characterized varying from one to 11 alleles per locus. Five loci exhibited greater than 50% heterozygosity within the 49 individuals genotyped. Furthermore, the primers also amplified the DNA from two additional genera of Neotropical parrots, indicating the potential utility of these markers for population-level studies and conservation efforts of Neotropical parrots.

Keywords: *Ara*, conservation, macaw, microsatellite, parrot, Psittaciformes

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The Blue-and-gold Macaw has one of the broadest geographical distributions among the Neotropical parrots. The current genetic status of wild populations was only addressed by one study based on DNA fingerprinting data (Caparroz *et al.* 2001). These authors pointed out that a population from southwestern Goiás, Brazil, possessed heterozygosity levels similar to those found in vulnerable bird populations. Thus, other populations that were not studied may also have low genetic variability. In order to assess such information and characterize the species' genetic structure, we isolated microsatellite loci. To date, these markers have been isolated from only two Neotropical parrots, *Forpus passerinus* (Hughes *et al.* 1998) and *Amazona guildingii* (Russello *et al.* 2001). In addition, these loci primers cross-amplified in other Neotropical parrots, including representative species from *Brotogeris*, *Aratinga*, *Amazona*, *Ara* and *Pionus* (Hughes *et al.* 1998; Russello *et al.* 2001).

Total genomic DNA was isolated from blood samples of two wild female individuals using standard proteinase K digestion followed by phenol:chloroform purification as described by Bruford *et al.* (1992) with modifications. DNA was simultaneously digested with *NheI*, *RsaI* and *HaeIII* (New England Biolabs) resulting in a majority of fragments within the 200–1000 bp size range. Microsatellite loci were then isolated following the enrichment protocol of

Hamilton *et al.* (1999). The resulting GT_n and CT_n enriched samples were cloned into dephosphorylated pBluescript II SK(+/-) phagenid (Stratagene) and used to transform Epicurian Coli® XL1-Blue MRF' supercompetent cells (Stratagene).

Colonies were lifted off Luria-Bertani plates on nylon membranes using a protocol modified from Sambrook *et al.* (1989), and clones containing inserts with GT_n and CT_n repeats were detected with the Phototope-Star Chemiluminescent Detection Kit (New England Biolabs). Positive colonies were selected and incubated at 100 °C for 10 min 200 µL TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0). Inserts were amplified by polymerase chain reaction (PCR) with T3 and T7 primers, and were sequenced manually using the Thermo Sequenase DNA Polymerase kit (USB) with radioactively labelled [α -³²P]-ddNTPs or automatically with the Thermo Sequenase DYEnamic Direct Cycle sequencing kit (Amersham Pharmacia). Six primer pairs flanking microsatellite regions of > 10 repeats were determined using Oligo 4.1 (National Biosciences) (Table 1). One primer in each pair was manufactured with a 5'-M13 tail (5'-CACGACGTTGTAAAACGAC) for use in the universal dye-labelling method described by Boutin-Ganache *et al.* (2001).

PCRs were performed using a PE 9700 thermal cycler (PE Biosystems) and carried out in a 12.5 µL volume containing: 20–50 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.1% gelatin, 160 µg/mL bovine serum albumin, 800 µM of each dNTP, 400 µM of each

Correspondence: R. Caparroz. Fax: (11) 3091 7553; E-mail: renatoz@yahoo.com.br

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Table 1 Microsatellite loci in Blue-and-gold Macaw. T_a is the annealing temperature. The number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E) and product size ranges were obtained from the analysis of 49 unrelated birds sampled from six Brazilian regions, except the monomorphic loco that were genotyped in only 12 individuals

Locus (GenBank Accession no.)	Primer sequences (5'-3')	Repeat structure	T_a	No. of alleles	H_O	H_E	Length
UnaCT21 (AYZ44483)	F: CTTTCCCATACTTAGCCATA R: AGACATTTCAGACCGTGCC	(GT) _n (CTT)(GT) _n	50	11	0.87	0.85	245–273
UnaCT32 (AYZ44484)	F: TCTTGCTTATTCTTCCCCAG R: ACCACCACCAGGAAGCACGG	(GT) _n	53	8	0.62	0.69	258–276
UnaCT41 (AYZ44485)	F: TCTTCATCCCCTGCCTACAG R: CAGCTAACATAAAATAT	(GT) _n (ATGTT)(GT) _n	50	1	—	—	134
UnaCT43 (AYZ44486)	F: TCATCCTATCACCAGAAGGG R: CTTGAGGACAGTGCAGAGGG	(GT) _n	60	9	0.88	0.87	199–219
UnaCT74 (AYZ44487)	F: CTGGACTGCTGCTCTTAACA R: AGCCTGAAGTGAAGTGCATG	(GT) _n	53	5	0.52	0.69	232–260
UnaGT55 (AYZ44488)	F: TCTGCCCTCTGTCTTATGCC R: ACTTTGGTTTGCCCTGC	(GT) _n (AT) _n	50	4	0.50	0.50	184–192

Table 2 Cross species amplification of microsatellites from one individual of three New World parrot species using the primers developed from *Ara ararauna*

Species	UnaCT21	UnaCT32	UnaCT41	UnaCT43	UnaCT74	UnaGT55
<i>Anodorhynchus hyacinthinus</i>	+	+	+	+	+	+
<i>Amazona brasiliensis</i>	–	–	–	+	+	+
<i>Amazona amazonica</i>	–	–	–	+	+	+

+, successful amplification without extra optimization; –, unsuccessful amplification; * indicates that the products observed were smaller than the ones observed in *A. ararauna*.

primer and 0.5 U of *Taq* polymerase (Pharmacia). Reaction conditions for all primers were initially performed using a common cycling program (10 min at 95 °C, 35 cycles of 95 °C for 60 s, 50 °C for 40 s and 40 s at 72 °C, followed by a final step of 72 °C for 10 min). Then, the annealing temperature for each microsatellite locus was varied until a single-band was visualized in agarose gels.

Preliminary assessment of polymorphism at each locus in 10–12 individuals from different Brazilian regions was performed by generating PCR products using the same quantity of the designed primer pairs and a fluorescent M13 sequencing primer (IRD 700 and 800, Li-Cor). The five polymorphic primers were used to study variation in 49 *A. ararauna* individuals from six Brazilian regions (Table 1). The PCRs were carried out in a 12.5 µL volume containing: 20–50 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 800 µM of each dNTP, 80 µM of forward primer, 80 µM of fluorescent (TET or HEX, Applied Biosystems) M13 sequence primer, 160 µM of reverse primer and 0.5 U of *Taq* polymerase (Pharmacia) using the cycling program described above. Alleles were separated on a 5% polyacrylamide gel and analysed in an ABI 377 DNA Sequencer with Genescan and Genotyper 2.1 (PE Biosystems).

All polymorphic loci exhibited heterozygosity greater than 50% and had four or more alleles. The Mendelian inheritance of these loci was confirmed by genotyping a captive pair of *A. ararauna* and their five offspring (data not shown).

The primers were tested in an individual from three additional Neotropical parrot species: *Anodorhynchus hyacinthinus*, *Amazona brasiliensis* and *A. amazonica* (Table 2). The first two species are endangered and have been the subjects of specific conservation programs. The microsatellite loci described here and those described in studies cited above demonstrate the potential utility of these primers to further population-level studies and conservation efforts of New World parrots.

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Anexo XIII

CHARACTERIZATION OF MICROSATELLITE LOCI IN THREE SPECIES OF *AMAZONA* (PSITTACIFORMES) USING HETEROLOGOUS PRIMERS

Renato Caparroz¹, Kelly C. E. Leite¹, Leandro A. Chinalia¹, Cristina Y. Miyaki², & Rosane G. Collevatti¹

¹Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Campus II, SGAN 916 Norte, Brasília, DF, 70790-160, Brazil.
 E-mail: renatocz@ucb.br

²Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil.

Caracterização de locos de microssatélite em três espécies de *Amazona* (Psittaciformes) por meio de iniciadores heterólogos.

Key words: *Amazona*, Psittaciformes, parrot, microsatellite, heterologous primers, population genetics.

The genus *Amazona* presents 31 Neotropical species (Collar 1997) of which 16 are threatened at various levels (IUCN 2006). Among these, two endemic species of the Atlantic forest, the Red-tailed (*A. brasiliensis*) and the Red-spectacled (*A. pretrei*) amazons, are classified as vulnerable. Habitat destruction and pet trade are the most important threats to these species. Although the Blue-fronted Amazon (*A. aestiva*) is not listed as a threatened species, it is intensively captured in the wild for pet trade (Seixas & Mourão 2000).

Microsatellites have been widely used as markers in population genetics, parentage and conservation assessment studies as they present codominant inheritance and relatively high levels of polymorphism, and are easily analyzed using the polymerase chain reaction (PCR) (Bruford & Wayne 1993). However, the development of polymorphic

microsatellite loci is usually time-consuming and expensive. Whenever cross-species amplification is possible, the cost of genotyping can reduce significantly and more population genetic and conservation studies can be done. This amplification is generally possible between closely related species as a consequence of the homology of the microsatellite flanking regions. The conservation of these regions has been detected in plants (e.g., Collevatti *et al.* 1999) and animals (e.g., Lau *et al.* 2004). Here we report the amplification of microsatellite loci in three *Amazona* species: *A. aestiva*, *A. pretrei* and *A. brasiliensis*, using heterologous primers designed for two other parrot species: *A. guildingii* and *Ara ararauna*.

METHODS

Total genomic DNA was extracted from

TABLE 1. Characterization of the 18 microsatellite loci for three *Amazona* species using heterologous primers.

Name	Repeat motif ^a	<i>A. aestiva</i> (N = 22)			<i>A. pretrei</i> (N = 23)			<i>A. brasiliensis</i> (N = 18)		
		T _A	N _A	Length (bp)	T _A	N _A	Length (pb)	T _A	N _A	Length (bp)
UnaCT21 ¹	(GT) _n (CTT)(GT) _n	-	-	-	-	-	-	-	-	-
UnaCT32 ¹	(GT) _n	-	-	-	-	-	-	60	1	248
UnaCT43 ¹	(GT) _n	52	6	199-211	52	5	186-204	66	4	202-208
UnaCT74 ¹	(GT) _n	50	1	211	54	3	224-228	60	2	212-214
UnaGT55 ¹	(GT) _n (AT) _n	50	1	166	50	1	166	54	1	166
AgGT02 ²	(GT) _n	-	-	-	?	?	?	54	1	171
AgGT04 ²	(GT) _n	48	4	254-262	?	?	?	54	4	252-278
AgGT07 ²	(GT) _n	50	12	257-283	58 to 50	11	250-288	50	8	261-279
AgGT08 ²	(GT) _n (GCGT) _n	52	1	304	50	1	302	50	2	322-330
AgGT12 ²	(GT) _n	58 to 50	15	299-333	52	7	296-308	56	4	297-307
AgGT17 ²	(GT) _n	58 to 50	1	419	58 to 50	5	420-430	60	1	414
AgGT21 ²	(GT) _n	50	16	306-340	58 to 50	5	299-309	54	11	310-340
AgGT22 ³	(CA) _n	59	5	190-198	59	3	184-190	60	7	186-200
AgGT29 ³	(CA) _n	59	12	190-224	59	9	193-211	56	8	187-221
AgGT72 ³	(CA) _n	50	14	270-302	58 to 50	7	276-288	50	6	272-293
AgGT81 ²	(GT) _n	50	5	332-344	?	?	?	52	7	330-346
AgGT83 ²	(GT) _n	58 to 50	17	231-275	54	18	233-273	56	8	245-269
AgGT90 ³	(GT) _n	58 to 50	13	196-232	52	11	195-227	50	7	192-210

¹Caparroz *et al.* (2003); ²Russello *et al.* (2001); ³Russello *et al.* (2005); ^aRepeat motif in the original species; T_A = the annealing temperature (°C) used in "touchdown" or standard PCRs; N_A = number of alleles; - = unsuccessful amplification; ? = not tested.

TABLE 2. Polymorphic 14 microsatellite loci characterized for three *Amazona* species using heterologous primers. The number of individuals genotyped for each species is showed between parentheses. Proportion of expected (H_E) and observed heterozygotes (H_O), paternity exclusion probability (Q), identity probability (I) are also showed. *Loriz* with deviation from Hardy-Weinberg equilibrium ($p < 0.05$) are indicated as *. Unsuccessful amplification is indicated as -.

Name	<i>A. aestiva</i> (N = 22)					<i>A. pretrei</i> (N = 23)					<i>A. brasiliensis</i> (N = 18)				
	H_E	H_O	Q	I	H_E	H_O	Q	I	H_E	H_O	Q	I	H_E	H_O	Q
UnaCT43	0.722	0.500*	0.493	0.206	0.638	0.478*	0.379	0.310	0.607	0.812	0.324	0.396	-	-	-
UnaCT74	-	-	-	-	0.124	0.130	0.062	0.780	-	-	-	-	-	-	-
AgGT04	-	-	-	-	-	-	-	-	0.583	0.500	0.305	0.410	0.726	0.778	0.509
AgGT07	0.838	0.810	0.687	0.079	0.803	0.565*	0.631	0.105	0.472	0.412	0.180	0.613	0.469	0.588	0.382
AgGT08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AgGT12	0.901	0.954	0.803	0.032	0.828	0.773	0.657	0.098	0.844	1.000	0.694	0.076	0.734	0.889	0.511
AgGT17	-	-	-	-	0.534	0.609	0.322	0.323	0.731	0.833	0.520	0.180	0.491	0.444	0.298
AgGT21	0.910	0.952	0.820	0.028	0.662	0.696	0.403	0.297	0.784	0.647*	0.591	0.132	0.797	0.823	0.601
AgGT22	0.678	0.500*	0.449	0.223	0.643	0.435*	0.351	0.353	0.731	0.833	0.520	0.180	0.491	0.444	0.298
AgGT29	0.830	0.682	0.683	0.074	0.855	0.869	0.708	0.072	0.731	0.833	0.520	0.180	0.491	0.444	0.298
AgGT72	0.896	0.905	0.790	0.038	0.628	0.565	0.412	0.242	0.784	0.647*	0.591	0.132	0.797	0.823	0.601
AgGT81	-	-	-	-	-	-	-	-	0.784	0.647*	0.591	0.132	0.797	0.823	0.601
AgGT83	0.915	0.952	0.828	0.026	0.925	1.000	0.849	0.020	0.797	0.823	0.601	0.132	0.797	0.823	0.601
AgGT90	0.830	0.818	0.687	0.072	0.855	0.304*	0.716	0.066	0.721	0.778	0.494	0.202	0.721	0.778	0.494
All loci			0.999	1.684 x 10 ⁻¹¹			0.999	1.908 x 10 ⁻⁹			0.999	2.289 x 10 ⁻⁸			

blood samples of 22 *A. aestiva* (from Mato Grosso do Sul), 23 *A. pretrei* (from Rio Grande do Sul), and 18 *A. brasiliensis* (from Paraná), using standard proteinase K digestion followed by phenol:chloroform purification as described by Bruford *et al.* (1992) with modifications. The relatedness among all individuals analyzed was unknown.

A total of 18 heterologous microsatellite primer pairs were tested (Table 1): 13 with *Amazona guildingii* (Russello *et al.* 2001, Russello *et al.* 2005), and 8 with *Ara ararauna* (Caparroz *et al.* 2003). PCR amplifications were carried out in a total reaction volume of 10 μ l containing 50–20 ng of template DNA, 10mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 μ M of each dNTP, 0.5 μ M of each primer, and 0.5 unit of Taq polymerase (Pharmacia, BR) in a PE 9700 thermal cycler (PE Biosystems). PCR conditions for the majority of the primers were: initial denaturation at 95°C for 10 min, 35 cycles of 95°C for 1 min, annealing ranging from 48 to 66°C for 1 min, and 72°C for 1 min, followed by 72°C for 20 min. Reaction conditions for some primers were optimized using the “touchdown” cycling program as described by Russello *et al.* (2001). For *A. brasiliensis*, the PCR products were fluorescently (FAM, HEX/TET or NED, Applied Biosystems) labeled using an M13 forward primer as described by Caparroz *et al.* (2003). For the other two species, we used fluorescent labeled forward primers.

Initially, PCR products for two individuals from each species were visualized in 1.5% TBE agarose gels stained with ethidium bromide. The loci that presented clear and reproducible products were amplified in all individuals and analyzed in 7% denaturing polyacrylamide gels in an ABI 377 DNA Sequencer using the GeneScan and Genotyper 2.1 (Applied Biosystems). For all positive amplifications, one homozygous individual was sequenced and the presence of microsatellites was confirmed.

The number of alleles per locus, the observed and expected heterozygosities, and the paternity exclusion and the genetic identity probabilities were estimated using Identity 1.0 (Wagner & Sefc 1999). Deviation from Hardy-Weinberg expectations and linkage disequilibrium were analysed using approaches as implemented in Genepop 3.4 (Raymond & Rousset 1996).

RESULTS AND DISCUSSION

Among the 18 primer pairs, 15 successfully amplified products in *A. aestiva* (11 polymorphic). In *A. pretrei* and *A. brasiliensis*, 13 and 17 primer pairs resulted in products (12 and 13 polymorphic), respectively (Table 2). Evidence of deviation from Hardy-Weinberg equilibrium was found for some loci (Table 2) probably due to the presence of null alleles. Null alleles are alleles that consistently do not amplify during PCR, such that many of the homozygotes are, in reality, heterozygotes (Pemberton *et al.* 1995). Nucleotide sequence variation in the primer annealing site flanking the microsatellite may be the main cause of the failure to amplify alleles by PCR (Callen *et al.* 1993). The presence of null alleles may result to homozygosity excess, leading to an underestimation of genetic diversity, population size and failure in determining genetic structure, migration rates, kinship and parentage (Marshall *et al.* 1998, Taberlet *et al.* 1999, Piggott & Taylor 2003, Chapuis & Estoup 2004, Dakin & Avis 2004). The loci AgGT07 and AgGT90 presented linkage disequilibrium ($P < 0.05$) in the three species. This was also observed between AgGT04 and AgGT21 in *A. brasiliensis*, and between AgGT12 and AgGT72 in *A. aestiva*. The number of loci developed for *A. guildingii* that successfully resulted in amplification products was higher for *A. brasiliensis* than for the other species. This result may be due to the phylogenetic relationship among these species, since *A.*

gouldingii is phylogenetically more related to *A. brasiliensis* than the other two species (Russello & Amato 2004). The same three heterologous primer pairs from *A. ararauna* showed amplification in the three species, but in *A. brasiliensis* an additional pair resulted in products.

The use of these heterologous microsatellite primers will allow the collection of population genetic data for the development of conservation strategies for these species.

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Anexo XIV

Genetic variation and population structure of the endangered Hyacinth Macaw (*Anodorhynchus hyacinthinus*): implications for conservation

Patrícia J. Faria · Neiva M. R. Guedes · Carlos Yamashita · Paulo Martuscelli · Cristina Y. Miyaki

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Abstract The Hyacinth Macaw (*Anodorhynchus hyacinthinus*) is one of 14 endangered species in the family Psittacidae occurring in Brazil, with an estimated total population of 6,500 specimens. We used nuclear molecular markers (single locus minisatellites and microsatellites) and 472 bp of the mitochondrial DNA control region to characterize levels of genetic variability in this species and to assess the degree of gene flow among three nesting sites in Brazil (Pantanal do Abobral, Pantanal de Miranda and Piauí). The origin of five apprehended specimens was also investigated. The results suggest that, in comparison to other species of parrots, Hyacinth Macaws possess relatively lower genetic variation and that individuals from two different localities within the Pantanal (Abobral and Miranda) belong to a unique interbreeding population and are genetically distinct at nuclear level from birds from the state of Piauí. The analyses of the five apprehended birds suggest that the Pantanal is not the source of birds for illegal trade, but their precise origin could not be assigned. The low genetic variability detected in the Hyacinth Macaw does not seem to pose a threat to the survival of this species. Nevertheless, habitat destruction and nest poaching are the most important factors negatively affecting their populations in the wild. The observed genetic structure emphasizes the need of protection of Hyacinth Macaws from different regions in order to maintain the genetic diversity of this species.

P. J. Faria · C. Y. Miyaki

Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, CEP 05508-090 São Paulo, SP, Brasil

P. J. Faria (✉)

School of Biosciences, Cardiff University, Cardiff CF10 3US, UK

e-mail: patriciajfaria@yahoo.com.br; fariap@cf.ac.uk

N. M. R. Guedes

Projeto Arara Azul/UNIDERP, Rua Klaus Sturhk 178, Campo Grande, MS, Brasil

C. Yamashita

IBAMA – Gerência de São Paulo, Núcleo de Fauna, Alameda Tietê 637, São Paulo, SP, Brasil

P. Martuscelli

Instituto Insularis, Rua Gravatá 387, Condomínio Quinta da Boa Vista, Mairiporã, SP, Brasil

Keywords *Anodorhynchus hyacinthinus* · Hyacinth Macaws · Macaws · Parrots · Psittacidae · Genetic variation

Introduction

The Hyacinth Macaw (*Anodorhynchus hyacinthinus*) is the largest macaw in the world (Forshaw 1989). It is listed in the Appendices I and II of The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and it is considered endangered by the International Union for the Conservation of Nature and Natural Resources (IUCN; BirdLife International 2004). The genus *Anodorhynchus* is currently represented by three species: Glaucous macaw (*A. glaucus*), Lear's macaw (*A. leari*) (both critically endangered, BirdLife International 2004), and Hyacinth Macaw (*A. hyacinthinus*, endangered, BirdLife International 2004).

Hyacinth macaws are highly specialized in their diet and choice of nest sites (Guedes and Harper 1995). In the Pantanal, they mainly feed on nuts from two palm trees: acuri (*Scheelea phalerata*) and bocaiúva (*Acrocomia totai*) and about 95% of their nests are found in manduvi trees (*Sterculia apetala*) (Guedes 1993). Nevertheless, in other regions of Brazil, such as in the northeast, it also breeds on cliffs (Collar 1997). These macaws have low reproductive rates, they breed every two years and on average, only one chick per pair survives as a high percentage of eggs and chicks are predated (Munn et al. 1989; Guedes 1993; Pinho and Nogueira 2003). These characteristics contribute to a slow potential for population recovery in the wild. Populations are mostly threatened by human-related factors, such as habitat destruction, cattle overgrazing, hardwood logging, African grass cropping, poaching and trapping of wild birds; in addition, in northern Brazil, they are hunted by indigenous people for food and handcrafts (Munn et al. 1989; Guedes and Harper 1995).

The Hyacinth Macaw occurs in Bolivia, Paraguay and Brazil (which has the largest population) (Collar 1997). Currently, there are about 6,500 Hyacinth Macaws living in three separate areas in Brazil (Guedes 2004): approximately 5,000 individuals in the Pantanal Matogrossense, 1,000 in Gerais (states of Maranhão, Piauí, Bahia, Tocantins, Goiás and Minas Gerais) and 500 in the Amazon Region (Pará and Amazonas states) (Munn et al. 1989; Collar et al. 1992; Sick 1997; Guedes 2004).

Effective species conservation programs require the evaluation of levels of intra-specific genetic diversity (Haig 1998). To examine the genetic variability in parrot and macaw species and consequently help their conservation programs, several molecular markers for population genetic analyses of this group of birds were selected in a previous study (Faria and Miyaki 2006). So far, no information is available about the distribution of the genetic variation in Hyacinth Macaws and our work aimed to fill this gap.

In this study, for the first time, we analyze genetic diversity in Hyacinth macaws from three different areas in Brazil (Abobral and Miranda—both in the Pantanal—and Piauí) using three kinds of molecular markers (two single locus minisatellite probes, two microsatellite loci and 472 bp of mitochondrial control region). We aim: (1) to characterize the levels of genetic variability of three Hyacinth Macaw populations in Brazil, (2) to assess the level of genetic structure among the two regions in Pantanal (Abobral and Miranda) and Piauí, (3) to verify the utility of our data set to identify the origin of five seized birds, and (4) to use our results for conservation programs. We predict that the genetic variation found in Hyacinth Macaws will be lower than that observed in other species of birds that are not threatened and/or have larger effective population sizes. We also expect to find no evidence of gene flow between the Pantanal and Piauí due to habitat

fragmentation and philopatric behavior. We discuss the conservation implications of our results and their forensic use.

Material and methods

Forty-three individuals were sampled in the wild and in captivity: 16 individuals from Abobral (19°22' S, 57°02' W), 16 from Miranda (19°56' S, 57°20' W), both these regions are within the Pantanal Matogrossense in Mato Grosso do Sul state, six from São Gonçalo da Gurguéia (10°01' S, 45°18' W) in Piauí state and five specimens from a seizure by the Brazilian wildlife authority (IBAMA) in January 2000 in Bahia state. The geographical distribution of Hyacinth Macaws in Brazil and the sampling sites are presented in Fig. 1.

Blood samples (100 µl from the brachial wing vein) were obtained from nestlings and only one chick per nest was used for the analysis. The samples were kept at −20°C in absolute ethanol and total DNA was extracted using the method described in detail by Bruford et al. (1992).

Single locus minisatellites

For each bird, 6 µg of genomic DNA were digested with *Mbo*I. The DNA fragments were transferred onto a nylon membrane by Southern blotting (Sambrook et al. 1989). The

Fig. 1 Geographical distribution of Hyacinth Macaw in three regions in Brazil: Amazon, Gerais and Pantanal. Black triangles show the sampling sites: (1) Piauí, (2) Pantanal do Abobral and (3) Pantanal de Miranda. Map modified from BirdLife International (2006) (available at: <http://www.birdlife.org>) according to suggestions of Paulo T. Z. Antas

membranes were hybridized with two single locus minisatellite probes separately, selected in a previous work (for further details see Faria and Miyaki 2006). The probes were labeled with [α P^{32}] dCTP by random priming and hybridization was carried out at 65°C for 24 h (in a solution of Na_2HPO_4 0.263 M; EDTA 1 mM; SDS 7% and BSA 1%). After the washes, the filters were X-rayed at –70°C using X-ray films (Kodak RX) with intensifying screens for 2–7 days.

Microsatellites

Two polymorphic microsatellite loci previously tested in this species (for details see Faria and Miyaki 2006) were amplified with the following primer pairs: HYA1172F (GAT CCTTTGCTTAAGACAGATGTC) and HYA1172R (GAGTGAAATACACATTCAGC TTCTG); and MAC436F (GCACCAAACACAACATCTTATTC) and MAC436R (TTGGGACACCAATGTAATTTG) (Scott Davis, pers. comm.). The amplifications were carried out using 35 cycles of 95°C for 60 s, 51–52°C for 30 s and 72°C for 40 s and the PCR products were separated in 6.5% acrylamide gels. Genotyping was performed through two different procedures: direct incorporation of [α P^{32}] dCTP during PCR and silver staining of polyacrylamide gel.

Mitochondrial DNA

Amplification of the first domain of the control region was performed in 10 μ l volumes of: 1 $\times$ amplification buffer (Pharmacia); 0.2 mM of each dNTP, 0.75 units of *Taq* DNA polymerase (Pharmacia), 10 pmol of each primer (CR522Rb: TGGCCCTGACYTAG GAACCAG and LGlu: GCCCTGAAARCCATCGTTG; Eberhard et al. 2001) and 20 ng of genomic DNA. A cycle of 95°C for 40 s, 53°C for 20 s and 72°C for 30 s was repeated 35 times.

The amplification products were checked using 1% agarose gels. Afterwards, the products were purified with 10 units of exonuclease I and 1 unit of shrimp alkaline phosphatase (Amersham/Pharmacia). Approximately 60 ng of the purified PCR product was sequenced using a Big Dye Terminator sequencing kit (Perkin Elmer) following the manufacturer's instructions and were loaded in an automated sequencer ABI 377 (Applied Biosystems). The sequences were edited in the program Sequence Navigator version 1.0 (Applied Biosystems).

Population analysis

ARLEQUIN (version 2.0; Schneider et al. 2000) was utilized to estimate mean observed and expected heterozygosities and allele frequencies, and to detect linkage disequilibrium and deviations from the Hardy–Weinberg equilibrium in the nuclear DNA data (mini and microsatellites). Population differentiation was assessed through pairwise comparisons of F_{ST} values. A Bayesian maximum likelihood approach to infer the number of populations without prior information of the sampling location was used through the software STRUCTURE (version 2.2; Pritchard et al. 2000; Falush et al. 2003). We assumed the admixture model with correlated allele frequencies, a burn-in of 50,000 interactions and 50,000 MCMC replications after burn-in. The program was run 10 independent times for

each number of populations (K) between 1 and 4. The significance of the results was estimated as described by Evanno et al. (2005) through the plot of the mean likelihood values ($\ln \Pr(X/K)$) over 10 runs for each K and the rate of change of the likelihood function with respect to K (ΔK). Assignment tests were also conducted using STRUC-TURE and the proportion of individuals assigned to the genetic clusters was estimated.

We combined mini and microsatellite results into a single dataset, and confined our analysis to allele frequency-based methods since the statistical power gained by combining markers is expected to outweigh the disadvantage of assuming a single evolutionary model for the nuclear markers (Nichols and Beaumont 1996).

The comparisons of heterozygosity values were carried out according to Nei and Kumar (2000). First, we calculated the difference in gene diversity for the i th locus between populations X and Y ($d_i = h_{xi} - h_{yi}$) for all polymorphic loci. The mean ($\bar{d}$) and the variance [$V(\bar{d})$] of d_i were estimated by the following formulae:

$$\bar{d} = \sum_{i=1}^{L'} d_i / L'$$

$$V(\bar{d}) = \sum_{i=1}^{L'} (d_i - \bar{d})^2 / [L'(L' - 1)]$$

where L' is the number of polymorphic loci.

Further, the significance of any difference of the values of heterozygosity in different populations was tested using a t -test:

$$t_{L-1} = d/s(d)$$

where $L - 1$ is the degrees of freedom, $s(d)$ is the square root of $V(d)$.

ARLEQUIN was also used to estimate the haplotype (H) and nucleotide diversity (π) (Nei 1987) of the mitochondrial DNA data. Population differentiation was assessed through pairwise comparisons of Φ_{ST} values and hierarchical analyses of molecular variance (AMOVA; Excoffier et al. 1992). Median joining networks were estimated using NETWORK 4.1.1.1 (Bandelt et al. 1999) (available at <http://www.fluxus-engineering.com/>).

Additionally, the demographic history, evidence for past population growth, decline or stability, were tested using two neutrality tests (Fu's F_s tests by Fu 1997 and Tajima's D by Tajima 1989) and by generating mismatch distributions. These tests were conducted in DNAsp (version 4.1; Rozas et al. 2003). ARLEQUIN was used to test the significance of the mismatch distribution (Rogers and Harpending 1992), through a parametric bootstrap test between the observed and expected mismatches, the sum of square deviations (SSD) (Schneider and Excoffier 1999).

Results

Single-locus minisatellite and microsatellite variation

Minisatellite loci GguA₆ and GguB₁₀ produced six and four alleles, respectively. Microsatellite loci presented two (HYA1172) to four (MAC436) alleles (Table 1). The values of H_e ranged from 0.06 to 0.84, whilst the values of H_o ranged from 0 to 0.80 (Table 1). Departures from the Hardy–Weinberg equilibrium were found only for the locus MAC436

Table 1 Number of alleles (A), expected (H_e) and observed (H_o) heterozygosities obtained with the minisatellite loci A₆ and B₁₀ and with microsatellites loci HYA1172 and MAC436 in each locality

Locality		A6	B10	HYA1172	MAC436
Abobral ($n = 16$)	A	3	2	1	2
	H_e	0.53	0.42	0.27	0.06
	H_o	0.33	0.43	0.31	0.06
Miranda ($n = 16$)	A	4	2	2	2
	H_e	0.59	0.48	0.48	0.06
	H_o	0.69	0.56	0.50	0.06
Piauí ($n = 6$)	A	4	2	2	2
	H_e	0.71	0.20	0.16	0.53
	H_o	0.40	0.20	0.16	0.16
Seizure ($n = 5$)	A	5	3	2	3
	H_e	0.84	0.60	0.20	0.71
	H_o	0.80	0.80	0.20	0

in the apprehended samples ($P = 0.011$). Linkage disequilibrium was found in Miranda samples between loci HYA1172 and MAC436 ($P = 0$), in Piauí for loci GguA₆ and GguB₁₀ ($P = 0$) and between loci MAC436 and GguA₆ for the seizure ($P = 0.034$) (data not shown).

The pairwise comparisons of the heterozygosity values did not reveal any significant value ($0.0015 < t < 2.047$) among all pairs of populations analyzed (data not shown). Consequently, the values of genetic diversity obtained in each locality are similar to each other.

Population genetic analysis showed no evidence for genetic differentiation between Miranda and Abobral ($F_{ST} = 0.05$; $P = 0.12$) and also between Piauí and the seized samples ($F_{ST} = -0.03$; $P = 0.39$) (Table 2). However, significant population differentiation was found between the Piauí and Pantanal populations ($F_{ST} = 0.33$; $P < 0.001$ and $F_{ST} = 0.25$; $P < 0.001$; Abobral and Miranda, respectively) and between the seized samples and the Pantanal ($F_{ST} = 0.25$; $P = 0.005$ and $F_{ST} = 0.17$; $P = 0.019$; Abobral and Miranda, respectively) (Table 2). Similar results were obtained using the Bayesian approach on STRUCTURE, where the most probable number of populations inferred was two (Fig. 2): genetic population 1 (red) was mainly constituted by individuals from Abobral and Miranda, while genetic population 2 (green) by Piauí and seizure. The mean likelihood value for 10 independent runs was great at $K = 2$ (-241.15). Most Hyacinth macaws from Abobral and Miranda were assigned to genetic population 1 (62.5% and 61.4%, respectively), while individuals from Piauí and seizure had higher probabilities of belonging to genetic population 2 (60.6% and 72.6%, respectively).

Table 2 Nuclear pairwise F_{ST} (below the diagonal) and mitochondrial DNA Φ_{ST} (above the diagonal)

Locations	Abobral	Miranda	Piauí	Seizure
Abobral	–	–0.04	0.18	0.18
Miranda	0.05	–	0.14	0.13
Piauí	0.33	0.25	–	0.16
Seizure	0.25	0.17	–0.03	–

Significant values at $P = 0.05$ are indicated in bold

Fig. 2 Bar plot of population assignment proportions for $K = 2$ generated by STRUCTURE. Each individual is represented by a single vertical line divided into the two inferred clusters: genetic population 1 in red and genetic population 2 in green

Mitochondrial DNA

Control region sequences of 472 bp revealed thirteen variable sites that defined eight haplotypes, their frequencies in each population are shown in Table 3. One haplotype was shared by all populations (haplotype 4), while haplotypes 1, 2 and 3 were found exclusively in Pantanal. Haplotypes 6, 7 and 8 were detected only in Piauí, whilst haplotype 5 was only found in seized samples. The values of nucleotide diversity (π) per site ranged from 0.002 to 0.008 (Table 3).

Heteroplasmic sites were observed, nevertheless there was strong evidence that these sequences are mitochondrial as opposed to nuclear copies. Long PCRs were carried out in a previous study (Tavares et al. 2004) to verify the authenticity of the sequences amplified by these primers (CR522Rb/LGlu) in the Hyacinth Macaw. Further, both strands were independently sequenced for all individuals and several individuals were sequenced more than once, in both situations the same result was obtained, without any ambiguities.

Non-significant pairwise Φ_{ST} values were obtained through all comparisons ($-0.04 < \Phi_{ST} < 0.18$; $0.08 < P < 0.69$) (Table 2), indicating a lack of genetic structure at mitochondrial level. Initially only one comparison was conducted in AMOVA (Abobral and Miranda versus Piauí and Seizure), as based on our assumptions, these two main groups

Table 3 Haplotypes and variable sites found in the control region sequences of the mitochondrial DNA of Hyacinth Macaw from three locations in Brazil and individuals from seizure

Haplotypes	Variable sites	Haplotype frequency			
		Abobral	Miranda	Piauí	Seizure
	1 2 5 6 6 7 2 3 0 6 0 6 0 9 5 4 8 9 7 3 1 6 8 4 1				
1	T A C T T G R T T C T W G	0.375	0.250	–	–
2	. G . C C . R . C . . . C	–	0.125	–	–
3	. G T C C . R . C . C W C	0.062	0.125	–	–
4	. G T C C A R . C . C W C	0.563	0.5	0.333	0.6
5	. G . C C A R C C . C W C	–	–	–	0.4
6	. G T C C A R . C . . W .	–	–	0.167	–
7	. G T C C A R . C T C W C	–	–	0.333	–
8	G G T C C A R . C . C W C	–	–	0.167	–
π		0.008	0.007	0.003	0.002
H		0.575	0.70	0.86	0.60

would be more plausible. Non-significant results were obtained in AMOVA and the genetic variation was distributed as follow: 18.54% between groups ($P = 0.33$), -2.84% among populations within groups ($P = 0.44$) and 84.31% within populations ($P = 0.10$). Afterwards, all possible combinations were tested, but once more non-significant results were obtained (data not shown).

The star shape of the network generated by control regions sequences (Fig. 3) indicates a population expansion. For the neutrality tests, Tajima's D and Fu's F_s were non-significant ($P > 0.30$) for all populations (Abobral: $D = 2.41$, Fu's $F = 5.63$; Miranda: $D = 1.68$, Fu's $F = 3.25$). Nevertheless, negative values for both neutrality tests were obtained for Piauí population ($D = -0.67$, $P = 0.30$; $F_s = -0.99$, $P = 0.13$). Mismatch distributions of Abobral and Miranda were ragged (Fig. 4), as expected in a constant population size model, and differed significantly from population expansion model ($SSD = 0.25$, $P = 0.10$; $SSD = 0.08$, $P = 0.25$, Abobral and Miranda, respectively). Despite the unimodal shape of Piauí's mismatch distribution, similar to the pattern observed in expanding populations, once more no statistical significance from stationary populations models was detected ($SSD = 0.01$, $P = 0.70$).

Fig. 3 Medium spanning network of Hyacinth Macaw control region (472 bp). Each circle represents a haplotype and the diameter scales to the haplotype frequency. Mutational steps are represented by black bars on lines connecting the haplotypes. Blank circles represents haplotypes exclusive from Piauí populations

Fig. 4 Mismatch distributions for Hyacinth Macaw control region sequences. Expected (solid lines) according to the sudden expansion model and observed (dotted lines) showing the frequencies of pairwise differences within (a) Abobral, (b) Miranda and (c) Piauí

Discussion

Genetic variation

The comparison of genetic diversity values among Hyacinth Macaws from three locations in Brazil did not result in significant differentiation. We predicted that, based on the larger population size of the Pantanal, genetic variability would be higher in this region when

compared to Piauí. Nevertheless, considering that Hyacinth Macaws are strong flyers, there could conceivably be gene flow between Piauí and the nearest population in the Amazon (that could not be studied here). This might increase the effective size of the Piauí population and its levels of genetic variability would be higher.

Despite the fact that drawing comparison of genetic diversity indices obtained for different species and by different molecular markers needs to be done with caution, this information can still help to understand the degree of genetic threat of a certain species. Our data show that, in comparison with other population genetic analyses of Psittacines (Nader et al. 1999; Wright and Wilkinson 2001; Caparroz 2003; Wright et al. 2005), the Hyacinth Macaw possesses relatively lower levels of genetic variability.

Analysis of 1,290 bp of the control region of 50 Blue and yellow Macaws (*Ara ararauna*) revealed 38 variable sites that defined 28 haplotypes, while the analysis of microsatellite loci produced three to 11 alleles with average heterozygosity of 0.604 (Caparroz 2003). Analysis of 680 bp of the control region of the Yellow-naped Amazon (*Amazona auropalliata*, $n = 75$) showed 59 variable sites that defined 19 haplotypes (Wright and Wilkinson 2001) and the microsatellite analysis detected three to 25 alleles with average heterozygosity of 0.59 (Wright et al. 2005). Faria and Miyaki (2006) evaluated the levels of genetic variation in five species of parrots and macaws, using different nuclear molecular markers, and found that among the three species of macaws analyzed (*Ara ararauna*, *A. chloroptera* and *Anodorhynchus hyacinthinus*), the Hyacinth Macaw possessed the lowest values of genetic variability.

One of the main predicted consequences of a loss of genetic variability is a reduction of the ability of a species to react to environmental changes, which operates over long-time scales (Frankham et al. 2002). Nevertheless, planning for the conservation of biodiversity is usually focused over short time-scales (Haig 1998; Amos and Balmford 2001). It has been pointed out that demographic factors, environmental fluctuations and natural catastrophes can drive populations to extinction before genetic factors impact them (Lande 1988; Amos and Balmford 2001; Frankham et al. 2002; Spielman et al. 2004).

Despite the low values of genetic variation detected in our study ($H_{\text{average}} = 0.35$; $2 < \text{number of alleles} < 6$ for nuclear data; 13 variable sites and eight haplotypes for mtDNA), they seem to be higher than those obtained in other endangered bird species such as the Andean condor (*Vultur gryphus*; Hendrickson et al. 2003), the whooping crane (*Grus americana*; Glenn et al. 1999), the great prairie-chicken (*Tympanuchus cupido*; Westemeier et al. 1998; Johnson et al. 2004) and the Spotted owl (*Strix occidentalis*; Barrowclough et al. 1999). Consequently, the levels of genetic variation presented by Hyacinth Macaws in our study do not seem to pose a current concern in terms of conservation (see Conservation Implications).

Genetic structure

A lack of population differentiation was found between the two populations from Pantanal (Abobral and Miranda) with nuclear and mitochondrial DNA markers ($F_{ST} = 0.05$ and $F_{ST} = -0.04$, respectively) and STRUCTURE results suggest the existence of a single population in Pantanal. Both locations are about 100 km distant from each other and there are no physical barriers among them. The monitoring of Hyacinth Macaws with radio tracking in Pantanal showed that juveniles are able to fly about 35 km after fledging (Seixas et al. 2002). Also, several field observations have suggested that adults were observed up to 100 km away from their natal areas (Yamashita, pers. comm.). These

results show, as expected, that Hyacinth Macaws are able to fly long distances; however, it does not mean that effective dispersal occurs. Banding and recapture data would be necessary in order to evaluate the philopatric behavior of Hyacinth Macaws. Nevertheless, to date these data are scarce, mainly due to the high level of disturbance involving the capture of adults. Guedes and Harper (1995) suggest that there is nest site fidelity for Hyacinth Macaws in Pantanal, and the same pair could be using the same tree cavity for breeding in consecutive years, but so far there is not enough evidence of philopatric behavior in Hyacinth Macaw.

Even though the two locations from Pantanal seem to constitute a single breeding population, as shown by our data, other locations from Pantanal need to be evaluated before conclusions about the population structure of this region can be drawn. Thus, it would be desirable to sample individuals from the other regions from Pantanal (mainly those from the north) and use additional molecular markers.

Restrictions to gene flow can occur due to geographical, ecological or behavioral isolation (i.e., philopatry) or population history. Parrots and macaws are capable of flying long distances; therefore their dispersal seems to be more constrained by the selective use of the habitat and the availability of natural forest and resources than mobility (Myers and Vaughan 2004; Evans et al. 2005). Their movements are mainly determined by spatial and temporal patterns of fruiting, seeding and water availability (Collar 1997; Renton 2002). Consequently, habitat fragmentation seems to be an important factor driving their distribution, choice of breeding sites and the level of gene flow among populations.

Based on this, we predicted the occurrence of strong genetic structure between Pantanal and Piauí. Our results show significant genetic differentiation between Pantanal and Piauí ($F_{ST} > 0.25$; $P < 0.05$) for nuclear DNA. These findings are strongly supported by the results from STRUCTURE that show two genetic populations: one constituted by Pantanal (Abobral and Miranda) and another by Piauí and seizure. Also, private alleles and haplotypes were found in Piauí for nuclear and mtDNA, respectively, and according to Slatkin (1985) they are good indicators of population structure and limited gene flow between populations.

Despite the detection of significant genetic structure at nuclear level, a lack of genetic differentiation was obtained using mtDNA control region sequences. Two potential explanations may arise from this data: female-biased gene flow and/or the nature of the molecular markers used. Birds usually present female-biased gene flow (Greenwood 1980), while males show higher levels of philopatry which result in population genetic structure at nuclear markers. Among the Psittacinae, sex-biased gene flow has been described for Yellow-Naped Parrots (*Amazona auropalliata*; Wright and Wilkinson 2001; Wright et al. 2005) and for Blue-and-Yellow Macaws (*Ara ararauna*; Caparroz 2003). On the first study, despite the lack of genetic structure among nine locations in Costa Rica and no correlation with dialects, the authors described the occurrence of female-biased gene dispersal (Wright et al. 2005). Contrastingly, Blue-and-Yellow Macaws presented male-biased gene flow (Caparroz 2003).

In our study, the migration rates ($N_e m$) obtained from the analysis of mtDNA control region sequences ($3.01 \leq N_e m < \infty$) was around six times higher than the estimative using nuclear DNA ($0.70 \leq N_e m < 6.46$), suggesting female-biased gene flow in Hyacinth Macaws. Nevertheless, our results need to be seen with caution and need to be further investigated with the use of more accurate methodology to estimate migration, the inclusion of demographic parameters and the estimative of direct dispersal. Besides, there are some additional reasons described below.

The differences in the level of genetic structure between nuclear and mtDNA detected in our study may also be the effect of the effective population size and mutation rates (Chesser and Baker 1996). Populations that diverged recently and have not experienced enough time to achieve the equilibrium between genetic drift, mutation and gene flow can retain ancestral variation that can produce misleading conclusions about the current levels of gene flow (Birky et al. 1989). Microsatellites present higher mutation rates than mtDNA, therefore they are more effective in revealing recent genetic structure than mtDNA. Nevertheless, since the effective population size of mtDNA is one-fourth of nuclear markers, mtDNA markers would achieve the equilibrium faster than nuclear markers and could potentially reflect more adequately the contemporary pattern of genetic structure. On the other hand, the assumption of smaller effective size of mtDNA is usually violated as it depends strictly of equal sex ratio, random mating and male reproductive success (Prugnolle and Meeus 2002).

Testing for mutation, drift and migration is a difficult procedure (Birky et al. 1989) and it could not be conducted here. Consequently, in our study it was not possible to discriminate among the alternative hypotheses that would better explain the population structure pattern obtained in Hyacinth Macaws. Besides, Prugnolle and Meeus (2002) pointed that, in order to discriminate the effects of sex-biased gene flow and molecular markers nature it is necessary to have demographic data.

The historical demography of Hyacinth Macaw populations is unknown. According to Yamashita (1997), the areas occupied by the macaws from the genus *Anodorhynchus* have changed considerably over the years due to its strict dependence on few species of palm seeds. Also, their range might have been drastically reduced after the megafauna extinctions in South America, around 10,000 years ago, as these large vertebrates were the main seed-dispersers of Neotropical trees (Yamashita and Valle 1993; Yamashita 1997). Furthermore, the introduction of domestic livestock in the XVIth century, themselves potential seed-dispersers, might have modified the scenario once more, and subsequently changed the areas occupied by Hyacinth Macaws (Yamashita 1997; Yamashita, pers. comm.).

Thus, it seems that the effective population size of Hyacinth Macaws has not been stable over the years. Mismatch distribution and neutrality tests were conducted in order to gather some information about this issue; however, only non-significant results were obtained. Despite that, the star shape of the network (Fig. 3) indicates a rapid and recent population expansion. The haplotypes at the tips of the network show few mutations and occur in individuals from Piauí. This fact, together with the negative values of neutrality tests suggest that Piauí's population might have been colonized by Pantanal and it is still expanding.

In sum, our study detected a significant genetic differentiation at the nuclear level between Pantanal and Piauí. Possible causes driving this differentiation were raised, including female biased-gene flow, current habitat fragmentation and recent colonization followed by population expansion in Piauí. In order to identify the most important feature and clarify this scenario, the demographic history of Hyacinth Macaws needs to be further examined with broader sampling and additional molecular markers. The effect of the current habitat fragmentation on the precise gene flow patterns of Hyacinth macaws could not be determined in this study and this information is necessary to plan conservation actions.

Origin of seized birds

The identification of the origin of seized specimens is potentially important to help authorities to identify the areas where those birds have been poached. According to our

results, the individuals from the seizure do not belong to the Pantanal. This result is corroborated by the presence of alleles exclusive to individuals from Piauí, significant F_{ST} values when compared to both regions of Pantanal (Abobral and Miranda) and the assignment of seizure individuals to genetic population 2. However, due to the absence of samples from other locations, mainly from the Amazon region, it is not possible to identify the origin of these birds, neither prove that they belong to the Piauí.

Conservation implications

The low genetic variation detected does not seem to pose a current concern to the Hyacinth Macaw conservation. On the other hand, the main threats seem to be the high specialization in diet and in nest site preference and low reproductive rates of the three species of the genus *Anodorhynchus*, and also human related factors. The decline of the Glaucous Macaw (*A. glaucus*) was possibly caused by the decline of palm groves (*Butia yatay*), its exclusive food source, as a consequence of the introduction of domestic herbivores (Yamashita and Valle 1993). The Lear's macaw (*A. leari*), an endemic species from the caatinga ecosystem in Bahia state, northeastern Brazil, is highly threatened because of wild animal traffic (BirdLife International 2006). Currently, there are only around 500 individuals in the wild (Amaral et al. 2005).

The results concerning the genetic structure of Hyacinth Macaw populations have implications for the conservation of this species. If the different locations represent discrete breeding populations, as suggested by nuclear DNA data, then effective protection is needed in all regions that shelter Hyacinth Macaws in order to preserve the overall genetic variability of this species. Thus, it is urgent to protect the populations that are not being permanently monitored (populations from North and Gerais of Brazil), since they are the possible sources of individuals for the illegal trade. Regarding the current captive individuals of Hyacinth Macaw, it would be desirable that they were managed according to their geographical origin.

Considering the results obtained here, it seems that the survival of Hyacinth Macaws, such as other species of parrots (González 2003; Myers and Vaughan 2004; Vaughan et al. 2005; Wright et al. 2005) depends mainly on the protection of natural habitat and work with local communities to reduce nest poaching. These measures can help guarantee the maintenance of the Hyacinth Macaw populations in the wild and their survival.

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Anexo XV

CONTRASTING PHYLOGEOGRAPHIC PATTERNS IN MITOCHONDRIAL DNA AND MICROSATELLITES: EVIDENCE OF FEMALE PHILOPATRY AND MALE-BIASED GENE FLOW AMONG REGIONAL POPULATIONS OF THE BLUE-AND-YELLOW MACAW (PSITTACIFORMES: *ARA ARARAUNA*) IN BRAZIL

RENATO CAPARROZ,^{1,4} CRISTINA Y. MIYAKI,² AND ALLAN J. BAKER³

¹Pós-graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Campus II SGAN 916 Norte,
 Av. W5, 70790-160, Brasília, DF, Brazil;

²Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, 277,
 Cidade Universitária, 05508-090, São Paulo, SP, Brazil; and

³Department of Natural History, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario M5S 2C6, Canada

ABSTRACT.—Comparing the patterns of population differentiation among genetic markers with different modes of inheritance can provide insights into patterns of sex-biased dispersal and gene flow. The Blue-and-yellow Macaw (*Ara ararauna*) is a Neotropical parrot with a broad geographic distribution in South America. However, little is known about the natural history and current status of remaining wild populations, including levels of genetic variability. The progressive decline and possible fragmentation of populations may endanger this species in the near future. We analyzed mitochondrial DNA (mtDNA) control-region sequences and six microsatellite loci of Blue-and-yellow Macaws sampled throughout their geographic range in Brazil to describe population genetic structure, to make inferences about historical demography and dispersal behavior, and to provide insight for conservation efforts. Analyses of population genetic structure based on mtDNA showed evidence of two major populations in western and eastern Brazil that share a few low-frequency haplotypes. This phylogeographic pattern seems to have originated by the historical isolation of Blue-and-yellow Macaw populations ~374,000 years ago and has been maintained by restricted gene flow and female philopatry. By contrast, variation in biparentally inherited microsatellites was not structured geographically. Male-biased dispersal and female philopatry best explain the different patterns observed in these two markers. Because females disperse less than males, the two regional populations with well-differentiated mtDNA haplogroups should be considered two different management units for conservation purposes. Received 4 November 2007, accepted 10 December 2008.

Key words: *Ara ararauna*, Blue-and-yellow Macaw, microsatellite, mitochondrial DNA, sex-biased dispersal.

Patrones Filogeográficos Contrastantes en ADN Mitocondrial y Microsatélites: Evidencia de Filopatría de las Hembras y Flujo Genético Sesgado hacia los Machos entre Poblaciones Regionales de *Ara ararauna* (Psittaciformes) en Brasil

RESUMEN.—Comparar los patrones de diferenciación poblacional entre marcadores genéticos con diferentes modos de herencia puede brindar información acerca de patrones de dispersión y flujo genético sesgados de acuerdo al sexo. *Ara ararauna* es un psitácido neotropical con una distribución amplia en Sur América. Sin embargo, se conoce poco acerca de la historia natural y el estado actual de las poblaciones silvestres remanentes, incluyendo sus niveles de variabilidad genética. La disminución progresiva y la posible fragmentación de las poblaciones podrían amenazar a esta especie en el futuro cercano. Analizamos secuencias de la región de control del ADN mitocondrial (ADNmt) y seis loci microsatélites de individuos muestreados a través de la distribución de la especie en Brasil para describir la estructura genética poblacional, para hacer inferencias sobre la demografía histórica y el comportamiento de dispersión, y para proveer información para los esfuerzos de conservación. Los análisis de la estructura genética basados en ADNmt mostraron evidencia de la existencia de dos poblaciones principales en el oeste y este de Brasil que comparten algunos haplotipos de baja frecuencia. Este patrón filogeográfico parece haberse originado tras el aislamiento geográfico de poblaciones de *A. ararauna* hace aproximadamente 374,000 años y ha sido mantenido por el flujo genético restringido y la filopatría de las hembras. En contraste, la

⁴E-mail: renatocz@yahoo.com.br

variación en los microsatélites (marcadores heredados biparentalmente) no estuvo estructurada geográficamente. La dispersión sesgada hacia los machos y la filopatría de las hembras son las mejores explicaciones de los patrones diferentes observados entre marcadores. Debido a que las hembras se dispersan menos que los machos, las dos poblaciones regionales con haplogrupos de ADNmt bien diferenciados deberían considerarse como dos unidades de manejo diferentes para propósitos de conservación.

CHARACTERIZING POPULATION STRUCTURE in a species requires an integration of evolutionary (i.e., phylogeographic) and contemporary (e.g., behavioral) components (Avice 1994). Interpretation of the relative influence of historical and contemporary factors solely on the basis of genetic data can be complicated by a host of factors, including sex-biased dispersal and philopatry (Scribner et al. 2001). Dispersal is a life-history characteristic that has relevant effects on both the dynamics and the genetics of species (Clobert et al. 2001). In many species of birds and mammals, there is considerable sexual variation in the extent of natal philopatry, the distance moved by an individual from its birth site to its breeding ground. In mammals, females tend to remain in close proximity to the natal area, whereas males disperse. Conversely, in birds, the reverse pattern is generally found (e.g., females disperse and males are philopatric; Greenwood 1980, Clarke et al. 1997).

In a species with sex-biased dispersal, the simultaneous use of markers with different modes of inheritance (i.e., uni- or biparental) can reveal the contrasting patterns of spatial distribution of the species' genetic variation. For example, Melnick and Hoelzer (1992) compared allozyme and mitochondrial DNA (mtDNA) data in macaque monkeys (*Macaca mulatta*). Stronger population structure was detected with the mtDNA than with nuclear DNA. This was in accordance with behavioral observations of males leaving the natal troop before sexual maturity and females staying with the troop for life. Similar patterns were observed in a variety of other species (Karl et al. 1992, FitzSimmons et al. 1997, Ishibashi et al. 1997), including birds (Gutierrez 1994, Gibbs et al. 2000). By contrast, in Willow Ptarmigan (*Lagopus lagopus*), behavioral studies indicated female-biased dispersal, but lower population differentiation was observed in mtDNA data than in microsatellite data, even though female-biased gene flow should homogenize both nuclear and mitochondrial variation simultaneously (Piertney et al. 1998, 2000).

The relevance of genetic information, including data on population genetic structure, in species conservation has long been recognized (e.g., Lande and Barrowclough 1987, Simberloff 1988). The rationale for this is based on the theoretical understanding of the relationship between genetic diversity and fitness in response to selection: loss of genetic diversity increases autozygosity and reduces the ability of a population to adapt to future selective pressures (Fisher 1930, Chesson et al. 1980, Lacy 1987). Thus, to devise adequate conservation and management strategies for any species, it is important to incorporate an understanding of its genetic structure.

The Blue-and-yellow Macaw (*Ara ararauna*) is a Neotropical parrot with a broad geographic distribution from eastern Panama south through Colombia to Bolivia, Paraguay, and Brazil (Forshaw 1989, Collar 1997). This species inhabits seasonally flooded forests and gallery forests, and in the northwest it occurs in deciduous forest (Collar 1997). The global population size of the Blue-and-yellow Macaw is unknown, but the species is described as "common" in parts of its range (Collar 1997) and is not considered threatened.

However, it is a very popular pet, and collecting pressures combined with habitat fragmentation have resulted in the extirpation of the species in Trinidad and its extirpation or decline in many areas of Brazil, Ecuador, and Colombia (Collar 1997). In Brazil, this species is considered critically endangered in the State of São Paulo (São Paulo State Government: 1998), nearly extinct in the State of Rio de Janeiro (Bergallo et al. 1999), and vulnerable in the State of Minas Gerais (Machado et al. 1998). Under this scenario of progressive decline and possible fragmentation of populations, it is important to have a conservation plan for this species.

At present, little is known about the natural history and current status of the remaining Blue-and-yellow Macaw populations. Consequently, data on the genetic variability of wild populations are practically nonexistent. There is one DNA fingerprinting study of wild Blue-and-yellow Macaws from the southwestern State of Goiás in Brazil (Caparroz et al. 2001), in which the population analyzed showed a high mean genetic similarity index, similar to those found in species considered vulnerable or endangered.

We characterized the entire mtDNA control region of the Blue-and-yellow Macaw and analyzed the variation of a large fragment from this region and six microsatellite loci of individuals sampled throughout the species' geographic range in Brazil. We describe the population genetic structure and population history of this species to make inferences regarding historical demography and dispersal behavior and to provide data for conservation efforts.

METHODS

Sample collection and DNA extraction.—Approximately 0.1 ml. of blood was collected from the brachial vein of 50 Blue-and-yellow Macaws (37 chicks found in natural nests and 13 adult captive birds caught in the vicinity) at six locations in Brazil (Table 1 and Fig. 1). All the samples were stored in absolute ethanol at -20°C and deposited in the tissue collection of the Laboratório de Genética e Evolução Molecular de Aves (LGEMA) at the University of São Paulo. Total genomic DNA was extracted using a standard proteinase K/SDS and phenol-chloroform protocol (Bruford et al. 1992).

Amplification and DNA sequencing.—A primer walking strategy was used to obtain new primers to sequence the entire mtDNA control region. The initial 1.6-Kb fragment was amplified for 10 individuals by polymerase chain reaction (PCR) with primers GluL₁₆₇₃₇ (Eberhard et al. 2001; Table 2) and CRH₁₂₄₈ (Tavares et al. 2004; Table 2), and this fragment was sequenced using primer GluL₁₆₇₃₇. A new forward primer (Table 2) was then designed in a conserved region ~ 500 base pairs (bp) from the 5' end of the sequence, and this new primer was used with CRH₁₂₄₈ to amplify a shorter fragment. Subsequent use of this procedure with sequential and partially overlapping PCR products using new forward primers not only helped sequence the entire mtDNA control region but also helped to confirm sequence alignment.

We used this primer set (Table 2) to amplify and sequence the entire mtDNA control region of 28 Blue-and-yellow Macaws.

TABLE 1. Sampling locations of Blue-and-yellow Macaw in Brazil, sample sizes, and numbers of mtDNA control-region haplotypes (*H*).

Population	Latitude, longitude	Sample size		
		Wild	Captive	<i>H</i>
Corginho, central Mato Grosso do Sul (CMS)	19°S, 54°W	7	2	6
Chapadão do céu, southwestern Goiás (SG)	18°S, 52°W	16	1	8
Chapada Gaúcha, northwestern Minas Gerais (NMG)	15°S, 45°W	2	2	3
Cavalcanti, northeastern Goiás (NG)	13°S, 47°W	7	3	6
Peixe, southeastern Tocantins (ST)	12°S, 48°W	3	2	3
Ilha do Marajó, northeastern Pará (wild samples) and Redenção, southern Pará (captive samples) (PA)	01°N, 49°W, 08°S, 50°W	2	3	4
Total		37	13	

FIG. 1. (A) Regions in Brazil where we sampled Blue-and-yellow Macaws (for abbreviations, see Table 1; adapted from a map by Instituto Brasileiro de Geografia e Estatística). Numbers indicate sample size at each sampling site. Differently shaded regions depict the main hydrographic basins in Brazil. (B) Genetic relationships among the six populations sampled, as inferred from hierarchical analysis of nucleotide diversity; the values of g_{ST} that are shown represent the proportion of the total genetic diversity within a cluster. The probability of observing higher values of g_{ST} by chance is also shown. Numbers in parentheses indicate the Φ_{ST} values estimated by AMOVA. Significant Φ_{ST} values ($P < 0.05$) are indicated with an asterisk.

TABLE 2. Primer pairs used to amplify and sequence the entire mtDNA control region of Blue-and-yellow Macaw. Primer names indicate the position of the primer in the flanking or control region.

Forward primer	Sequence (5'→3')	Reverse primer	Sequence (5'→3')	Fragment size (bp)
Lglu ₁₆₇₃₇ ^a	TCTTGGCAKCTTCAGTACCATGCTTT	H ₅₂₂ ^a	TGGCCCTGACYTAGGAACCAG	650
Lcr ₄₅₆ ^b	CACGAGAGATCAYCAACCCGGTGT	H ₁₀₂₈ ^b	GTGTAAAACAAAGTGCATCAGGGT	650
Lcr ₈₄₀ ^c	TCATTTTMRCACTGATGCTTG	Hcr ₁₃₂₂ ^c	GAGATAGTTGAGGCATAAGTGATTAA	400
Lcr ₁₂₀₉ ^c	CACCTACAAACACYACAAACAAC	Hcr ₁₂₄₈ ^a	TCTTGGCAKCTTCAGTACCATGCTTT	350

^aEberhard et al. 2001.

^bTavares et al. 2004.

^cPresent study.

Amplifications were performed in 10 μ L with 20–50 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.8 mM dNTPs, 1 μ M each primer, and 0.5 U *Taq* polymerase (Pharmacia). Each reaction consisted of an initial step of 95°C for 5 min followed by 30 cycles of 95°C (1 min), 53°C (40 s), and 72°C (40 s), and a final extension of 72°C for 5 min. The PCR products were loaded on 0.8% agarose gels to check their concentration and the presence of a unique band. The products were purified by digestion with 5 U of exonuclease I (USB) and 0.5 U of shrimp alkaline phosphatase (USB) at 37°C (1 h) and then at 80°C (20 min). Both DNA strands were sequenced using the Big Dye terminator sequencing kit (Perkin-Elmer, Waltham, Massachusetts), following the manufacturer's instructions. Sequencing products were loaded in 5% polyacrylamide denaturing gels on an ABI 377 automated sequencer (Applied Biosystems, Foster City, California). The sequences were aligned using the computer program SEQUENCE NAVIGATOR (Applied Biosystems) and were deposited in GenBank (accession nos. EF446619–EF446668).

For population genetic analyses, we sequenced only the first 1,290 bp of the 5' portion of the control region (first three primer pairs in Table 2) and increased the sample size to 50 Blue-and-yellow Macaws. Homologous portions from one Blue-throated Macaw (*A. glaucogularis*; GenBank accession no. EU038111) and one Red-bellied Macaw (*Orthopsittacus manilata*; GenBank accession no. EU038110) were sequenced and used as outgroups in the phylogenetic analysis. The first species is sister to *A. ararauna* (Oliveira-Marques 2006), and the latter is closely related to *A. ararauna* (Tavares et al. 2006).

Population genetic analyses based on mtDNA.—We used the D^* and F^* tests (Fu and Li 1993) in DNASP, version 4.10.9 (Rozas et al. 2003), to test the hypothesis that the mutations detected in the control-region sequences in each population were selectively neutral. Statistical significance of D^* and F^* values were determined by comparing the observed values with the critical values described in Fu and Li (1993).

We used two approaches to study the population genetic structure of Blue-and-yellow Macaws. First, we performed a hierarchical analysis of nucleotide diversity (Holsinger and Mason-Gamer 1996). The statistical significance of support values at each node is tested by creating a null distribution of values generated by random resampling and comparing the observed value with this null distribution. The most valuable feature of this approach is that it allows any pattern to emerge from the data rather than using an *a-priori* hypothesis of the population structure (Holsinger and Mason-Gamer 1996).

The second method we used was hierarchical analysis of molecular variance (AMOVA) using Φ_{ST} (Excoffier et al. 1992) as implemented in ARLEQUIN, version 2.0 (Schneider et al. 2000). The Φ_{ST} analyses were performed using a matrix with Tamura (1992) distances between sequences. In contrast to the previous method, this approach requires an *a-priori* hypothesis of sets of populations at different hierarchical levels. Using the results of the Holsinger and Mason-Gamer (1996) analysis, we tested the hypothesis that two populations are present: western (SG+CMS+PA) and eastern (ST+NG+NMG) (Fig. 1).

Relationships among haplotypes were obtained by computing a median-joining network using the algorithm described by Bandelt et al. (1999) and implemented in NETWORK, version 4.1.1.0

(Fluxus Technology, Clare, United Kingdom). Additionally, haplotype trees were inferred by maximum parsimony (MP) and maximum likelihood (ML) using PAUP*, version 4b8 (Swofford 1999). These trees were rooted with two outgroups (*A. glaucogularis* and *O. manilata*). The MP tree was obtained by branch-and-bound search. Starting trees were obtained via stepwise addition with random addition of sequences. A hierarchical likelihood-ratio test performed in MODELTEST, version 3.7 (Posada and Crandall 1998), indicated that the best-fit evolutionary model was the Hasegawa-Kishino-Yano (HKY; Hasegawa et al. 1985) with a gamma distribution ($\alpha = 0.0156$) and transition:transversion ratio of 5:32. This model was used in the ML analysis that was performed using a heuristic tree search with starting trees obtained via stepwise addition with 10 random-addition sequence replicates. Statistical support of tree nodes was obtained by nonparametric bootstrap (1,000 replicates).

Microsatellite genotyping.—Five dinucleotide microsatellite loci (UnaCT21, 32, 43, 74, and UnaGT55) isolated from the Blue-and-yellow Macaw (Caparroz et al. 2003) and one (AgGT21) isolated from the St. Vincent Amazon (*Amazona guildingii*; Russello et al. 2001) were used to genotype all samples. The forward primer from each pair was synthesized with a 5' M13 tail that allowed use of the universal dye-labeling method described by Boutin-Ganache et al. (2001). The PCR amplifications were carried out in a final volume of 12.5 μ L containing 20–50 ng of DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 80 μ M M13 fluorescently labeled primer (TET or HEX; Applied Biosystems), 160 μ M forward primer, 80 μ M reverse primer, and 0.5 U *Taq* polymerase (Pharmacia). Each reaction consisted of an initial step of 95°C for 10 min followed by 35 cycles at 95°C for 60 s, 50 to 60°C for 40 s and 72°C for 40 s, and a final step at 72°C for 10 min. The PCR products were electrophoresed in 5% denaturing acrylamide gels and analyzed in an ABI 377 DNA Sequencer with GENESCAN and GENOTYPER, version 2.1 (Perkin-Elmer).

Statistical analyses of microsatellites.—Observed (H_O) and expected (H_E) heterozygosities were calculated for each locus and for each population using ARLEQUIN. Each locus was tested for deviation from Hardy-Weinberg equilibrium expectation using an exact test based on the Markov-chain method of Guo and Thompson (1992), as implemented in GENEPOP, version 3.3 (1,000 dememorization, 1,000 batches, 1,000 iterations; Raymond and Rousset 1995). Linkage disequilibrium between all pairs of loci among populations and within each population was calculated with Fisher's exact test using the same program (1,000 dememorization, 300 batches, 1,000 iterations). Type I error rates for tests of linkage disequilibrium and departure from Hardy-Weinberg expectations were corrected for multiple comparisons using the sequential Bonferroni procedure (Rice 1989).

Analysis of gene and genotype frequency differentiation among all pairs of populations was done with a contingency table test as implemented in GENEPOP. Probability values (P) for each pairwise comparison were estimated by a Markov-chain method using the same program (1,000 dememorization, 100 batches, 1,000 iterations). P values were adjusted using sequential Bonferroni correction.

We also quantified differentiation between pairs of populations by calculating the fixation indices (F_{ST} and R_{ST}). F_{ST} values were calculated assuming Kimura and Crow's (1964) infinite

alleles model (IAM) of mutations. However, because microsatellites may evolve in a stepwise fashion via DNA polymerase slippage and the gain or loss of one or a few repeat units, statistics that assume Ohta and Kimura's (1973) stepwise mutation model (SMM) may be more appropriate for microsatellites. Therefore, we present subpopulation fixation indices based on both the IAM (F_{ST} , estimated as described by Weir and Cockerham [1984]) and the SMM (R_{ST} , estimated as described by Slatkin [1995]). We used FSTAT (Goudet 2001) to calculate F_{ST} and RSTCALC (Goodman 1997) to calculate R_{ST} , and permutation procedures in both programs to test whether particular values were significantly different from zero. Table-wide critical values were adjusted using the sequential Bonferroni correction.

To test for population structure based on the shared ancestry of microsatellite alleles, we used STRUCTURE, version 2.1 (Pritchard et al. 2000). Following the suggestions of those authors, we ran the entire data set (without any prior information) to determine the most likely number of clusters. Using 10^5 estimation steps after 30,000 burn-in steps, we ran five replicates of each value of K (number of clusters) from 1 to 6.

Demographic history.—The hypothesis of demographic stability was tested for the eastern and western groups by comparing the observed pairwise nucleotide site differences in mtDNA (mismatch distribution) and the expected values for a stable population using DNASP. We also estimated Ramos-Onsins and Rozas's (2002) R_2 statistic and the confidence interval (assuming a large constant population size; Hudson 1990) to distinguish population growth from constant population size using the same program.

We used the program IM (Hey and Nielsen 2004) to distinguish the contributions of isolation and migration to the observed patterns of genetic divergence between western and eastern groups (see below) on the basis of mtDNA data alone, because structuring of female lineages would be obscured by possible male-biased gene flow in microsatellites in a combined analysis. IM employs integrated likelihood to simultaneously estimate posterior probabilities for the following population parameters that are scaled by the neutral mutation rate, following the notation in Hey (2005). θ_{west} , θ_{east} , and θ_A are the standardized mutation rates for the western, eastern, and ancestral populations, respectively; m_{west} and m_{east} are the migration rates into the western and eastern populations, respectively; u is the neutral mutation rate for the entire sequence; $t = tu$ is the number of mutations since the populations split; $N_{e_{west}}$, $N_{e_{east}}$, and N_{e_A} are the effective number of individuals at the time of population divergence in the western group, eastern group, and ancestral population, respectively; $N_{e_{f_{west}}}$ and $N_{e_{f_{east}}}$ are the number of female migrants per generation into western and eastern populations, respectively; and t is the time of population splitting in millions of years and the expected time to the most recent common ancestor (TRMCA) for each pair of populations being compared. We used a burn-in period of 200,000 steps, when the chain became independent of the starting point, and then monitored the parameter values of the simulation for 7 million updates. We used a procedure for swapping among multiple heated chains to ensure that the distributions we obtained reflected the stationary distributions. Metropolis-coupling runs used 10 coupled chains that varied over a range of heating values ($g_1 = 0.7$ and $g_2 = 0.85$). The peaks of the resulting distributions were taken as estimates of the parameters. Credibility intervals

were assessed for each parameter as the 90% highest posterior density (HPD) intervals, which are the boundaries of the shortest span that includes 90% of the probability density of a parameter. We used a mutation rate of $\mu = 5.77 \times 10^{-5}$ substitutions locus⁻¹ generation⁻¹ and a generation time of 9 years to convert parameter estimates to more easily interpreted units. The estimate of the control-region mutation rate was based on the divergence time between *A. ararauna* and *O. manilata* of 14 mya (Tavares et al. 2006) divided by the maximum pairwise Tamura-Nei distance between these species (90 substitutions lineage⁻¹).

For the microsatellite data, we examined the signature of population expansion on genetic variability by estimating the imbalance index (β) derived by Kimmel et al. (1998) for each population. To obtain a mean estimate and standard error of β for each locality, we used locus-specific values of Θ_v and Θ_p to calculate β for each locus and then averaged these values across loci. Gene flow between the western and eastern populations was estimated using the maximum-likelihood method in MIGRATE (Beerli and Felsenstein 1999, 2001). This method does not assume the equilibrium island model in which gene flow is symmetrical among populations (gene flow can be asymmetrical). Search parameters were as follows: 10 short chains totaling 500,000 trees, of which 10,000 were recorded, followed by three long chains totaling 2,500,000 trees, of which 50,000 were recorded. We used adaptive heating with initial relative temperatures of 1, 1.5, 3.0, and 1,000 to avoid fatal attraction to $M = 0$. Nucleotide frequencies were estimated from the data, and initial estimates of theta and gene flow were obtained using F_{ST} . The analysis was run four times with different random numbers, and the results were averaged.

RESULTS

Control-region features.—Nuclear copies of mitochondrial genes are known to occur in birds (Quinn 1997). We considered that all the sequences analyzed in the present study should be of mitochondrial origin for the following reasons: (1) no contaminating products were detected after PCR; (2) there were no double peaks in sequence electropherograms; (3) there were perfectly matching sequences in overlapping PCR products; (4) there was variation among individuals in sequence without any evidence of variation within individuals (see [2] above); and (5) several conserved sequence blocks were present that are characteristic of the avian control region (details below).

The Blue-and-yellow Macaw control region has 1,484 bp and falls within the size range of those of many other bird species (1,000–1,500 bp; Baker and Marshall 1997, Ruokonen and Kvist 2002). We identified many of the conserved features typically found in the avian control region: C-, D-, and F-boxes, conserved sequence blocks (CSB 1 and 2), and termination-associated sequences (TAS-TATAT and TACAT) (data not shown). The average base composition found in the Blue-and-yellow Macaw control region was A = 25.7%, C = 27.5%, G = 15.0%, and T = 31.8%. The lower guanine frequency observed in the control region of this species was described as a characteristic of the L-strand of vertebrate mtDNA (Baker and Marshall 1997).

Control-region variation.—When outgroups were included, 209 (16.2%) polymorphic sites were observed within the 1,290 bp sequences. When only the 50 Blue-and-yellow Macaws were

FIG. 2. Median-joining network showing relationships among the Blue-and-yellow Macaw mtDNA haplotypes (1,290 bp of control region). Sizes of circles are proportional to the number of individuals carrying each haplotype. Lines connecting haplotypes represent single-mutation steps. If more than one substitution separates two haplotypes, the number is represented by cross bars on the line. Populations are abbreviated as in Table 1.

considered, 39 polymorphic sites (3.0%) were found and only one indel of 1 bp (site 1,281) was observed in the alignment, whereas the remaining polymorphic sites had two or more nucleotide substitutions. The average transversion:transition ratio varied from 1:4 to 1:13, depending on the inclusion or exclusion of outgroup sequences, respectively. Values for D^* and F^* computed across all combined populations were -1.18 and -0.72 , respectively. Both values were not significantly different from zero ($P > 0.1$). Therefore, we assume that the variation observed in the control region of Blue-and-yellow Macaw is selectively neutral.

A total of 28 haplotypes was found among the 50 Blue-and-yellow Macaw samples. Pairwise distances calculated as the proportion of different sites between two haplotypes averaged 0.78% (range: 0.08–1.55%). Most haplotypes were restricted to only one population, and only haplotypes H2 and H8 were found in more than one population (Fig. 2). Differentiation among haplotypes as depicted in a median-joining network was moderate to large, with haplotypes being separated by 1–20 substitutions (Fig. 2). There were three main haplotype clusters in the network: cluster I, comprising most (85%) of the haplotypes found in the two western sampling sites (SG and CMS); cluster II, composed exclusively of

haplotypes in the western sampling site (PA); and cluster III, comprising 65% of haplotypes found in most eastern sampling sites (ST, NG, and NMG). Haplotype clusters I and II were separated by 5 nucleotide substitutions, whereas haplotype clusters I + II were separated by ≥ 11 nucleotide substitutions from cluster III. Thus, haplotypes from clusters I and II tended to occur in populations in western Brazil (hereafter, "western haplogroup"), and those from cluster III in populations of eastern Brazil ("eastern haplogroup"). However, low-frequency haplotypes H14, H17, and H28 in haplotype cluster I of western populations were sampled in the eastern populations, and haplotypes H4, H5, H6, H9, and H20 from haplotype cluster III of eastern populations were sampled at low frequencies in western populations.

Phylogenetic relationships among Blue-and-yellow Macaw haplotypes estimated by MP and ML (trees not shown) were similar. However, they showed the same relationships as the unrooted network (Fig. 2), and outgroups rooted the network between the western and eastern haplogroups. These results confirmed the presence of two main haplogroups and that the PA haplotypes, excluding H20, were in the western haplogroup, as in the network. The bootstrap support values for the divergence of the two major

haplogroups were 100% in both MP and ML trees, and each haplogroup was also well supported. Within the western haplogroup, the three PA haplotypes found in northwestern Brazil formed a distinct sub-clade (bootstrap values >82% in both analyses).

Microsatellite variability.—Genetic variation at the six microsatellite loci analyzed in the six sampled populations is described in the Appendix. Three to 11 alleles locus⁻¹ were observed. The observed heterozygosity locus⁻¹ ranged from 0.23 (AgGT21) to 0.88 (UnaCT43). No significant deviation from Hardy-Weinberg equilibrium was detected in any locus. Furthermore, there was no evidence of nonrandom association of genotypes ($P > 0.05$) in any of the 15 tests for linkage disequilibrium performed on all possible pairwise comparisons of the loci analyzed.

Population structure.—Although many of the mitochondrial haplotypes detected in our samples are represented by a single individual and were found in only one locality (Fig. 2), geographic patterns of haplotype distribution were observed. Hierarchical analysis of nucleotide diversity detected the same groups described above. Approximately 25% of the total genetic diversity in the samples was apportioned between western and eastern populations. Additionally, within the western population a significant component of variance (16%) was identified between PA and the two southwestern localities (SG and CMS).

Conversely, variation in microsatellite loci was not structured geographically. No significant P values ($P > 0.05$) were observed in pairwise comparisons among populations across all loci in either genic or genotypic compositions, which indicates that the allelic and genotypic distributions were similar across localities. In addition, the overall F_{ST} value estimated with AMOVA was not statistically significantly different from zero ($F_{ST} = 0.00069$; $P > 0.05$), and all individuals were assigned to one cluster using STRUCTURE.

Demographic patterns.—The observed mismatch distribution for western and eastern populations was bimodal and, thus, not congruent with a model of population expansion. Distribution is usually multimodal in demographically stable populations but is expected to be unimodal in populations that experienced a recent demographic expansion (Slatkin and Hudson 1991, Rogers and Harpending 1992). The R_2 values were 0.4 and 0.12 for western and eastern haplogroups, respectively. In both cases, the R_2 values were within the associated confidence interval, which suggests that the two regional populations have not undergone a recent demographic expansion and can be considered to be in demographic equilibrium.

The coalescent analysis of the control-region sequences of Blue-and-yellow Macaws in IM produced parameter estimates that had unimodal posterior distributions (not shown). Similar estimates of effective population size for all populations (ancestral, western, and eastern) were consistent with stable population size since the split from the ancestral population (Table 3). Estimates of demographic parameters with their 90% highest posterior density (HPD) intervals indicate that the western and eastern populations split ~0.37 mya (HPD = 0.19–0.86) and that female gene flow between them is low to moderate (Table 3). Posterior probabilities of TMRCA peaked at 7.94 (HPD = 4.53–14.67), which indicates that haplotypes coalesced to a common ancestor ~1.24 mya (HPD = 0.71–2.29).

The mean values of β for each of the six sampled populations based on microsatellite data ranged from 0.58 ± 0.42 (PA) to 1.41 ± 1.84 (ST) (data not shown). All population-specific values have 95% confidence intervals that overlap with an equilibrium value of 1.0. This supports the idea that these populations are close to or at demographic equilibrium. Estimates of gene flow based on microsatellite data using the coalescent approach in MIGRATE were symmetric between western and eastern populations (western to eastern $Nm = 1.01$, eastern to western $Nm = 1.04$ immigrants per generation). This level of gene flow is sufficient, in theory, to prevent significant differentiation in neutral microsatellites by genetic drift in large populations (Mills and Allendorf 1996).

DISCUSSION

Analyses of population genetic structure in Blue-and-yellow Macaws based on the mtDNA control region showed evidence of two major regional populations: SG+CMS+PA (western haplogroup) and NG+ST+NMG (eastern haplogroup). However, these regional populations also showed evidence of low to moderate gene flow between them, given that they shared few haplotypes and that some closely related haplotypes are found in the two geographic regions (Fig. 2). This pattern can be attributed to low to moderate levels of female gene flow (Table 3) between historically isolated populations. Although the relatively small sample sizes and high diversity of mtDNA haplotypes make it somewhat difficult to draw many conclusions, it seems that much of the movement of mtDNA lineages between regions has occurred over a long period, as evidenced by the lack of sharing between the geographic groups of specific mtDNA haplotypes other than two common ones. Our analysis with IM also indicated that female gene flow occurred

TABLE 3. Coalescent estimates (MLE) and the 90% highest posterior density (HPD) intervals of demographic parameters based on mtDNA, assuming the splitting of western and eastern groups from an ancestral population. Population parameters that are scaled by the neutral mutation rate (θ_{west} , θ_{east} , and θ_A) are the standardized mutation rates for western, eastern, and ancestral populations, respectively; m_{west} and m_{east} are the migration rates into the western and eastern populations, respectively; $t = tu$ is the number of mutations since the populations split, where u is the annual locus-mutation rate; Ne_{west} , Ne_{east} , and Ne_A are the effective number of individuals in the western, eastern, and ancestral populations at the time of population divergence; $Ne_{(f)}m_{west}$ and $Ne_{(f)}m_{east}$ are the number of female immigrants per generation into western and eastern populations, respectively; and t is the time of the population split in millions of years.

	θ_{west}	θ_{east}	θ_A	m_{west}	m_{east}	$t = tu$	Ne_{west}	Ne_{east}	Ne_A	$Ne_{(f)}m_{west \text{ to east}}$	$Ne_{(f)}m_{east \text{ to west}}$	t
MLE	32.0	33.2	27.1	0.58	0.001	2.40	138,648	143,848	117,418	0.01	4.64	0.374
Lower HPD	15.2	16.9	6.4	0.17	0.000	1.22	65,858	73,224	27,730	0.00	0.65	0.190
Upper HPD	71.6	73.3	117.2	2.70	0.092	5.50	310,226	317,591	507,799	1.68	48.33	0.858

asymmetrically and mainly from the eastern to the western haplogroup (Table 3). Although direct studies of dispersal (e.g., radiotelemetry) are not available for Blue-and-yellow Macaw, the species is known to fly long distances and is potentially able to disperse over broad areas (Forshaw 1989).

In contrast with these results based on mtDNA control-region sequences, the two haplogroups showed no significant genetic differentiation based on microsatellite loci. There are three possible reasons for the differences in phylogeographic patterns observed between these two DNA markers: (1) the haplogroups are not in demographic equilibrium, and the high similarity in microsatellite loci is mainly attributable to recent sharing of ancestral polymorphisms rather than to gene flow; (2) differences in the effective population size and mutation rate of each type of marker result in mtDNA showing inherently higher levels of differentiation; or (3) male-biased gene flow and large effective population sizes have prevented microsatellite, but not female-specific mtDNA, differentiation between eastern and western haplogroups. We consider each of these possibilities below.

Hypothesis of common ancestry.—A high level of similarity among populations based on microsatellite data can result from a high level of retained ancestral polymorphism within populations (Gibbs et al. 2000). However, microsatellite data showed that in all populations the confidence interval for the imbalance index included the expected equilibrium value of 1. This suggests that the populations sampled are close to or at equilibrium (for details, see Kimmel et al. 1998). Additionally, the IM-estimated divergence time of the western and eastern haplogroups seems to be sufficiently old for the sorting of ancestral polymorphisms by genetic drift. Thus, recent common ancestry is an unlikely explanation for lack of differentiation observed on the basis of microsatellite data.

Hypothesis of genetic characteristics of markers.—Comparison of levels of variation between mtDNA and microsatellite data is not easy, because one is based on DNA sequence and the other on allele frequencies, which requires different measures of differentiation. As noted by Seielstad et al. (1998), comparisons of levels of differentiation among different classes of genetic markers need to take into account genetic characteristics that could bias such comparisons. Specifically, the fourfold difference in effective population size between organelle and autosomal markers (Birky et al. 1989) means that mtDNA may inherently show higher levels of differentiation at equilibrium than microsatellites, even though the microsatellite mutation rate is roughly an order of magnitude higher. However, the faster rate of mutation in microsatellites should have been sufficient to produce detectable allele frequency differences between eastern and western populations since the time of population divergence of females estimated with mtDNA.

Hypothesis of sex-biased dispersal.—Mitochondrial DNA is maternally inherited in birds; if females are more philopatric than males, this alone could result in greater population structure than that obtained by our analysis of data from biparentally inherited microsatellites. We found that female gene flow is asymmetric and low to moderate between western and eastern haplogroups, resulting in immigrant mtDNA haplotypes occurring at low frequency in both populations. Estimates of gene flow using microsatellites in MIGRATE were symmetric and showed about two migrants generation⁻¹ between the two populations, which

would theoretically prevent significant differentiation in allele frequencies, given selective neutrality and the large effective population sizes (see Mills and Allendorf 1996). Thus, the results suggest that males disperse over longer distances than females and that male-biased dispersal is at a rate high enough to explain the lack of population structure in nuclear DNA. Alternative explanations are different selective sweeps on mtDNA (Ballard and Whitlock 2004) in both populations or a random phylogeographic division without any clear geographic barrier to dispersal (Irwin 2002). However, these hypotheses are not supported by neutrality tests or by the exchange and persistence of immigrant haplotypes in western and eastern haplogroups.

Discrepancies in population structure obtained by markers with different inheritance patterns were observed in several organisms, including mammals (Melnick and Hoelzer 1992, Ishibashi et al. 1997), turtles (FitzSimmons et al. 1997), and birds (Scribner et al. 2001, Gay et al. 2004). In all cases, these discrepancies are concordant with the expected dispersal behavior of the sexes in these species. Male-biased dispersal in the Blue-and-yellow Macaw differs from the general pattern observed in birds of natal and breeding dispersal as either female-biased or equal with no differences between the sexes (Greenwood 1980, Clarke et al. 1997). However, most bird species for which dispersal data are available are passerines, whereas ~73% of the nonpasserines showed evidence of male-biased dispersal (Clarke et al. 1997).

Numerous hypotheses have related sex biases in dispersal to differences in the advantages that philopatry conveys to males and females in competition for mates or breeding resources, or to the influence of male-female differences on the fecundity cost of dispersal (Greenwood 1980, Dobson 1982, Johnson 1986, Pusey 1987, Wolff and Plissner 1998, Perrin and Mazalov 2000). However, predictions based on these different hypotheses depend on the mating system of the species considered. Greenwood (1980) hypothesized that female-biased dispersal in birds is associated with monogamy, resource defense by males, and local competition for mates among females, whereas mammalian male-biased dispersal is associated with polygyny, resource defense by females, and male defense of mates. Considering that competition for mates may be the most important factor in the evolution of dispersal patterns, male-biased dispersal observed in the Blue-and-yellow Macaw may be related to local competition for mates among males. However, little is known about the natural history of the Blue-and-yellow Macaw, so an explanation of sex-related differences in dispersal must await future ecological and genetic studies.

Demographic history.—The phylogeographic pattern observed in mtDNA sequences of the Blue-and-yellow Macaw can be interpreted as a relatively ancient isolation of individuals in western and eastern populations ~374,000 years ago, strong female philopatry, and male-biased dispersal among these populations. The paleogeological events that may have contributed to this contrasting phylogeographic pattern could possibly be related to the origin and formation of the main hydrographic basins in Brazil. The three haplotype clusters (Figs. 1 and 2) are geographically distributed in three different basins: cluster I in the Prata river basin, cluster II in the Amazonas river basin, and cluster III in the Araguaia-Tocantins river basin. Blue-and-yellow Macaws nest in holes in dead palm trees mainly of the genus *Mauritia* (Forshaw 1989, Collar 1997). These palm species occur in seasonally flooded

nascent and gallery forests that border rivers and streams, and the origin and formation of the watercourses could have shaped the geographic distribution of this species.

Implications for conservation.—The microsatellite survey indicates no population structure among the Blue-and-yellow Macaws we studied, which suggests that they should be considered a panmictic population; yet mtDNA data indicate two partially isolated groups. However, given that females disperse less than males, these two groups should be considered two different management units (Moritz 1994b) for conservation purposes. Particularly, if females do not move much, local populations could be extirpated by anything that increased female mortality or reduced their reproductive success. However, this result should be viewed with caution, because the Blue-and-gold Macaw has a continental geographic distribution, occurring throughout most of South America, and the results presented here involved few sampled localities and few individuals. Thus, more extensive sampling is required to better understand the historical and current processes of population structuring in this species. In addition, study and monitoring of local populations are important to better understand the population dynamics and to establish conservation strategies for this species.

Our results also highlight a potential problem in using mtDNA sequences alone to identify conservation units (e.g., Moritz 1994a, b; Holland and Hadfield 2002; Vieites et al. 2006). Several mitochondrial genome characteristics—such as its small size, ubiquity, maternal inheritance with no or rare recombination (Horai et al. 1995; Arnason et al. 1996), and faster evolutionary rate than nuclear DNA (Brown et al. 1982)—make this marker attractive for identifying units for conservation and designing management plans. However, caution should be used when defining units for conservation on the basis of any single character. As discussed above, there are some characteristics intrinsic to markers (effective population sizes) or to the biology of the species (male-biased gene flow) that can lead to misinterpretation of data. Thus, comparisons based solely on mtDNA may not represent the true genetic relations among populations. This does not mean that mtDNA data should be ignored in the identification of conservation units (e.g., Holland and Hadfield 2002); rather, we suggest that it is important to obtain corroborating evidence from nuclear markers to support conclusions from mtDNA. Finally, our study reinforces the observation that male-biased dispersal occurs in nonpasserine birds (Clarke et al. 1997) and that this can have a significant role in determining the genetic structure of bird populations.

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APPENDIX. Genetic variation at six microsatellite loci of Blue-and-yellow Macaws from six populations (*n* = number of individuals genotyped; range = size range of alleles in base pairs; *H_E* and *H_O* are expected and observed heterozygosities, respectively).

Locality ^a	Locus					
	UnaCT21	UnaCT32	UnaCT43	UnaCT74	UnaGT55	AgCT21
CMS						
<i>n</i>	8	8	8	8	8	8
Number of alleles	6	7	8	4	4	3
Range	247–257	258–276	199–219	232–246	184–192	308–312
<i>H_E</i>	0.867	0.800	0.908	0.533	0.592	0.342
<i>H_O</i>	0.750	0.750	1.000	0.375	0.750	0.375
SG						
<i>n</i>	17	17	17	17	17	17
Number of alleles	8	4	7	3	4	3
Range	247–261	262–272	199–217	240–246	184–192	308–312
<i>H_E</i>	0.866	0.618	0.829	0.520	0.403	0.171
<i>H_O</i>	0.882	0.647	0.882	0.412	0.353	0.118
NMG						
<i>n</i>	4	4	4	4	4	4
Number of alleles	4	3	5	3	2	1
Range	245–259	262–268	205–217	240–246	184–190	308
<i>H_E</i>	0.821	0.750	0.857	0.750	0.607	—
<i>H_O</i>	1.000	0.500	1.000	0.750	0.500	—
NG						
<i>n</i>	10	10	10	10	10	10
Number of alleles	8	2	7	4	4	3
Range	247–273	262–264	199–217	232–246	184–192	308–312
<i>H_E</i>	0.874	0.505	0.842	0.721	0.489	0.363
<i>H_O</i>	0.800	0.200	1.000	0.600	0.600	0.300
ST						
<i>n</i>	5	5	5	5	5	5
Number of alleles	4	2	4	3	2	2
Range	249–255	262–264	199–215	240–246	184–190	308–312
<i>H_E</i>	0.800	0.533	0.889	0.844	0.356	0.200
<i>H_O</i>	1.000	0.800	0.600	0.400	0.400	0.200
PA						
<i>n</i>	5	5	5	5	5	5
Number of alleles	6	6	6	4	3	2
Range	247–261	262–276	205–217	232–246	184–190	308–310
<i>H_E</i>	0.889	0.911	0.889	0.800	0.533	0.356
<i>H_O</i>	0.800	0.800	0.800	0.600	0.400	0.400

^aLocalities abbreviation as in Table 1.

Anexo XVI

Phylogeography of *Xiphorhynchus fuscus* (Passeriformes, Dendrocolaptidae): vicariance and recent demographic expansion in southern Atlantic forest

GUSTAVO SEBASTIÁN CABANNE¹, FABRÍCIO R. SANTOS² and CRISTINA YUMI MIYAKI^{1*}

¹Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, 05508-090, São Paulo, SP, Brazil

²Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31270-901, Belo Horizonte, MG, Brazil

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Knowledge of the evolutionary processes that shaped a biota is important for both academic and conservation purposes. The objective of the present study is to analyse the mitochondrial genetic variation of *Xiphorhynchus fuscus* (Aves: Dendrocolaptidae) from the southern Atlantic forest in Brazil and Argentina, and to discuss whether the results support different hypotheses regarding the local intraspecific diversification of this species. We sequenced 575 bp of the control region of 114 specimens collected in the Brazilian states of Bahia, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, and Santa Catarina, and in the province of Misiones in Argentina. We studied the population genetic structure with analysis of molecular variance and the demographic history with multiple regression analysis, coalescence simulations, and demographic tests. *Xiphorhynchus fuscus* presented a significant population genetic structure ($\Phi_{st} = 0.57$). Three mitochondrial lineages were described, one associated with *Xiphorhynchus fuscus tenuirostris* and the others with *Xiphorhynchus fuscus fuscus*. The data did not support the primary influence of geographical barriers or rivers in the intraspecific diversification of *X. fuscus* in the southern Atlantic forest. Instead, the data supported the influence of isolation by geographical distance, recent vicariance events, and demographic expansions apparently related to Pleistocene and Holocene forest dynamics. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, 91, 73–84.

ADDITIONAL KEYWORDS: coalescence simulations – forest refuges – isolation by distance – mitochondrial control region – multiple regression – Neotropics – Pleistocene – riverine barriers – Valley of the Paraíba do Sul river.

The Atlantic forest is distributed along eastern Brazil, eastern Paraguay, and north-eastern Argentina (Gusmão Câmara, 2003). Its unique biota is probably the result of a complex evolutionary history; however, few studies have attempted to clarify the biogeographical processes that shaped it (Muistrangi & Patton, 1997; Costa *et al.*, 2000; Geise, Smith & Patton, 2001; Pellegrino *et al.*, 2005). The knowledge of these evolutionary processes is important for both academic and conservation purposes (Moritz, 2002). Among the several hypotheses on the diversification of rainforest bio-

tas (Moritz *et al.*, 2000), the evolution in palaeorefuges and the influence of geographical barriers are two of the most discussed ones.

In the Neotropics, the refuge theory was originally proposed to explain speciation during the Pleistocene mainly in the Amazon basin (Haffer, 1969; Vanzolini & Williams, 1970; Brown & Ab'Saber, 1979; Haffer & Prance, 2001). This theory proposes that during the glaciations the rainforests were reduced to refuges isolated by open areas, and that organisms isolated in these refuges could have diverged and originated new lineages. Then, in the next interglacial period, the forest expanded and the new clades would be in contact. This hypothesis requires the expansion and contrac-

*Corresponding author. E-mail: cymiyaki@ib.usp.br

tion of open areas and the formation of forest refuges. Palynological (Ledru *et al.*, 1998; Behling & Negrelle, 2001; Behling, 2002) and other types of studies (Brown & Ab'Saber, 1979) propose that open areas dominated the Atlantic forest's landscape during the maximum of Late Pleistocene glaciations, suggesting that the refuge theory can be important to understand the biological diversification of the biome.

Pellegrino *et al.* (2005), based on a phylogeographical study of the gecko *Gymnodactylus darwini* (Gekkonidae, Squamata), proposed that rivers play an important role in the diversification of Atlantic forest biota. Other authors proposed that the tectonic activity associated with the formation of geographical landmarks could be relevant to biodiversity modelling, as suggested by Silva & Straube (1996) who observed that the geographical range of some passerines was limited by a graben, the valley of the Paraíba do Sul

river (VPSR). Those tectonic episodes could have been important for the biota in the southern Atlantic forest, where a complex relief exists with many mountain ranges and valleys, and where neotectonic activity was described (Petri & Fulfaro, 1983; Riccomini *et al.*, 1989).

Xiphorhynchus fuscus is an Atlantic forest endemic member of the Family Dendrocolaptidae (Aves) with a broad distribution. It occurs in eastern Brazil (from the state of Ceará to the state of Rio Grande do Sul), eastern Paraguay and north-eastern Argentina (Marantz *et al.*, 2003). There are four subspecies defined by slight variations in colour and morphological measurements (Marantz *et al.*, 2003). *Xiphorhynchus fuscus tenuirostris* and *Xiphorhynchus fuscus fuscus* inhabit the eastern and the southern range of the species, respectively (Fig. 1). The other subspecies inhabit the interior of the Brazilian state of

Figure 1. Study area, sampling localities, and distribution of *Xiphorhynchus fuscus* subspecies and mtDNA lineages. Locality numbers correspond to Table 1. BA, Bahia; ES, Espírito Santo; GO, Goiás; MG, Minas Gerais; MI, Misiones; MT, Mato Grosso do Sul; PR, Paraná; RJ, Rio de Janeiro; RS, Rio Grande do Sul; SC, Santa Catarina; SP, São Paulo. Approximate distribution limits of the two subspecies indicated by broken lines (Zimmer, 1947; Marantz *et al.*, 2003). A: *X. fuscus tenuirostris*, B: *X. fuscus fuscus*.

Table 1. Localities, sample sizes (*N*), haplotypes, and nucleotide diversity (π) in percentage

Locality	<i>N</i>	Haplotypes ^j	$\pi\%$ (SD%)
1. Minas Gerais (MG), Jequitinhonha, left bank of the Jequitinhonha river, 16°20'S, 41°00'W	5 ^a	T2 ¹ , T4 ¹ , T6 ¹ , T7 ²	0.5186 (0.2198)
2. MG, Salto da Divisa, left bank of the Jequitinhonha river, 16°05'S, 40°02'W	6 ^a	T1 ⁴ , T5 ¹ , T7 ¹	0.4606 (0.1785)
3. Bahia, Porto Seguro, 17°22'S, 40°17'W	1 ^b	T3 ¹	–
4. MG, Nova Lima, 19°59'S, 43°49'W	1 ^a	N8 ¹	–
5. MG, Marliéira, 19°43'S, 42°44'W	1 ^a	N9 ¹	–
6. MG, Caratinga, 20°50'S, 42°05'W	1 ^a	N12 ¹	–
7. MG, Simonésia, 20°07'S, 42°00'W	1 ^a	N13 ¹	–
8. MG, Araponga, 20°40'S, 42°31'W	3 ^a	N2 ¹ , N10 ¹ , N11 ¹	0.4710 (0.2374)
9. Rio de Janeiro, Itatiaia National Park, 22°25'S, 44°36'W	10	N2 ⁶ , N6 ¹ , N14 ¹ , N15 ²	0.2274 (0.1239)
10. São Paulo (SP), Bananal State Park, 22°41'S, 44°19'W	2 ^c	N3 ¹ , N6 ¹	0.7109 (0.3368)
11. SP, Picinguaba, 22°31'S, 44°50'W	4	N2 ⁴	0.0
12. SP, Caraguatatuba, 23°37'S, 42°26'W	2	N2 ¹ , N14 ¹	0.1760 (0.1771)
13. SP, Morro do Diabo State Park, 22°30'S, 52°18'W	1	S1 ¹	–
14. Paraná (PR), Ortigueira, 24°12'S, 50°55'W	1	S8 ¹	–
15. PR, Wenceslau Braz, 22°51'S, 49°47'W	7 ^d	S1 ⁵ , S8 ²	0.1677 (0.1237)
16. SP, Barreiro Rico, 22°38'S, 48°13'W	3 ^e	N2 ² , S8 ¹	0.9479 (0.3195)
17. SP, Itaberá State Park, 23°51'S, 49°08'W	7	S4 ³ , S6 ³ , S10 ¹	0.2014 (0.1154)
18. SP, Buri State Park, 23°39'S, 48°32'W	9 ^f	S4 ⁴ , S7 ¹ , S8 ³ , S9 ¹	0.1760 (0.1098)
19. SP, Caboclos, 24°28'S, 48°35'W	8	S4 ⁵ , S8 ³	0.0943 (0.0949)
20. SP, São Roque, 23°34'S, 47°09'W	9	N1 ³ , N2 ¹ , S4 ⁵	0.9199 (0.2815)
21. SP, Morro Grande State Park, 23°42'S, 46°59'W	14	N1 ⁵ , N2 ³ , S5 ¹ , S6 ² , S7 ³	0.8830 (0.2589)
22. SP, Juquitiba, 23°53'S, 47°00'W	7 ^g	N1 ¹ , N4 ² , S4 ¹ , S6 ¹ , S7 ¹ , S8 ¹	1.0172 (0.3025)
23. SP, Serra do Mar State Park, Station Curucutú, 23°58'S, 46°44'W	4 ^h	N2 ¹ , N4 ¹ , N5 ¹ , N7 ¹	0.4424 (0.1858)
24. Argentina, Misiones (MI), Campo San Juan State Park, 27°22'S, 55°39'W	3	S1 ¹ , S2 ²	0.1173 (0.1065)
25. MI, Yabotí Biosphere Reserve, 26°48'S, 53°55'W	3	S1 ² , S3 ¹	0.1173 (0.1181)
26. Santa Catarina, Botuverá, 27°13'S, 49°03'W	1 ⁱ	S1 ¹	–

^aTissue samples deposited at the Universidade Federal de Minas Gerais; all the other samples are deposited at the Universidade de São Paulo.

Samples with museum voucher or specimen field (f) numbers: ^bMuseu Paraense Emílio Goeldi fAA568; ^cMuseu de Zoologia da Universidade de São Paulo (MZUSP) f76, f91; ^dMZUSP fITA257, fITA302, fITA283; ^eMZUSP f31, f59, f60, ^fMUZUSP 76134, MZUSP 75588; ^gMZUSP fITA141, fITA171, fITA182, fITA152, fITA172, fITA157, 75565; ^hMZUSP f9; ⁱMZUSP 75032.

^jNumbers in superscript indicate the number of individuals with the corresponding haplotype.

Bahia (*Xiphorhynchus fuscus brevirostris*) and the north-eastern part of the country (*Xiphorhynchus fuscus atlanticus*). The distribution limits of these subspecies are not well known. Given its geographical distribution and deep-forest dependency, *X. fuscus* is a good model to study the diversification of the Atlantic forest biota.

The objective of the present study is to analyse the mitochondrial DNA variation of populations of *X. fuscus* from the southern part of the Atlantic forest and to discuss whether the results support different hypotheses regarding the local intraspecific genetic diversification of this bird.

MATERIAL AND METHODS

STUDY AREA AND SAMPLES

Samples (blood or muscle, *N* = 114) were collected between 2000 and 2004 in the Brazilian states of Bahia, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, and Santa Catarina, and in the province of Misiones in Argentina (Fig. 1, Table 1). The collection localities are covered by dense ombrophilus, mixed or semideciduous forest, which are the main forest types of the Atlantic forest (Veloso, 1991). The relief in the study area is complex, especially in the eastern portion due to the presence of the Serra do Mar and the

Serra da Mantiqueira coastal ridges, which can surpass 2000 m a.s.l.

Blood was collected (approximately 0.1 mL) with insulin syringes from the largest vein in the right cervical region. Muscle was obtained from specimens which were deposited at the Museu de Zoologia da Universidade de São Paulo. Each bird was captured with mist nets, photographed and marked with an aluminium ring. All tissue samples are deposited at the Laboratório de Genética e Evolução Molecular de Aves (Instituto de Biociências, Universidade de São Paulo, Brazil), or at the Laboratório de Biodiversidade e Evolução Molecular (Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais, Brazil).

MOLECULAR METHODS

Total DNA was obtained from blood or muscle samples by a conventional proteinase K–SDS digestion, organic extraction with phenol–chloroform, and ethanol precipitation (Bruford *et al.*, 1992). To diminish the possibility of amplifying unexpected copies of nuclear mtDNA inserts, we amplified an approximately 1600-bp fragment with the primer pair LXfB2 (5'-TCAATTCCAAACAACTAGGAGG-3', present study)/HPR0 (5'-GCTTTGGGAGTTGGAGATAA AGG-3', present study). This amplicon contained the complete control region (total size of 1275 bp, determined in the present study). The PCR reaction (10 µl) contained 20–40 ng of total DNA, 1X of *Taq* buffer (Pharmacia Biotech), 200 µM of each dNTP, 1 µM of each primer, and 0.1 U of *Taq* polimerase (Pharmacia Biotech). Amplifications were performed with an initial step at 95 °C for 4 min and 37 cycles of 45 s at 94 °C, 45 s at 53.5 °C, and 110 s at 72 °C, followed by a final extension of 10 min at 72 °C. PCR products were purified with shrimp alkaline phosphatase and exonuclease I. Sequencing reactions were performed with Big Dye Terminator Kit, version 3.0 (Applied Biosystems Inc.) using the amplification and the internal primers (HXfRC1 5'-GGGGAAAATAAACGTTTATTA AGTG-3', HXfRC2 5'-CAAGATGGACATGTTTCGAC ACCG-3', present study, and L537 5'-CCTCTGGTTC CTCGGTCAG-3'; Sorenson *et al.*, 1999). The sequencing reactions were precipitated with isopropanol 75% and ethanol 70%, and analysed in an automatic sequencer ABI Prism 377 (Applied Biosystems Inc.).

ANALYTICAL METHODS

The complete control region (1275 bp) and flanking genes (tRNA^{Thr} 70 bp, tRNA^{Phe} 70 bp) from one *X. f. fuscus* sample (tissue IBUSP P652) were sequenced. The analysis of 1048 bp of the control region (positions 57–1104) from 20 individuals revealed that the most variable portion encompasses 575 bp (positions 57–631); thus, this segment was

used in the phylogeographical analyses. Identification and simulation of the secondary structure of the tRNA sequences were carried out with the program tRNAscan-SE 1.21 (<http://www.genetics.wustl.edu/eddy/tRNAscan-SE/Lowe & Eddy, 1997>). All sequences are deposited in GenBank under the access numbers AY948386–AY948394, AY948396–AY948415, and DQ144622–DQ144636.

Sequences were aligned with the program Clustal X (Thompson *et al.*, 1997). The likelihood ratio test as implemented in the software Modeltest, version 3.7 (Posada & Crandall, 1998), was used to select the best fit evolutionary model [HKY85 with the proportion of invariable sites (I) of 0.8404 and a discrete gamma distribution ($\alpha = 0.6467$); Hasegawa *et al.*, 1985]. We reconstructed the phylogenies by Neighbour-joining (NJ; Saitou & Nei, 1987) with the software PAUP*, version 4.0b10 (Swofford, 2001) and by maximum likelihood (ML) with PHYML Online (Guindon & Gascuel, 2003; Guindon *et al.*, 2005). The starting tree for the ML analysis was obtained with PHYML Online. For both analyses, the best fit evolutionary model was used. A sequence of *Xiphorhynchus pardalotus* (tissue IBUSP P264) was used to root the resulting trees. The support of the nodes was evaluated by nonparametric bootstrap (100 replicates; Felsenstein, 1985).

In all further population analyses, we used the Tamura & Nei (1993) model of evolution, which is the closest one to the HKY85 model available in the software we used. The haplotype network was constructed in accordance with Templeton, Crandall & Sing (1992) with the program TCS, version 1.13 (Clement, Posada & Crandall, 2000). The net divergence between sets of sequences was obtained in MEGA, version 3.0 (Kumar, Tamura & Nei, 2004) with the formula $\delta = \delta_{xy} - [0.5(\delta_x + \delta_y)]$, where δ_{xy} is the mean divergence between all the sequences, and δ_x and δ_y are the mean divergences within each group of sequences *x* and *y*, respectively (Wilson *et al.*, 1985). The mutation rate used to estimate the divergence time between the lineages was $\mu = 8.72 \times 10^{-8}$ changes per nucleotide, as used by Miot, Gibbs & Hobson (2000) for the analysis of the first domain of the mtDNA control region of the passerine *Dendroica petechia*. We assumed a generation time of 1 year (Klicka & Zink, 1999).

To test whether the *X. f. fuscus* gene genealogy is the result of the neutral coalescence process within a panmictic population, we followed Knowles (2001) and applied a gene-tree population-tree approach using the program MESQUITE 1.02 (Maddison & Maddison, 2004). In this approach, gene trees (300 replicates) were simulated by neutral coalescence under a null model of a unique panmictic *X. f. fuscus* population whose effective population size (N_e) was invariable until total coalescence. Four arbitrary N_e were tested: 100 000, 50 000, 25 000, 12 000, and 6000. The

discordance between these gene trees and the alternative model of two isolated populations (one in the north and the other in the south of the Valley of the Paraíba do Sul river, VPSR; Fig. 1) was measured using statistic s (Slatkin & Maddison, 1989). The discordance between the reconstructed gene tree (NJ tree of *X. f. fuscus* sequences) and the two populations' model was also evaluated with the statistic s and this value was compared with the expected distribution of s -values obtained from the neutral simulations. If the s -value of the reconstructed gene tree is significantly lower than the values from the simulated gene trees ($\alpha = 0.05$), the null model is rejected. We used the nucleotide diversity (π , Nei & Kumar, 2000) calculated in Arlequin, version 2.0 (Schneider, Roessli & Excoffier, 2000) to approximate theta (Θ) and to calculate the empirical N_e according to the relationship $\pi = \Theta = 2N_e\mu$ (Watterson, 1975; Nei & Kumar, 2000).

We estimated the nucleotide diversity for each locality where two or more individuals were available using Arlequin 2.0. The analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was used to study the population genetic structure as implemented in Arlequin, version 2.0. We performed global (all the localities) and regional analyses (subgroups of localities). The Φ_{ST} was obtained using corrected distances and its statistical significance was estimated by a nonparametric permutation test (Excoffier *et al.*, 1992).

We performed a partial regression analysis (Smouse, Long & Sokal, 1986) to test the effect of two independent variables (linear geographical distance among the pairs of localities and their geographical location) on the average corrected genetic distances among individuals from pairs of locations. Genetic distances were estimated with the program MEGA, version 3.0. The matrix of geographical location was constructed as a dummy variable (Quinn & Keough, 2002) that indicated whether each pair of localities were within the same main geographical region (north-western Minas Gerais plus southern Bahia, south-eastern Minas Gerais plus southern Rio de Janeiro, and south from the VPSR). The magnitude of the partial regression coefficients indicates the relative importance of the different independent variables. If genetic distances reflect simple isolation by distance, geographical distance would be the best predictor. If vicariance were the most important process, the geographical location matrix would be the best predictor of genetic distances. Each matrix was transformed to a mean of zero and a variance of one before the analyses (Smouse *et al.*, 1986). The partial regression analyses were performed in the program Fstat, version 2.9.3.2 (Goudet, 2002), using 10 000 permutations to obtain the probability values.

Demographic expansion was inferred by calculating F_s (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) using DnaSP, version 4 (Rozas & Sánchez-Del Barrio, 2003). Significantly negative F_s and low values of R_2 indicate demographic expansion. For these calculations, the three major lineages recovered in the genealogy were defined as populations, following Cheviron, Hackett & Caparella (2005). Significance was determined based on 1000 coalescent simulations under a model of population stability using empirical sample sizes and estimates of Θ . The parameters of exponential population growth (Θ_0 , Θ_1 , and τ), according to the model of Rogers & Harpending (1992), were estimated in the program Arlequin, version 2.0. The expansion time was estimated according to $t = \tau/2u$ using lower and upper values of 95% confidence interval of the parameter τ , where u is the mutation rate per generation per haplotype (Rogers & Harpending, 1992).

RESULTS

CHARACTERISTICS OF THE SEQUENCES

The sequences obtained revealed evidence of being of mitochondrial origin: (1) the empirical base composition of the sequences used in the phylogeographical study (A, 27.7%; C, 25%; G, 16.3%; and T, 31%) is compatible with the expected one for avian mitochondrial DNA (Baker & Marshall, 1997); (2) the amplicon (1600 bp) contained the complete control region (1275 bp), flanked by a tRNA^{Thr} at the 5' end (70 bp) and a tRNA^{Pro} at the 3' end (70 bp), and this gene arrangement is the expected one for Suboscines (Mindell, Sorenson & Dimcheff, 1998); (3) the inferred secondary structure and the presence of expected anticodons (UGU and UGG) suggested that these tRNA genes are functional (data not shown); and (4) the amplicon analysed is longer than the average size of translocated copies of mitochondrial genes in an avian nuclear genome (Pereira & Baker, 2004). The 575 bp of the control region of 114 individuals of *X. fuscus* presented 32 haplotypes (Table 1, Figs 2, 3), with 31 polymorphic sites (5.39% of all positions), 30 transitions and two transversions, and no indels.

GENEALOGY, DIVERGENCE, AND GEOGRAPHICAL DISTRIBUTION OF *X. FUSCUS* MITOCHONDRIAL LINEAGES

The haplotype network presented three major clades (Fig. 2): one associated with *X. f. tenuirostris* and the others with *X. f. fuscus* [northern lineage (N) and southern lineage (S)]. The *X. f. tenuirostris* lineage occurred in samples from north-eastern Minas Gerais and southern Bahia. Haplotypes of the *X. f. fuscus* northern lineage only occurred in south-eastern Minas Gerais, southern Rio de Janeiro and north-eastern São

Figure 2. Haplotype network of *Xiphorhynchus fuscus* based on 575 bp of the control region. The size of each figure is proportional to the frequency of the corresponding haplotype in the total sample. Haplotype identification: T1 to T7, *Xiphorhynchus fuscus tenuirostris* lineage; N1 to N15, *X. f. fuscus* northern lineage; S1 to S10, *X. f. fuscus* southern lineage.

Paulo; whereas sequences of the southern lineage occurred in all localities in southern and central São Paulo, Paraná, Santa Catarina, and Misiones. Based on coalescence principles, the TCS algorithm (Crandall & Templeton, 1996) selected the haplotype S4 (*X. f. fuscus* lineage S) as the most likely ancestral one. The phylogenetic analysis by NJ and ML resulted in similar topologies and recovered the same three main lineages. Thus, we only present the NJ tree with the bootstrap supports obtained by both methods (Fig. 3).

Mean $\pm$ SD Tamura & Nei (1993) distance between *X. pardalotus* and *X. fuscus* was $12.366 \pm 1.481\%$. The distance between *X. f. fuscus* and *X. f. tenuirostris* was $2.368 \pm 0.561\%$, and between *X. fuscus* lineages N and S was $1.493 \pm 0.400\%$. The net divergence between the two *X. f. fuscus* lineages was $1.228 \pm 0.432\%$, which corresponds to $73\,500 \pm 24\,500$ years of divergence. The low number of samples of *X. f. tenuirostris* did not allow us to estimate the net divergence between this clade and *X. f. fuscus*; thus, the estimated divergence time between these clades

($135\,000 \pm 32\,000$ years) was based on the mean distance between groups ($2.368 \pm 0.561\%$).

COALESCENCE SIMULATIONS

Sequences within *X. f. fuscus* grouped in two main lineages that were strongly associated with specific geographical regions, suggesting that each lineage possibly evolved in allopatry. Given that the divergence between lineages is low and that the stochastic nature of the coalescence process can produce many different genealogies, we applied a gene-tree population-tree analysis to test whether a single historical population could have originated these results. The *s*-value (nine) of the reconstructed genealogy was significantly lower than the values obtained by the simulated gene trees, regardless of the N_e used ($P < 0.01$); thus, the null model of a single population was rejected. Also, the empirical N_e was 52 000 and is compatible with the range of N_e (6000–100 000) used in the simulations.

Figure 3. Neighbour-joining (NJ) tree based on 575 bp of the control region of *Xiphorhynchus fuscus*. The numbers at nodes show NJ and maximum likelihood bootstrap values above 50%, respectively. *Xiphorhynchus pardalotus* was used to root the tree. Haplotype identification: T1 to T7, *Xiphorhynchus fuscus tenuirostris* lineage; N1 to N15, *X. f. fuscus* northern lineage; S1 to S10, *X. f. fuscus* southern lineage.

POPULATION GENETIC STRUCTURE

A global AMOVA indicated that 57.7% ($\Phi_{ST} = 0.577$, $P < 0.01$) of the genetic variation in the samples of *X. fuscus* is allocated among the geographical regions where the main lineages occur (north-eastern Minas Gerais plus southern Bahia, south-eastern Minas Gerais plus southern Rio de Janeiro, and south of the VPSR). A regional AMOVA without the samples from the *X. f. tenuirostris* lineage (north-eastern Minas Gerais and southern Bahia) was performed subdividing each main region in two subregions. The subregions of the first region are Minas Gerais and Rio de Janeiro, whereas the subregions of the second region are São Paulo and south of São Paulo. This analysis indicated that 30.84% ($P < 0.01$) of the genetic diversity within *X. f. fuscus* was found among regions, that 12.93% ($P < 0.01$) occurred among the subregions within regions, and that 56.23% ($P < 0.01$) was allocated within subregions. The Φ_{ST} of this analysis was 0.43.

RELATIONSHIPS BETWEEN GENETIC DISTANCES AND PREDICTOR VARIABLES

The partial regression analysis indicated that 40.5% of the variation in genetic distances can be predicted by the linear geographical distance among pairs of localities (partial regression coefficient = 0.585, $P < 0.01$) and the geographical location (partial regression coefficient = 0.251, $P < 0.01$).

HISTORICAL DEMOGRAPHY

The F_s value for *X. f. tenuirostris* is nonsignificant, suggesting that there was demographic stability (Table 2). The F_s values are significant and negative for both *X. f. fuscus* lineages, suggesting the presence of past demographic expansion. Because the R_2 test is more suitable for the analysis of small samples than the F_s test (Ramos-Onsins & Rozas, 2002), we analysed *X. f. tenuirostris* ($N = 12$) using the R_2 test. The result was nonsignificant ($R^2 = 0.1800$, $P = 0.6960$), indicating that this taxa presented past demographic stability. Based on the estimations of τ , the demographic expansions of the northern and southern lineages of *X. f. fuscus* started approximately 57 000 and 19 000 years ago, respectively.

DISCUSSION

PHYLOGEOGRAPHICAL STRUCTURE OF *X. FUSCUS* IN THE SOUTH-EASTERN ATLANTIC FOREST

The study of the control region of *X. fuscus* revealed a significant population genetic structure, which is in accordance with other Neotropical birds (Bates, 2000,

ON THE ORIGIN OF THE PHYLOGEOGRAPHIC STRUCTURE OF *X. FUSCUS*

The partial regression analysis suggested that both isolation by geographical distance and the history of vicariance (geographical location) were important in shaping the population genetic structure of *X. fuscus*. The coalescence simulations supported the idea that *X. f. fuscus* lineages evolved in two populations instead of in a single one. At least two main vicariant phenomena were detected: one that separated the two subspecies lineages and the other which resulted in the divergence between the two *X. f. fuscus* phylogroups. The latter divergence is focussed on below.

Evolution in isolation and secondary contact provides a possible explanation for the phylogeographical structure of *X. f. fuscus*. Two vicariant events could have separated the ancestral population: the geological episodes that formed the VPSR or natural forest fragmentation.

The VPSR is a 173 km long and up to 2 km deep graben that separates the Serra da Mantiqueira and the northern Serra do Mar (Petri & Fulfaro, 1983) (Fig. 1). It is a contact area for birds (i.e. *Lepidocolaptes squamatus* and *Lepidocolaptes falcinellus*, Silva & Straube, 1996; *Heliobletus contaminatus contaminatus* and *Heliobletus contaminatus camargoi*; Silva & Stotz, 1992) and marsupials (i.e. *Marmosops paulensis* and *Marmosops incanus*; Mustrangi & Patton, 1997), and it separates mitochondrial lineages of the gecko *G. darwinii* (Pellegrino *et al.*, 2005). Also, morphologically distinct populations of the passerine *Scytalopus speluncae* (Giovanni, 2005) come into contact in the same geographical sector where the two *X. f. fuscus* lineages meet. Silva & Straube (1996) postulated that the tectonic process that opened the valley and the consequent alteration of the forest cover could have been a significant vicariant event for forest species. Assuming that this proposition is correct, the two lineages of *X. f. fuscus* that meet near to the valley could have evolved in isolation after the formation of the valley and, when the conditions for dispersion improved, they came into contact. The predictions of this hypothesis are that populations at each side of the valley are differentiated, and that the separation time between the lineages is compatible with the valley's age. The first prediction is supported by morphological characters that diagnose each of the bird populations from Minas Gerais and from São Paulo (Albuquerque, 1996). The prediction regarding the age of the valley, however, is not accepted. According to Petri & Fulfaro (1983), the formation of the valley started in the Miocene-Pliocene (approximately 15 Mya) and lasted until the Early Pleistocene. The estimated divergence time of the two *X. f. fuscus* lineages was 50 000–100 000 years ago, which is too recent to match the

formation of the valley. Thus, our data do not support the formation of the current VPSR as the vicariant event that produced the two *X. f. fuscus* lineages.

Brown & Ab'Saber (1979) suggested that the forest cover along the VPSR was not discontinued in the last glacial period. Based on this hypothesis, Silva & Straube (1996) suggested that the putative forest fragmentation that resulted in the distribution pattern of *L. falcinellus* and *L. squamatus* could not be related to this historical climatic oscillation. Clapperton (1993), however, suggested that, in the last glacial maximum, there were two main forest refuges in this area: one in the northern Serra do Mar and the other in the Serra da Mantiqueira and part of the Serra do Espinhaço in Minas Gerais; and these refuges were separated by a grassland area coincident with the VPSR. Under the scenario of Clapperton (1993), the palaeoreference hypothesis could explain the diversification of clades in *X. f. fuscus*, and thus the current contact area of the *X. f. fuscus* lineages should be secondary (Moritz *et al.*, 2000). A prediction of this hypothesis is that clades involved in the secondary contact should exhibit evidence of range expansion (Hewit, 2000; Moritz *et al.*, 2000; Cheviron *et al.*, 2005) and concomitant demographic expansion. The demographic analysis supports this prediction for the two *X. f. fuscus* lineages. Furthermore, the contact area between *X. f. fuscus* lineages has a high proportion of derived haplotypes, especially from the northern lineage (i.e. haplotypes N1, N4, and N5; Table 1, Fig. 2), which is also compatible with population expansion and secondary contact (Templeton, Routman & Phillips, 1995).

According to Behling (1998, 2002), grassland dominated the southern and south-eastern Brazilian landscape during the Late Pleistocene, where diverse forest ecosystems exist today. The current southern limit of the Atlantic forest biome contacts the grasslands biome of southern Brazil at latitude 28–27°S in the north of Rio Grande do Sul, and Behling (2002) concluded that, during the Late Pleistocene, this limit extended 750 km northwards, at least to latitude 20°S (southern Minas Gerais and northern São Paulo). Behling (2002) also proposed that the modern forest cover of south and south-east Brazil was established only in the Late Holocene. Under this historic scenario of dynamic forest cover, it is expected that forest dependent organisms presented strong demographic expansion in response to the forest advance in the Late Holocene, especially in regions southwards to the Late Pleistocene northern limit of grassland. Thus, we suggest that the demographic expansion of *X. f. fuscus* lineages is related to their geographical expansion that followed the forest advance in the Holocene, as this is compatible with the estimated expansion dates. The southern lineage possibly expanded from the Serra do Mar mountain range or from forest refuges along the Paraná river (Ledru

et al., 1998). On the other hand, *X. f. tenuirostris* presented no demographic expansion, possibly because their forest habitat was less affected by Pleistocene climatic alterations (Behling, 1998, 2002).

The hypothesis of refuges, the influence of geography, and river barriers are among the most discussed models in the study of Neotropical diversification. In conclusion, our data did not support the primary influence of geographical barriers (VPSR) or rivers in the divergence between the two main mitochondrial lineages of *X. f. fuscus* of the south-eastern Atlantic forest. Instead, the data supported the influence of isolation by geographical distance, recent vicariance events, and demographic expansions in shaping the phylogeographical structure of *X. fuscus*. These vicariance and expansions events appear to be related to recent natural forest landscape dynamics. There are more than 1000 bird species in the Atlantic forest, many of them distributed along the same geographical range of *X. fuscus* and with comparable ecological requirements. It is plausible that the same forest dynamics that appear to have modelled the phylogeographical structure of *X. fuscus* could have also affected other forest-dependent birds. More studies are needed to understand the importance of those evolutionary and demographic factors in shaping the Atlantic forest biota.

CONSERVATION IMPLICATIONS

The present study has revealed a strong phylogeographical structure in *X. f. fuscus* that defined two divergent populations in different geographical areas with dissimilar histories in the southern Atlantic forest: (1) south-eastern Minas Gerais plus southern Rio de Janeiro and (2) São Paulo plus southwards regions. As indicated by AMOVA, genetic differences between the two regions explained approximately 31% of the mitochondrial variation of *X. f. fuscus*. Unfortunately, current taxonomy does not recognize this diversity within *X. f. fuscus*, and even though the taxon is not threatened, this data is important for local conservation. It is possible that other forest birds present a similar condition. Thus, the importance of the characterization of the distribution of the genetic diversity of threatened species is further reinforced by the present data, as divergences not yet detected may be ignored in their conservation plans.

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Anexo XVII

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Nuclear and mitochondrial phylogeography of the Atlantic forest endemic *Xiphorhynchus fuscus* (Aves: Dendrocolaptidae): Biogeography and systematics implications

Gustavo S. Cabanne^{a,*}, Fernando M. d'Horta^a, Eloisa H.R. Sari^{b,c}, Fabrício R. Santos^b, Cristina Y. Miyaki^a

^a Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, 05508-090 São Paulo, SP, Brazil

^b Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil

^c Department of Biology, University of Missouri-St. Louis, 223 Research Building, Saint Louis, MO 63121-4499, USA

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ABSTRACT

We studied the intraspecific evolutionary history of the South American Atlantic forest endemic *Xiphorhynchus fuscus* (Aves: Dendrocolaptidae) to address questions such as: Was the diversification of this bird's populations associated to areas of avian endemism? Which models of speciation (i.e., refuges, river as barriers or geotectonism) explain the diversification within *X. fuscus*? Does the genetic data support subspecies as independent evolutionary units (species)? We used mitochondrial ($n = 34$) and nuclear ($n = 68$) DNA sequences of *X. fuscus* to study temporal and spatial relationships within and between populations. We described four main monophyletic lineages that diverged during the Pleistocene. The subspecies taxonomy did not match all the evolutionary lineages; subspecies *atlanticus* was the only one that represented a monophyletic and isolated lineage. The distribution of these lineages coincided with some areas of endemism for passerines, suggesting that those areas could be regions of biotic differentiation. The ancestor of *X. fuscus* diverged ~3 million years ago from Amazonian taxa and the phylogeographic pattern suggested that *X. fuscus* radiated from northeastern Brazil. Neither the riverine nor the geotectonic vicariance models are supported as the primary cause for diversification of geographic lineages, but rainforest contractions and expansions (ecological vicariance) can explain most of the spatial divergence observed in this species. Finally, analyses of gene flow and divergence time estimates suggest that the endangered subspecies *atlanticus* (from northeastern Brazil) can be considered a full species under the general lineage species concept.

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1. Introduction

The Atlantic forest (AF) (Fig. 1A) is the second richest Neotropical biome and one of the most endangered in the world (Galindo Leal and Câmara, 2003; Myers et al., 2000; Tabarelli et al., 2005). According to a model of forest stability during the last 21,000 years (Carnaval and Moritz, 2008) and to geological and palinological studies (i.e., Böhling, 2002; Böhling and Negrelle, 2001; Brown, 2005; Brown and Ab'Saber, 1979; Ledru et al., 2005, 1998), three historically different regions can be defined in the AF (Fig. 1B): (i) northern AF (NAF), with high forest stability in the eastern coastal region and short periods of rainforest expansions in areas currently covered by a type of dry forest (caatingas) (Veloso, 1991); (ii) central region (CAF), characterized by stability in the east and short periods of instability in the west and (iii) southern AF (SAF), distinguished by strong instability (regression and fragmentation and expansion at the mid and late Holocene).

According to the ecological vicariance model of diversification instability of forests can be important for speciation (reviewed in Marroig and Cerqueira, 1997; Moritz et al., 2000). Rainforest taxa may evolve in allopatry within rainforest relicts (refuges) that are caused and isolated by expansion of open areas (i.e., grasslands, savannas or dry forest). Predominant evolutionary forces are drift and divergent selection. In the Neotropics, this model was initially known as the theory of the Pleistocene forest refuges (Brown and Ab'Saber, 1979; Haffer, 1969; Haffer and Prance, 2001; Vanzolini and Williams, 1970). Specifically, the refuges' theory considers that speciation was driven by forest fragmentation that occurred during the maximum of the global Pleistocene glaciations. However, the ecological vicariance model may also be considered for interglacial periods, such as the Holocene. For example, caatingas currently cover most of northeastern Brazil, from Ceará to northern Minas Gerais (Veloso, 1991), and rainforest organisms are restricted to small rainforest relicts (brejos), or to coastal forests, which are likely to be remnants of a past continuous rainforest that was fragmented with the advance of caatingas (Ab'Saber, 1977; Carnaval and Bates, 2007; Carnaval and Moritz, 2008; Oliveira et al., 1999;

* Corresponding author. Fax: +55 11 3091 7553.

E-mail address: gscabanne@yahoo.com (G.S. Cabanne).

Fig. 1. Distribution of the Atlantic forest (AF), endemic bird areas, sampling locations and distribution of *Xiphorhynchus fuscus* and its subspecies. (A) Distribution of AF biome. NAF, northern AF; CAF, central AF; SAF, southern AF. (B) Historically stable areas for the AF during the last 21,000 years. Modified from Carnaval and Moritz (2008). The darker the area the higher the forest stability; i.e., black areas present the maximum likelihood to have maintained forest. The circle indicates the area of highest forest stability (Bahia refuge). (C) Areas of endemism of birds in the AF. SMC, Serra do Mar Center; PRC, Paraná Center; AAP, Atlantic slope of Alagoas and Pernambuco; DB, deciduous forest of Bahia; DMGG, deciduous forest of Minas Gerais and Goiás; AL, AF lowlands; AM, AF Mountains; Pe, Pernambuco; CB, Central Bahia; CoB, Coastal Bahia; SM, Serra do Mar. (D) Approximate distribution of *X. fuscus* (gray line) and approximate core distribution of subspecies shown by gray areas (Ridgely and Tudor, 1996; Marantz et al., 2003; Cabanne unpublished). Collection localities indicated by numbers (see Appendix B for details). States, PI, Piauí; CE, Ceará; RN, Rio Grande do Norte; PA, Paraíba; AL, Alagoas; PE, Pernambuco; BA, Bahia; ES, Espírito Santo; MG, Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul; Mi, Misiones (Argentina).

Wang et al., 2004). Therefore, these *brejos* are today's forest refuges and isolation by dry landscapes could have contributed to the evolution of the noteworthy endemic biota that characterizes the NAF (Silva and Casteleti, 2003).

The riverine barriers model of diversification (reviewed in Marroig and Cerqueira, 1997; Moritz et al., 2000) has also received attention to explain AF evolution. Some AF rivers limit regional faunas and therefore they may represent gene flow barriers and be important to model the biota distribution. This is the case of Doce river, which limits the range of many birds (Silva et al., 2004), mammals (Costa et al., 2000) and butterflies (Brown, 2005). Also, Lacerda et al. (2007) presented genetic data that suggests a role of the Jequitinhonha river for separating populations of the passerine *Thamnophilus ambiguus*. Finally, Pellegrino et al. (2005) found that some phylogroups of the gecko *Gymnodactylus darwini* are endemic to specific inter basins and proposed that rivers played an important role in the diversification of the AF biota.

The late Tertiary and early Pleistocene geotectonic activity associated to the formation and remodeling of geological landmarks of SAF (i.e., Serra do Mar mountain range) (Petri and Fulfaro, 1983; Riccomini et al., 1989) was suggested to be important for the evolution of the AF biota (Grazziotin et al., 2006; Lara and Patton, 2000; Leite, 2003; Mustrangi and Patton, 1997; Silva and Straube, 1996). For example, Silva and Straube (1996) observed that the range of some passerines were apparently limited by the geologic valley of the Paraíba do Sul river (Fig. 1) and proposed that the tectonic activity that opened the valley was important to split popu-

lations. However, our previous study of the mitochondrial DNA (mtDNA) of the passerine *Xiphorhynchus fuscus* did not support this geotectonic valley as a primary barrier (Cabanne et al., 2007).

The aforementioned hypotheses try to explain the origin of a highly diverse and heterogeneous AF (Silva and Casteleti, 2003). AF birds are good models for addressing these hypotheses given their high level of endemism (199 endemics, Stotz et al., 1996) and also because three different sets of endemic bird areas have been proposed (Fig. 1C). Areas of endemism are important to generate hypotheses about the history of geographical units and their biotas (Cracraft, 1985). These areas may be common regions of biotic differentiation (historical entities of a biome, Cracraft, 1985; Haffer, 1985), representing areas with past stability (refuges), regions isolated by rivers or geotectonic events, areas with different types of forest that define divergent selection, etc. The three groups of AF endemic bird areas are not totally congruent (Fig. 1C) and it is not known which one could best represent the AF biogeographic history. Regardless the process that lead to diversification of diagnostic taxa of each area of endemism, if areas of endemism were common regions of biotic differentiation, monophyletic populations from widespread and humid forest taxa are expected to be associated to those areas. And, phylogenetic relationship among these populations can help to understand the evolution of the areas' biotas. Alternatively, if areas of endemism only represent fortuitous assemblages of taxa without any historical significance, monophyletic lineages of widespread rainforest taxa should not be associated to these areas.

Fig. 2. Maximum likelihood (ML) tree based on 2471 bp of the cytb, ND2 and ND3 (mtDNA, dataset 1). Model: Tamura and Nei (1993) + I ($I = 0.6036$) + G ($\alpha = 2.1493$). Estimated base frequencies: A = 0.32, C = 0.33, G = 0.11, T = 0.24. Bootstrap values (percentage of 500 replicates) at the nodes: ML/maximum parsimony. The maximum parsimony analysis resulted in eight most parsimonious trees with 1807 steps.

Xiphorhynchus fuscus (Aves: Dendrocolaptidae) is a good model for biogeographic studies because it occurs in well preserved forest of most of the AF's range, from sea level up to 1200 m a.s.l. (Ridgely and Tudor, 1996) (Fig. 1D). Four subspecies (*atlanticus*, *tenuirostris*, *brevirostris* and *fuscus*) are described according to plumage variation and overall body size (Fig. 1D) (reviewed in Marantz et al., 2003). It is not clear if these subspecies represent real natural entities as no obvious diagnosis exist when series of specimens were compared, except for *atlanticus* (Cabanne, unpublished; Marantz et al., 2003). Subspecies *atlanticus* inhabits the most endangered forests of the biome (Galindo Leal and Câmara, 2003): coastal NAF, north to the São Francisco river and some of the brejos of Ceará (Baturité and Ibiapaba) (Fig. 1D). Those forests are naturally isolated from southern portions of the biome by caatingas and therefore we hypothesize that *atlanticus* is genetically isolated. The subspecies *atlanticus* is diagnosed by plumage, overall size and song (Cabanne, unpublished; Marantz et al., 2003) and since it is endangered (IBAMA, 2003), a genetic analysis is a priority study to help defining its evolutionary and taxonomic status.

We have previously described the mtDNA phylogeographic structure of *X. fuscus* at SAF (Cabanne et al., 2007). Now, our main goal is to study *X. fuscus* populations in the entire AF to address the following questions: (i) is the mtDNA phylogeographic pattern congruent with the nuclear DNA pattern? (ii) Was the intraspecific diversification of *X. fuscus* associated to any area of avian endemism? Which are those areas? (iii) Does the genetic structure of *X. fuscus* support the forest stability model of Fig. 1B? (iv) Which models of speciation (i.e., refuges, river as barrier, etc) can explain the diversification within *X. fuscus*? and (v) Does the genetic data support subspecies as independent evolutionary units (species)? In order to achieve these goals and to test predictions for some models of diversification (reviewed in Lara et al., 2005; Marroig and Cerqueira, 1997; Moritz et al., 2000; Patton and da Silva, 2005), we first analyzed the monophyly of *X. fuscus* based on mtDNA and then addressed evolutionary and spatial relationships

among the *X. fuscus*' lineages by using mtDNA and nuclear sequences.

Fig. 3. Median joining networks based on (A) mitochondrial DNA of *X. fuscus* (2499 bp of concatenated cytb, ND2 and ND3, dataset 2, $n = 34$ sequences) and on (B) FIB5 of *X. fuscus* (547 bp, dataset 2, $n = 68$ sequences). Number of aminoacidic changes are shown. See Table 1 for summary statistics of all the mtDNA and FIB5 dataset.

2. Materials and methods

2.1. Samples and sequences for the monophyly study (dataset 1)

We followed the taxonomic arrangement of Marantz et al. (2003). We tested the monophyly of *X. fuscus* using 29 terminals: 10 *X. fuscus*, 16 other taxa of the genus *Xiphorhynchus*, one *Lepidocolaptes angustirostris*, one *Campiloramphus trochilirostris* and, to root the phylogeny, *Sittasomus griseicapillus*. The sequences for this dataset were: ~1000 bp of cytochrome *b* (*cytb*), ~1000 bp of NADH dehydrogenase subunit 2 (*ND2*) and ~450 bp of NADH dehydrogenase subunit 3 (*ND3*). Some sequences other than of *X. fuscus* were obtained from GenBank (Aleixo, 2002). See Appendices A and B for details of the origin of sequences.

2.2. Samples and sequences for the intraspecific study (dataset 2)

For the intraspecific phylogeny we used 34 samples of *X. fuscus* collected in 22 localities from all over the species' range (Fig. 1D and Appendix B) and six samples of closely related outgroups (*X. spixii*, *X. ocellatus* and *X. pardalotus*). *X. guttatus* was used to root the intraspecific tree. This group of samples, without the outgroup taxa, was also used to obtain the sequence networks and for the population genetic analyses (Section 2.6). The DNA sequences obtained were: mtDNA (same genes used for dataset 1) and 547 bp of the intron 5 of the β -fibrinogen gene (*FIB5*). See Appendices A and B for details on origin of the samples, type of tissue, GenBank access, etc. From some specimens of *X. fuscus* we only collected blood because the species was either locally not abundant or endangered (i.e., subspecies *atlanticus*).

2.3. DNA sequencing, neutrality tests and recombination

Total DNA was obtained from blood or muscle following Bruford et al. (1992). The following primers were used for amplification and sequencing: for *cytb*—primers L14841 and H16065 (Lougheed et al., 2000), for *ND2*—primers LMET (Ribas et al., 2005) and H6313 (Sorenson et al., 1999), for *ND3*—primers L10755 and H11151 (Chesser, 1999) and for *FIB5*—primers *FIB5* and *FIB6* (Marini and Hackett, 2002). The sequences were obtained in sequencers ABI Prism 377 (Applied Biosystems) or MegaBACE 1000 (Amersham-Biosciences).

To identify haplotypes of heterozygous we used a Bayesian approach implemented in the program PHASE 2.0 (Stephens and Donnelly, 2003; Stephens et al., 2001). Heterozygous nucleotide positions were identified by double peaks in the electropherograms. Heterozygous indels positions were identified by an abrupt transition in the electropherogram from clean to unintelligible or a series of double peaks. All resolved positions received a posterior probability of one in the phasing analyses.

We used the PHI test in the program SPLITSTREE (Breen et al., 2006; Huson and Bryant, 2006) to check for recombination in the *FIB5*. The PHI test is powerful for detecting recombination and has been shown to be less sensitive to recurrent mutation and homoplasy than other traditional tests (i.e., Hudson and Kaplan, 1985). The neutrality tests of Tajima (1989) and of McDonald and Kreitman (1991) (MK test) were performed in DNASP 4.0. The MK test used *X. spixii* as outgroup.

2.4. Phylogenetic and genealogical analyses

Two different phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) approaches. Firstly, we evaluated whether *X. fuscus* was monophyletic using mtDNA sequences (dataset 1). Secondly, we studied relationships among the intraspecific *X. fuscus* lineages using a to-

tal evidence approach (concatenated dataset 2, mtDNA plus *FIB5*). Only for the second phylogeny, polymorphic positions in *FIB5* were coded using the IUPAC ambiguity codes.

MP heuristic searches were performed in PAUP' 4.0 b10 (Swofford, 2001) with 100 replicates of random stepwise addition and a limit of 1,000,000 rearrangements of TBR branch swapping per replicate. We summarized MP results in a majority rule consensus tree. ML heuristic searches were performed in PHYML 2.4.4. (Guindon and Gascuel, 2003). The likelihood-ratio test was used as implemented in MODELTEST 3.7 (Posada and Crandall, 1998) to select the best-fit model of molecular evolution to be used in ML analyses. We did 500 MP bootstrap replicates in PAUP' and 500 ML bootstrap replicates in PHYML. The partition homogeneity test (Farris et al., 1994) was performed in PAUP' using 1000 replicates and tested for incongruence between mtDNA and *FIB5* data.

We also explored relationships among haplotypes of dataset 2 using median joining networks (Bandelt et al., 1999) obtained in NETWORK 4.1.0.8 (<http://www.fluxus-engineering.com>). These analyses included all *X. fuscus* ($n = 34$) and one outgroup (*X. spixii*). Summary statistics for sequences were obtained using ARLEQUIN 3.1 (Excoffier et al., 2006).

2.5. Shimodaira and Hasegawa (1999) test

We used the Shimodaira and Hasegawa (1999) test (SH test) to assess whether a specific phylogenetic tree obtained in a constrained heuristic search and a non-constrained tree are equally good explanations of the data (Goldman et al., 2000). The constraints for the heuristic searches were derived from the three set of areas of endemism for AF's birds (Fig. 1C). If areas of endemism were historical units in the evolution of *X. fuscus* (i.e., refuges), we predicted to find reciprocally monophyletic populations in each of them. Thus, constraints forced monophyly (backbone option) of samples associated to each area of endemism depicted in Fig. 1C. Constraints did not force any specific relationship among areas, except in one specific case for the areas of Silva et al. (2004) [-(PE, ((CB, SM), COB))]- see areas in Fig. 1]. Backbones only included samples collected in localities mapped within areas of endemism. Appendix B shows the assignment of samples to corresponding areas. Because *X. fuscus atlanticus* is relatively differentiated from the other subspecies, we also used the SH analysis to test the null hypothesis of paraphyly of *X. fuscus*, with *atlanticus* forced to group with other species of *Xiphorhynchus*. SH tests were performed in PAUP' 4.0 b10 using the intraspecific phylogeny of *X. fuscus* obtained with dataset 2 (see Fig. 4), and applying the resampling estimated log-likelihood (RELL) option and 1000 replicates of non-parametric bootstrap to obtain the distribution of the statistics of the test.

2.6. Population analyses, divergence times and gene flow

We used the likelihood-ratio test, as implemented in MODELTEST, to test the null hypothesis of constancy of evolutionary rates. This was done by comparing the log-likelihood values of maximum likelihood trees with and without enforcing a molecular clock.

Rates of nucleotide change (sensu Ho et al., 2005) show variation depending on the date used to calibrate it (age of calibration); the relationship is not linear because rates tend to be faster when calibration points are recent. Ho et al. (2005) analyzed several calibrations of mtDNA molecular clock rates and developed a mathematical approach to directly relate DNA sequence distances to divergence dates. To obtain divergence dates (in million years, MYr) from mtDNA distances, we used the model for protein-coding sequences of the avian mtDNA and resolved numerically the Eq. (7) of the Ho et al.'s paper. To approximate a mtDNA rate of change to be used for the isolation migration analyses, we transformed the

Fig. 4. Phylogenetic relationships, genetic diversity and demography of intraspecific lineages of *X. fuscus*. (A) Maximum likelihood (ML) tree based on a total evidence analysis of mtDNA and FIB5 (dataset 2, total alignment of 3046 bp, cytb, ND2, ND3 and FIB5). Model of evolution: Tamura and Nei (1993) + I (I = 0.501) + G (α = 0.8132). Estimated base frequencies: A = 0.31, C = 0.29, G = 0.13, T = 0.27. Bootstrap values (percentage of 500 replicates) at the nodes: ML/maximum parsimony. The maximum parsimony tree had 639 steps. Numbers within parentheses indicate sampling locality (see Fig. 1 and Appendix B). (B) Distribution of the main lineages of *X. fuscus*. Based on the present work and in Cabanne et al. (2007). (C) Estimation of genetic diversity (Θ) and demographic signature (g) of samples of *X. fuscus*.

observed mtDNA divergence (*p*-distance) to absolute time by the aforementioned method and then related both distance and time to obtain a rate of change. The mtDNA calibrations that resulted after applying this method were 2.46% of divergence per MYr for the study of the divergence between AF and the Amazon forest (dataset 1) and 4–4.6% per MYr for the population study (dataset 2). The substitution rate adopted for FIB5 was 0.72% divergence/MYr, according to a specific calibration for other intron in chicken (see Axelsson et al., 2004).

We used the isolation–migration (IM) model (Hey and Nielsen, 2004; Nielsen and Wakeley, 2001), implemented in the program IM (ver. April 21, 2008), to estimate gene flow rates and final divergence times between lineages of *X. fuscus*. IM estimates the effective population size parameters of two populations (θ_1 and θ_2 , theta per marker) that diverged from an ancestral population (θ_a) time ago in the presence of gene flow (*m*). Units of analysis (Table 1) were selected according to specific phylogenetic clades with geographic and or morphological correspondence, and not strictly following the subspecies taxonomy. For all analyses we used the complete se-

quences of mtDNA and FIB5 together (dataset 2). We used an inheritance scale of 0.25 for the mtDNA, the HKY model of evolution and assumed $\theta_1 = \theta_2 = \theta_a$ and $m_1 = m_2$. For testing whether a model of isolation without gene flow fitted to the data better than a model with gene flow, we repeated the former analyses constraining $m = 0$ and used the Akaike information criterion to compare models according to Nielsen and Wakeley (2001). For each analysis, we measured the distribution of the average time of migration events. Inspecting the posterior distribution of migration times allows a qualitative evaluation of the gene flow scenario. A high concentration of migration events near the present is consistent with secondary contact and posterior gene flow after allopatric divergence, while a broad distribution of migration times is consistent with recurrent gene flow since the time of population splitting (Niemiller et al., 2008; Won and Hey, 2005). We made preliminary runs to adjust priors and at least five subsequent analyses with different starting points to check for parameter convergence. We used 500,000 iterations for burn-in and analyses were stopped when the smallest effective sample sizes (ESS) were higher than 50 and parameter

Table 1
Summary statistics of the mtDNA (concatenated cytb, ND2 and ND3) and FIB5 sequences of the complete dataset and intraspecific lineages of *X. fuscus*

<i>X. fuscus</i> , complete dataset 2		Lineages (geographic region)							
		atlanticus (NAF)		brevirostris/tenuirostris (CAF)		fuscus N (SAF)		fuscus S (SAF)	
		mtDNA	FIB5	mtDNA	FIB5	mtDNA	FIB5	mtDNA	FIB5
N	34	68	10	20	11	22	8	16	5
K	23	28	6	8	8	15	6	7	3
π%	1.51	0.693	0.0961	0.612	0.2778	0.735	0.0837	0.61	0.1436
D	1.019 ^{NS}	-1.24 ^{NS}	-0.856 ^{NS}	0.841 ^{NS}	-0.541 ^{NS}	-0.977 ^{NS}	-0.632 ^{NS}	0.039 ^{NS}	-0.807 ^{NS}

N, number of sequences; K, number of haplotypes; π%, nucleotide diversity (Nei and Kumar, 2000) in percentage based on *p*-distance; D, Tajima's D (1989). NS, non-significant (*P* > 0.05). NAF, northern AF; CAF, central AF; SAF, southern AF.

trend lines stabilized after four to 30 million iterations. For parameters in which the complete posterior distribution was completely estimated, we presented the highest posterior density interval. We transformed the time parameter t into time in years using the formula $t = t/u$, where u is the geometric mean of mutation rates per marker and generation (Hey and Nielsen, 2004), and the migration parameter m into the effective number of diploid individuals migrants per generation using the formula $M = (\theta m)/4$. We assumed a generation time of 1 year for *X. fuscus*.

To estimate migration rates and genetic diversity (θ , theta per site) for each independent marker (mtDNA and FIB5) and to further evaluate recombination at the FIB5 we used the maximum likelihood coalescent method implemented in LAMARC 2.1.2b (Kuhner, 2006). Unlike IM, LAMARC assumes migration equilibrium. In order to compare mtDNA and nuclear (FIB5) migration, we corrected the difference between the markers' effective size by applying an mtDNA effective size scalar equal to 4. We also used LAMARC to evaluate the demographic signature of *X. fuscus*' lineages by estimating the exponential population growth rate g ($\theta_t = \theta_{now}e^{-gt}$, where θ_{now} is the current θ and θ_t is the value of the parameter t time ago) using mtDNA and FIB5 together. Positive g -values indicate population growth and negative values, population decline. All final LAMARC analyses used the F84 model of sequence evolution with empirical base frequencies and transition/transversion ratios and ran 10 short chains (500 sampled trees every 50 iterations and burn-in of 1000 trees) followed by two long chains (20,000 sampled trees every 50 iterations and burn-in of 1000 trees).

Finally, we compared the migration rates obtained by IM and LAMARC with those obtained by assuming the island model of Wright (1940). Global Φ_{st} and its 95% bootstrap confidence interval (c.i. 20,000 replicates) for each marker were obtained by AMOVA (Excoffier et al., 1992) in ARLEQUIN and gene flow estimations were obtained using the formula $M = (1 - \Phi_{st})/2\Phi_{st}$ (Hedrick, 2000).

3. Results

3.1. Molecular variation

A mtDNA sequence matrix of 2471 bp [dataset 1: 785 variable positions, 522 (66.5%) informative for parsimony] was obtained for the *X. fuscus* monophyly study. The alignment for the total evidence intraspecific study resulted in 3047 bp [dataset 2: 2499 bp of mtDNA: 445 variable positions, 303 (68%) informative for parsimony and 547 bp of FIB5: 37 variable positions, 23 (62.1%) informative for parsimony]. Summary statistics for all the *X. fuscus*' sequences (dataset 2) are presented in Table 1. The MK test for the *X. fuscus*' mtDNA (2499 bp, $n = 34$) was marginally significant (G -test with William's correction, $P = 0.046$) due to the existence of an excess of non-synonymous polymorphism. This result is expected for mtDNA (Nachman, 1998) and since Tajima's D -test (Tajima, 1989) was non-significant (Table 1) we interpreted this pattern as a result of demographic history. FIB5 sequences of *X. fuscus* did not present evidence of recombination, according to the PHI test ($P = 0.848$) and LAMARC (recombination parameter $r < 0.0001$).

3.2. Phylogenetic and genealogical analyses

The topologies obtained by MP and ML were similar, and only ML trees are shown. *X. fuscus* samples grouped in a well supported monophyletic clade in the mtDNA analysis (Fig. 2). Subspecies *atlanticus* was monophyletic, and the other three subspecies formed another monophyletic clade, with *tenuirostris* and *brevirostris* grouped in one well supported lineage, and *fuscus* forming two separate lineages. The median joining network of *X. fuscus* mtDNA (Fig. 3A) resulted in four main lineages congruent with the phylogenetic analysis of Fig. 2 and confirmed that *atlanticus* is monophy-

letic and the most divergent lineage. FIB5 network did not corroborate all the mtDNA lineages (Fig. 3B). The total evidence topology (mtDNA + FIB5, Fig. 4A) did not differ from the one obtained with mtDNA alone (Fig. 2) and was in accordance with the mtDNA network (Fig. 3). The null hypothesis of *X. fuscus* paraphyly was rejected by the SH test (Table 2, line 1). The incongruence between mtDNA and FIB5 suggested by the networks (Fig. 3) was confirmed by the partition homogeneity test ($P = 0.005$). This result was expected because the dataset have a high number of sequences from the same species, and it is not surprising to find incongruence between loci at the intraspecific level, especially when one of the markers (FIB5) presents higher effective size and lower evolutionary rate. This was confirmed when the number of *X. fuscus* sequences in dataset 2 was reduced and the partition homogeneity test was not significant ($P > 0.01$), indicating congruence between mtDNA and FIB5 among the species of *Xiphorhynchus* analyzed (data not shown).

The SH tests showed that monophyletic intraspecific lineages of *X. fuscus* were only associated to some of the areas endemism of Silva et al. (2004) ($P > 0.01$, Table 2), rejecting the hypotheses that the other areas of endemism would represent centers of diversification.

In summary, four well supported main lineages with geographic correspondence were revealed by both mtDNA only (dataset 1, Fig. 2) and combined markers (dataset 2, Fig. 4): *atlanticus*, the clade of *tenuirostris* and *brevirostris*, and the two lineages of *fuscus* (N and S, for north and south). These four lineages, or subsamples of them, were used as units of analysis for the population genetic studies (Table 1).

3.3. Mitochondrial genetic distances

The null assumption of constancy of molecular evolution rates for the mtDNA phylogeny data (dataset 1) was not rejected ($P > 0.05$). The mtDNA corrected genetic distance between *X. fuscus* and its sister clade containing the Amazonian *X. spixii*, *X. elegans*, *X. ocellatus* and *X. pardalotus* (Fig. 2), was 0.0836 (SD = 0.006) and according to the method of Ho et al. (2005) the distance corresponded to 3.07 (SD = 0.13) MYr of divergence. Table 3 presents uncorrected mtDNA distances between intraspecific lineages of *X. fuscus* (dataset 2).

3.4. Population divergence, gene flow and demography

Divergence among *X. fuscus*' lineages obtained by IM ranged from 0.4 to 0.8 MYr (Table 4). The Akaike information test rejected the model of divergence without gene flow for all the comparisons (data not shown). Migration rates obtained by IM were slightly lower than those obtained by LAMARC. However, both methods showed a similar scenario. In general, migration rates between *atlanticus* and any other lineage, and between Ceará and Alagoas, were the lowest. Distribution of migration events obtained by IM peaked between 0.3 and 0.15 MYr and suggested that migration

Table 2
Results of Shimodaira and Hasegawa (1999) test based on a total evidence tree (Fig. 4) to evaluate the monophyly of *X. fuscus* and to test if monophyletic clades are associated to Atlantic forest endemic bird areas

Hypothesis	-lnL constrained tree	delta -lnL	IP
<i>X. fuscus</i> paraphyletic	7701.48898	52.53429	0.000
Cracraft (1985)	7696.28494	47.33024	0.000
Stattersfield et al. (1998)	7770.7244	113.26381	0.000
Silva et al. (2004)	7657.45863	8.50392	0.085
Silva et al. (2004) with hierarchy*	7664.90740	27.44877	0.018

-lnL of the unconstrained tree is 7648.95470.
* (PE, ((CB, SM), COB)), see areas in Fig. 1.

Table 3
Uncorrected genetic distances between intraspecific lineages of *X. fuscus* based on 2499 bp of the mtDNA (concatenated cytb, ND2 and ND3) above the diagonal and standard errors below the diagonal

	atlanticus (NAF)	brevirostris/tenuirostris (CAF)	fuscus N (SAF)	fuscus S (SAF)
atlanticus (NAF)		0.026	0.025	0.026
brevirostris/tenuirostris (CAF)	0.003		0.011	0.013
fuscus N (SAF)	0.003	0.002		0.012
fuscus S (SAF)	0.003	0.002	0.002	

Geographic regions indicated between parentheses.
NAF, northern Atlantic Forest; CAF, central Atlantic forest; SAF, southern Atlantic forest.

was not concentrated at the end of the divergence as would be expected in a model of complete allopatric divergence followed by a secondary contact, except for the pair *atlanticus* × *tenuirostris*/*brevirostris*, which also presented recent gene flow (Fig. 5).

The nuclear marker presented higher migration rates than the mitochondrial marker in all the comparisons with LAMARC (Table 4). However, when mtDNA migration estimations considered the difference between markers' effective size, the confiability intervals overlapped and therefore the disparities were not important. The population genetic structure among the four lineages of *X. fuscus* was smaller with the FIB5 than with the mtDNA ($\Phi_{stFIB5} = 0.147$, c.i.: 0.08–0.215, $P < 0.01$ and $\Phi_{stmtDNA} = 0.916$, c.i.: 0.88–0.93, $P < 0.01$). Based on the island model of Wright (1940), the overall M_{FIB5} was 2.9 (c.i.: 1.82–5.75) and the M_{mtDNA} was 0.045 (c.i.: 0.037–0.07). Migration rates obtained by the Wright (1940) island model are slightly higher from those obtained by the IM model (average $M = 0.39$, $n = 6$) but similar to those obtained for the FIB5 by LAMARC (average $M = 2.92$, $n = 6$).

The highest levels of genetic diversity (Θ -values) obtained in LAMARC were found at CAF, while Θ -values at NAF and SAF were similar (Fig. 4C). The exponential growth rates (g) indicated signatures of demographic growth in all the samples other than Ceará, which showed decline (Fig. 4C). Notwithstanding some confiability intervals of g included stability ($g = 0$), all the demographic tendencies were clear as stability was a very marginal situation.

Table 4
Population divergence times and migration between populations of *X. fuscus* based on the isolation-migration model of Hey and Nielsen (2004) and the model of LAMARC 2.0 (Kuhner, 2006)

Estimation	Divergence with gene flow, IM (90% c.i.)			Migration per marker, LAMARC (95% c.i.)		
	θ	t (MYr)	M	M_{FIB5}	M_{mtDNA}	$M_{mtDNA-4Ne}$
Inter lineages (AF region)						
1 [atlanticus] × [brevirostris/tenuirostris] (NAF × CAF)	12.31 (8.62–18.28)	0.47 (>0.25) ¹	0.15 (0.01–0.65)	1.25 (0.16–6)	0.08 (0.00–0.89)	0.33 (0.01–3)
2 [atlanticus] × [fuscus N] (NAF × SAF)	7.11 (4.64–11.37)	0.79 (>0.45) ¹	0.08 (0.00–0.39)	0.64 (0.06–3.0)	0.06 (0.00–0.75)	0.23 (0.01–3.57)
3 [atlanticus] × [fuscus S] (NAF × SAF)	6.58 (3.96–10.42)	0.79 (>0.44) ¹	0.03 (0.00–0.32)	0.46 (0.01–3.66)	0.06 (0.00–0.69)	0.24 (0.01–2.66)
4 [brevirostris/tenuirostris] × [fuscus N] (CAF × SAF)	11.55 (8.17–17.52)	0.43 (0.08–1.66)	0.65 (0.11–3.12)	6.18 (1.48–10.71)	0.14 (0.01–0.70)	0.58 (0.02–6.12)
5 [brevirostris/tenuirostris] × [fuscus S] (CAF × SAF)	12.77 (8.62–19.25)	0.44 (0.13–1.34)	0.30 (0.03–1.88)	4.44 (1.35–7.98)	0.14 (0.00–1.50)	0.55 (0.02–6)
6 [fuscus N] × [fuscus S] (SAF)	5.53 (3.30–8.94)	0.39 (>0.25) ¹	0.40 (0.04–2.58)	4.59 (0.8–16.8)	0.10 (0.00–0.91)	0.39 (0.01–5.09)
Intra lineages ^a						
7 atlanticus [CE] × [AL]	2.73 (1.31–5.16)	0.11 (>0.04) ¹	0.21 (0.00–1.78)	0.56 (0.02–5.25)	0.38 (0.01–5.40)	1.52 (0.02–21.6)
8 brevirostris/tenuirostris [CH] × [COB]	9.53 (5.96–16.22)	0.13 (0.05–0.61)	0.36 (0.00–5.67)	3.09 (0.57–16.2)	0.49 (0.02–6)	2 (0.08–24)

Confiability intervals (c.i.) are shown between parentheses. Divergence times (t) in million years (MYr) and migration (M) in effective number of individuals per generation. θ , theta value for all the markers in the IM model.

M , M_{FIB5} , M_{mtDNA} , migration rates for all the markers in the IM model, and for FIB5 and mtDNA in LAMARC, respectively. $M_{mtDNA-4Ne}$, migration value obtained with the mtDNA and forcing a fourfold effective size.

^a CE and AL, populations of *atlanticus* of Ceará and Alagoas, respectively (see Fig. 1D). CH and COB, populations of *brevirostris/tenuirostris* of the Chapada Diamantina range (see Fig. 1A) and of the coastal region of Bahia plus northeastern Minas Gerais, respectively. NAF, northern AF; CAF, central AF; SAF, southern AF.

¹ Maximum value of the interval not presented because the likelihood distribution was flat at the end.

Fig. 5. Distributions of migration events for each pairwise comparison between geographically contiguous lineages of *X. fuscus* obtained from the IM program (Won and Hey, 2005), averaged across markers.

4. Discussion

The analyses showed that *X. fuscus* is monophyletic (Figs. 2 and 4). Even though FIB5 and mtDNA sequences were congruent at the level of the genus *Xiphorhynchus*, FIB5 sequences did not evidence a strong phylogeographic structure as shown by the mtDNA (Fig. 3). This result is not contradictory because nuclear markers are less variable and have longer coalescence times than the mtDNA. Thus, and also because mtDNA patterns are robust indicators of population history (Zink and Barrowclough, 2008), we consider our results unbiased and reliable.

The differences between migration values obtained by LAMARC and IM were not unexpected. IM does not consider migration equilibrium and is able to distinguish between shared polymorphism originated by gene flow and incomplete lineage sorting (Nielsen and Wakeley, 2001; Pinho et al., 2008). On the other hand, both LAMARC and the F_{ST} -based methods may retrieve higher migration val-

ues because they interpret shared polymorphism as gene flow; thus we considered M-values obtained by those approaches as overestimations. A mtDNA phylogeographic pattern more structured than the FIB5 pattern, as observed in *X. fuscus*, may be caused by diminished female gene flow. However, LAMARC gene flow analyses did not support this scenario and therefore the incongruence between markers (Fig. 3) at the population level could be explained as consequence of the difference between the markers' effective sizes.

4.1. Congruence between genetic structure and subspecies

The subspecies taxonomy did not match all the evolutionary lineages of *X. fuscus* (Figs. 3 and 4). *X. f. atlanticus* was the only subspecies that represented a monophyletic and isolated lineage. A completely resolved relationship among all *X. fuscus* lineages was not obtained, possibly due to a rapid and recent diversification (Fig. 4). IM analyses indicated divergence times greater than zero between all the lineages (Table 4). These results, the geographic partition of lineages and the congruence of genetic and morphological differences between *atlanticus* and all the other lineages indicated altogether that the results evidenced organismal lineages rather than clades, which are consequence of stochastic coalescence of sequences (Dolman and Moritz, 2006; Irwin, 2002). Since the subspecies other than *atlanticus* lack an obvious diagnosis (genetic or morphological), a detailed study is needed to reevaluate the geographic variation of external characters and to understand the correspondence of such variation with the genetic pattern.

4.2. Contact between the Amazon and the Atlantic forest

Several evidences support a historical contact between the Amazon and the Atlantic forest (i.e., Bates et al., 1998; Behling et al., 2000; Costa, 2003; Oliveira et al., 1999; Silva, 1996; Wang et al., 2004) and our results provided additional data to better understand about this contact. The ancestral *X. fuscus* is likely to be Amazonian, as almost all species of *Xiphorhynchus* are restricted to this region or to Central America. The oldest split within *X. fuscus* separated NAF from other regions (Fig. 4 and Table 4) and the most basal FIB5 haplotype is from NAF (Fig. 3B), suggesting that the ancestor of *X. fuscus* arrived in the AF at northeastern Brazil, perhaps through extinct rainforests in the coast or in the currently dry inland. A southern dispersion scenario, for example through the Paraguay river basin (Nores, 1992), is less parsimonious as it implies a long distance dispersion from SAF towards NAF before the first intraspecific divergence occurred.

Few studies addressed the timing of the connection between the two forests. Most of the palinological and geological studies only cover the last 200,000 years and evidence several short periods of forest expansion that could have linked the two biomes (i.e., Behling et al., 2000; Oliveira et al., 1999; Wang et al., 2004). The divergence between *X. fuscus* and its Amazonian sister clade (~3 MYr) also suggests older connections (Pliocene), which is in accordance with other studies (i.e., Costa, 2003; Ribas and Miyaki, 2004). The radiation of the clade of *X. fuscus*, compared to its Amazonian sister clade, showed shorter branches (Fig. 2) and lower genetic distances among terminals (data not shown). This pattern suggests lower diversification rates in AF, and could be product of higher speciation rates in Amazon forest or higher extinction rates in AF (Ricklefs, 2007). More studies are necessary to address this issue.

4.3. Areas of endemism

Areas of endemism are important to generate hypotheses about the history of geographical units and their biotas. The areas of endemism for AF passerines described by Silva et al. (2004) were not rejected as regions of differentiation for *X. fuscus* (Table 2).

However, test significances were low because two of the propositions of Silva et al. (2004) were not supported: (i) coastal Bahia plus northern Espírito Santo as an independent area from inland Bahia plus part of northeastern Minas Gerais (Figs. 1C and 4) and (ii) the region south to the Doce river (area SM) as a homogeneous biogeographic unit. Since organisms can react idiosyncratically to specific biogeographic events (Prum, 1988) other patterns could emerge from other organisms. Notwithstanding our results are among the first steps for understanding the AF evolution, they suggest that the areas of Silva et al. (2004) may represent a good hypothesis about biogeographical units of the biome. However, only corroboration of our results with other taxa would show if these areas have a general biogeographic significance.

4.4. Intraspecific pattern of *X. fuscus* and its evolution: geotectonism and rivers

The subspecies *X. fuscus atlanticus* is differentiated and isolated in the NAF while the other lineages are connected by moderate migration rates. Interestingly, even though the ancestral *X. fuscus* seems to be pre-Pleistocene, the current lineages only diverged in a relatively short period at the mid-late Pleistocene (Table 4).

The proposition of Silva and Straube (1996) about the primary biogeographic role of the valley of the Paraíba do Sul river (Fig. 1D) was not supported for *X. fuscus* in our previous study (Cabanne et al., 2007). In that study we only analyzed mtDNA data and discussed whether the tectonic formation of the valley initiated the divergence of lineages *fuscus* N and S, which get into contact near the valley (Fig. 1D). Even though divergence times obtained in the present study (Table 4) are slightly older than the first estimates (probably because migration equilibrium was not assumed now) they confirm previous conclusions. The geotectonic valley is not supported as a primary barrier for the divergence between *X. fuscus* lineages because the valley's age (>15 MYr; Souza et al., 2005) does not match the divergence between lineages (~0.4 MYr) and because the lineages' contact is outside and perpendicular to the valley (Fig. 4). See Cabanne et al. (2007) for a detailed discussion on this issue.

Rivers and their valleys can be primary or secondary barriers to gene flow (Moritz et al., 2000; Patton and da Silva, 2005). A primary barrier is one that imposed itself on an existing taxon range and separated populations that became sister clades. A secondary barrier is the encounter point of clades that evolved elsewhere. A river can also act as a barrier when episodes of overwater dispersal occurred, which can be recognized when haplotypes from each bank present a paraphyletic relationship (see Patton and da Silva, 2005). Lineages *brevirostris/tenuirostris* and *fuscus* N meet at the lower portion of the Doce river in Espírito Santo (Fig. 4), thus the river could have been a significant barrier for gene flow. Assuming that the divergence time between *X. fuscus* and its Amazonian sister clade (Pliocene) is the earliest date of arrival of the ancestral *X. fuscus* at the AF and considering that the current fluvial system of eastern Brazil was already established at that period (Dominguez et al., 1987; Lundberg et al., 1998; Potter, 1997; Ribeiro, 2006), the Doce river appears to be a secondary barrier. The relationships among the three lineages at eastern Brazil are not well resolved (Fig. 4), possibly as a consequence of rapid diversification. This lack of resolution did not allow us to distinguish between a secondary contact and overwater dispersal (Patton and da Silva, 2005). However, the signal of population expansion for the lineages (Fig. 4C) that meet at the Doce river is congruent with a secondary barrier (Cheviron et al., 2005). An alternative view related to a sort of primary barrier effect of the Doce river proposes that birds could have dispersed along the river from its headwaters in the Espinhaço mountain range in central Minas Gerais and reached the mouth of the stream. Later, downstream populations in each

margin may have diverged due to the isolation by the river. This model is compatible with the population expansion signals that we have found (this work and Cabanne et al., 2007). However, it implies that the headwaters of the Doce river are not an effective barrier and we would expect to find at the springs intermediary haplotypes between the two lineages currently separated by the river. This prediction was not corroborated by the mtDNA data since the lineage *fuscus* N is found at the two margins of the river in its springs (Fig. 4 and Cabanne et al., 2007).

The São Francisco is the largest river in the study area and could have isolated populations of *X. fuscus*. However, this river runs mostly in the middle of a dry landscape (Caatinga) unsuitable for rainforest birds. Thus, it is difficult to detach the effect of the river from the effect of caatingas in the isolation of forest organisms. Also, the São Francisco river is ancient and runs over the tectonically stable Brazilian shield (Lundberg et al., 1998; Potter, 1997; Ribeiro, 2006), thus if it acted as barrier it should be secondary.

4.5. Evolution of the intraspecific pattern of *X. fuscus*: ecological vicariance

Part of the controversy related to the evolution in refuges lies on whether past climatic cycles fragmented forests significantly (i.e., Colinvaux et al., 2000). Main predictions for the hypothesis of refuges are demographic expansions and secondary contacts of forest taxa (Moritz et al., 2000). According to these predictions and to the model of Fig. 1B, the following general pattern is expected: (i) low genetic diversity and signatures of stability and/or demographic decline in NAF, especially in interior regions where rainforest regressed during the Holocene; (ii) high genetic diversity and signature of stability or weak expansion in CAF (high forest stability) and (iii) low genetic diversity and strong signatures of demographic expansion in populations of SAF (low forest stability).

Overall genetic diversity levels are in accordance with the predictions mentioned (Fig. 4C). For both markers, the highest genetic diversity was found in CAF, suggesting that this region maintained a larger population of *X. fuscus* than the other regions (NAF and SAF). Despite the fact that SAF presents today (before human impact) a similar or even larger range of rainforest than the CAF (Fig. 1A), the difference in genetic diversity levels between both regions suggest that SAF has had smaller populations of *X. fuscus* over the long term. This comparison showed the impact of the forest history on the genetic diversity of organisms. The demographic signatures suggest population growth for all the samples, except for Ceará where decline was evidenced (Fig. 4C). The decline at Ceará is compatible with the existence of a past large rainforest in the interior of the current Caatinga and thus supported the model depicted in Fig. 1B. The signature of expansion in Alagoas, the other sample from NAF, contrasts with the one of Ceará and with our expectations. Even though the coastal region of Alagoas and Pernambuco, known as the Pernambuco refugia (Carnaval and Moritz, 2008), is likely to have maintained forest over the long term (Fig. 1B), the results suggest that this refugia was smaller than the current distribution of rain forest in the region. A signature of demographic growth in CAF similar to the ones observed at SAF (lineages *fuscus* N and *fuscus* S) was not expected (Fig. 4C). However, even though sample sizes of each lineage other than *fuscus* S are relatively similar (Table 1), the confidence interval of the CAF *g*-value is smaller than the other intervals. This result may indicate a violation of the LAMARC model (constant growth) in regions other than CAF (M. Kuhner personal communication), which would be compatible with the notion of high forest instability at those regions. Fluctuation in the forest range and connectivity related to global glacial cycles could have alternated short periods of growth with periods of stability or population decline in NAF and in SAF, producing the ob-

served demographic patterns. Since CAF forest are likely to have been more stable during the late Pleistocene climatic cycles (Carnaval and Moritz, 2008), any demographic trend could have been maintained longer than in NAF and SAF. However, as the behavior of methods to study historical demography (e.g., LAMARC or the ones based on summary statistics, Ramos-Onsins and Rozas, 2002) are not well understood when the constant population trend is violated (M. Kuhner personal communication), our comparative discussion of the demography of *X. fuscus* should be interpreted with caution.

Secondary contacts also evidence demographic fluctuations compatible with refuges. Our previous study of *X. fuscus* mtDNA describes a secondary contact between lineages *fuscus* N and S along no evident barrier to gene flow in central São Paulo (Cabanne et al., 2007). However, the migration-time graphic did not evidence recent gene flow between these lineages (Fig. 5), perhaps because the contact is very recent or the analysis lacked statistical power. Interestingly, phylogeographic gaps in other vertebrates also occur in central and eastern São Paulo (Grazziotin et al., 2006; Muirangi and Patton, 1997; Pessoa et al., 2006), supporting the idea that common events affected a great proportion of the biota, as could have happened during past global climatic fluctuations. Event though populations are currently isolated, the migration-time graph showed recent gene flow for the pair *atlanticus*–*tenreiroi* (Fig. 5). Gene flow is also suggested by the FIBS network (Fig. 3), which showed one common haplotype shared between interior CAF (*brevirostris*) and NAF, a pattern likely to be originated by population contact (Omland et al., 2006). These results support the model of Fig. 1B, where short rainforest expansions connected in the past NAF with CAF, and suggested a stronger biogeographic link between interior CAF and NAF than between coastal CAF and NAF.

NAF and CAF populations of *X. fuscus* diverged ~0.5 MYr ago while Ceará and Alagoas populations diverged more than 0.1 MYr ago (Table 4). Even though rainforests are likely to have expanded historically in the interior of the continent and to have survived at the NAF coastal region during the last 21,000 years (Fig. 1B), our results suggest that NAF has not been totally covered by rainforest during the recent past (i.e., the last global glaciation). We believe that this spatial and temporal isolation may be responsible for the extraordinary endemism observed at NAF (Galindo Leal and Câmara, 2003). For example, the southern range border of *atlanticus* coincides with a strip of caatingas along the northern basin of the São Francisco river (Fig. 1D) and this region could have acted as a gene flow barrier and isolated the ancestral of *atlanticus*.

Additionally, divergence at NAF may be currently ongoing. This appears to be evidenced by the sample from Ceará that presented only one mtDNA haplotype, but regular diversity at the nuclear marker, different from the ones at Alagoas (Figs. 3 and 4). This genetic diversity pattern was not observed in any other sample of comparable size (i.e., *atlanticus* from Alagoas or *fuscus* S) and may have been caused by strong genetic drift, a major factor occurring in allopatric divergence and expected to be strong in small and isolated populations such as the one at Ceará (Serra de Baturité, ~125 km²). Our study agrees with the report of Carnaval and Bates (2007) on the phylogeography of two frogs from northeastern Brazil. They found monophyletic populations with low genetic diversity at brejos of Ceará that diverged from coastal Pernambuco and Alagoas more than 0.1 MYr ago. Furthermore, our results and those from others (Carnaval and Bates, 2007; Leite, 2003) support the view that the formation of northernmost forest enclaves (i.e., Serra de Baturité, Fig. 1D) could have been a consequence of the early expansion of caatingas (mid Pleistocene) (Ab'Saber, 1977) and that, in opposition to southern relicts of Ceará (i.e., Serra de Araripe), where *X. fuscus* does not occur, were not extinct at the end of the Pleistocene.

Contrarily to NAF, isolation by caatingas did not result in a phylogeographic gap between the Chapada Diamantina rainforest relict and coastal rainforest in CAF (Fig. 1D), possibly because the relict is large and recent (Table 4). Samples from the Chapada Diamantina grouped with birds from coastal Bahia and Espírito Santo (Fig. 4), a pattern that supports a recent forest connection between both regions and is in accordance with the model of Fig. 1B. Our results show the magnitude of the dynamism of the ecotone rainforest-caatinga (Oliveira et al., 1999; Wang et al., 2004). In some regions, such as interior Bahia (CAF), this dynamism allowed sporadic connection of forests and prevented significant divergence, but in other regions, such as NAF, this dynamism did not allow reconnection of forests and homogenization in the short term.

Our genetic diversity and demographic results (Fig. 4C) matched the general predictions of the model of Carnaval and Moritz (2008). However, the existence of *X. fuscus* lineages, as well as other taxa or phylogroups (Brown, 2005; Costa et al., 2000; Graziotin et al., 2006; Pessoa et al., 2006; Silva et al., 2004), endemic to SAF suggests that the model failed to predict large areas with stable rainforest at SAF. It might be proposed that those *X. fuscus* lineages (*fuscus* N and S) dispersed from the stable CAF after forests expanded in SAF. Under this scenario, the SAF lineages should be derived phylogenetically from the CAF lineage or be present in CAF, but none of these predictions are supported (Fig. 4). Perhaps a small range of stable forest, as predicted for the western SAF (Fig. 1B), might explain the *X. fuscus* lineages endemic to SAF. However, the high number of other taxa endemic to the eastern SAF (i.e., in Serra do Mar at eastern São Paulo, Paraná and Santa Catarina, Costa et al., 2000; Silva et al., 2004) cannot be explained by these small forests. We believe that the persistence of forest blocks has been essential for the evolution of endemic taxa and phylogroups at eastern SAF.

The IM analyses and the Akaike information criteria (Nielsen and Wakeley, 2001) ruled out fully allopatric divergence. Allopatric models are often used to explain biogeographic patterns, especially those with reciprocal monophyly. However, isolation with gene flow can also result in monophyletic clades (Hey and Nielsen, 2004; Nielsen and Wakeley, 2001; Nosil, 2008). If isolation–migration models were more frequently considered, minor geographic features that are traditionally considered inefficient barriers would become important to explain biogeographic patterns. From this standpoint, climatic changes could be central since they would modulate the effect of gene flow barriers. For example, a river may have a stable course but changes in its water flow induced by climatic alterations could modify its effect as a barrier, or a mountain range could be geologically stable but an alteration of its vegetation cover could also modulate its effect as a barrier. The transition of *X. fuscus* lineages at eastern São Paulo occurs in proximity of the Paraíba do Sul valley and the mountain ranges of Serra da Mantiqueira and Serra do Mar (Fig. 4B) (Cabanne et al., 2007). This region is topographically and phytogeographically complex. There are steep altitudinal (>1000 m) and climatic transitions and the vegetation types are various, such as tropical evergreen forest, semideciduous forests, enclaves of Cerrado (South American Savanna, Veloso, 1991) and grasslands at mountains' tops. Besides, palynological studies suggest the existence during the late Pleistocene and early Holocene of a larger range of cerrados and grasslands in southeastern Brazil (Behling, 1998); therefore indicating that local past landscapes were complex as well. Thus, these landscape characteristics may present a partial barrier effect for the gene flow of forest organisms that could have been modulated by climatic oscillations. Hot and dry periods could have expanded cerrados and cold periods could have narrowed forest corridors at hillsides by modifying the grassland–forest line at mountains. This partial barrier effect may be considered together with more widespread changes in the forest distribution during glaciations (Fig. 1B) to explain divergence and transitions of lineages in SAF (Fig. 4).

4.6. Systematics and conservation of *X. fuscus atlanticus*

Based on our results and on the distinctiveness of the subspecies (Cabanne, unpublished; Marantz et al., 2003), we followed the general lineage species concept (de Queiroz, 1998) and propose to recognize *X. fuscus atlanticus* as a full species. The mtDNA *p*-distance between *atlanticus* and any other genetic lineage (>2.6%, Table 3) is compatible with mtDNA divergences found between well recognized species of the genus *Xiphorhynchus* (Aleixo, 2002). Gene flow between *atlanticus* and other lineages was generally low ($M < 1$) and likely not enough to avoid allele fixation and strong divergence (Hedrick, 2000; Wang, 2004; Wright, 1931). This gene flow level supports the condition of independent evolutionary lineage for *atlanticus*. Besides, *atlanticus* is diagnosed by five aminoacidic changes at the mtDNA, whereas the other lineages are only characterized by synonymous or at most one non-synonymous change (Fig. 3). *X. fuscus atlanticus* is monophyletic at the mtDNA, but it shares one FIB5 haplotype with interior Bahia birds (Figs. 2 and 3). This condition is not unexpected for a nuclear marker and should not be overvalued for taxonomical discussions. Particularly in this case, the specific population is isolated, diagnosable by external characters and monophyletic at mtDNA. All isolated populations will eventually acquire reciprocal monophyly, as stated by the coalescence theory (Hudson, 1990). However, as not all genetic markers fixate new mutations at the same rate, it is expected to find intermediate states of polyphyly in recently isolated populations (Baker et al., 2003; Omland et al., 2006; Zink and Barrowclough, 2008). Our data did not support the other three lineages (*tenuirostris/brevirostris*, *fuscus* N and *fuscus* S) as independent evolutionary entities. Those lineages are differentiated at the mtDNA, but gene flow among populations was relatively high ($M > 1$) and diagnosis by external characters is not obvious.

If *atlanticus* is recognized as a species it will add a new globally threatened taxon to the region, since the population is already locally endangered. Notwithstanding the shallow genetic divergence (i.e., mtDNA *p*-distance 0.0012), our results indicated that the two populations of *atlanticus* studied (Alagoas and Ceará), should be considered different evolutionary units (Moritz, 1995) and treated as different management units for conservation purposes. Gene flow between Ceará and Alagoas was very low (Table 4), which indicated low genetic exchangeability between them (Crandall et al., 2000). This result was expected as the two regions are isolated by c. 500 km of caatinga and no stepping stone populations are known.

4.7. Conclusions

Some monophyletic lineages of *X. fuscus* were associated to the areas of endemic passerines described by Silva et al. (2004). Since the genetic data of *X. fuscus* supported in some degree the palaeobotanic model of the AF (Fig. 1B), those endemic bird areas appear to be reflecting zones with long term forest stability (see also Carnaval and Moritz, 2008).

According to its biological composition and late Pleistocene and Holocene history, the AF can be divided in at least three areas: NAF and SAF, which are instable in terms of range and connectivity, and the more stable CAF. Perhaps, the stability is one of the reasons why CAF shares a considerable amount of taxa with lowland Amazon forest (i.e., Bates et al., 1998; Ridgely and Tudor, 1996) that appears to be more stable in the long term (Colinvaux et al., 2000). The main forces driving instability at AF are apparently the climatic alterations related to global glacial cycles (Carnaval and Moritz, 2008). A traditional view about diversification driven by glacial cycles is that it occurs in pulses and creates topologically symmetric phylogenies (Marroig and Cerqueira, 1997). However, there would not be such pulses of diversification in the AF as a whole because

instability at NAF and at SAF are not completely synchronic. Forest fragmentation at NAF is maximum during interglacial periods (i.e., Holocene), while fragmentation at SAF is maximum during the peaks of glaciations. Therefore, if ecological vicariance is important for the diversification of the AF biota, these asynchronic cycles of fragmentation would create a constant rhythm of diversification and phylogenies that may not be symmetric. To support this hypothesis, we would expect to find more signatures of population decline and divergence in isolated forest at NAF and additional secondary contact regions at SAF.

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Appendix A

GenBank accession numbers of sequences other than those of *Xiphorhynchus fuscus* used for phylogenetic analyses (see Appendix B for *X. fuscus* sequences)

Taxon	cytb	ND2	ND3	FIB5	Reference [sample ID]
<i>Sittasomus griseicapillus</i>	<u>AY089796</u>	<u>AY089834</u>	<u>AY089894</u>	—	Aleixo (2002)
<i>Lepidocolaptes angustirostris</i>	<u>AY089811</u>	<u>AY089838</u>	<u>AY089881</u>	—	Aleixo (2002)
<i>Campylorhamphus trochilirostris</i>	<u>AY089822</u>	<u>AY089857</u>	<u>AY089906</u>	—	Aleixo (2002)
<i>Xiphorhynchus flavigaster</i>	<u>AY089828</u>	<u>AY089849</u>	<u>AY089896</u>	—	Aleixo (2002)
<i>X. flavigaster</i>					
<i>X. guttatus guttatus</i>	<u>AY089808</u>	<u>AY089869</u>	<u>AY089908</u>	EF190698	Aleixo (2002), this work [LGEMA P238 = LSU35582] ^{*1}
<i>X. g. eytoni</i>	<u>AY089794</u>	<u>AY089845</u>	<u>AY089884</u>	—	Aleixo (2002)
<i>X. g. guttatusoides</i>	<u>AY089791</u>	<u>AY089866</u>	<u>AY089882</u>	—	Aleixo (2002)
<i>X. obsoletus obsoletus</i>	<u>AY089823</u>	<u>AY089868</u>	<u>AY089913</u>	—	Aleixo (2002)
<i>X. chunchotambo chunchotambo</i>	<u>AY089815</u>	<u>AY089844</u>	<u>AY089915</u>	—	Aleixo (2002)
<i>X. ch. Brevirostris</i>	<u>AY089793</u>	<u>AY089846</u>	<u>AY089885</u>	—	Aleixo (2002)
<i>X. ocellatus ocellatus</i>	<u>AY089804</u>	<u>AY089861</u>	<u>AY089909</u>	EF190699	Aleixo (2002), this work [LGEMA P249 = LSUB35600] ^{*1}
<i>X. o. wealdiellii</i>	<u>AY089820</u>	<u>AY089859</u>	<u>AY089878</u>	—	Aleixo (2002)
<i>X. pardalotus pardalotus</i>	<u>AY089831</u>	<u>AY089848</u>	<u>AY089910</u>	EF190701	Aleixo (2002), this work [LGEMA P265 = LSUB35635] ^{*1}
<i>X. p. pardalotus</i>	EF190607	EF190632	EF190669	EF190700	This work [LGEMA P264 = LSUB35634] ^{*1}
<i>X. elegans elegans</i>	<u>AY089805</u>	<u>AY089852</u>	<u>AY089899</u>	—	Aleixo (2002)
<i>X. e. juruanus</i>	<u>AY089824</u>	<u>AY089874</u>	<u>AY089883</u>	—	Aleixo (2002)
<i>X. e. ornatus</i>	<u>AY089812</u>	<u>AY089841</u>	<u>AY089889</u>	—	Aleixo (2002)
<i>X. susurrans</i>	<u>AY089800</u>	<u>AY089863</u>	<u>AY089914</u>	—	Aleixo (2002)
<i>X. triangularis bangsi</i>	<u>AY089826</u>	<u>AY089864</u>	<u>AY089918</u>	—	Aleixo (2002)
<i>X. spixii</i>	EF190605	EF190630	EF190667	EF190696	This work [FMNH 391312]
<i>X. spixii</i>	EF190606	EF190631	EF190668	EF190697	This work [FMNH 391313]

Underlined accession numbers indicate sequences obtained from GenBank. All samples are muscle. Cytb, cytochrome b; ND2, NADH dehydrogenase subunit 2; ND3, NADH dehydrogenase subunit 3; FIB5, intron 5 of the β -fibrinogen gene. ^{*1}, sample deposited in two institutions. Tissue collections: LGEMA—Laboratório de Genética e Evolução Molecular de Aves, Universidade de São Paulo, São Paulo; LSU—Louisiana State University Museum of Natural Science, Baton Rouge; FMNH—Field Museum of Natural History, Chicago.

Appendix B

Collection localities (all in Brazil, except when indicated) of *Xiphorhynchus fuscus* and areas of endemism (Cracraft, 1985; Stattersfield et al., 1998; Silva et al., 2004) to which samples are associated, tissue identification and GenBank accession numbers

Locality	Sub-species	N	Cracraft (1985)	Stattersfield et al. (1998)	Silva et al. (2004)	Sample ID ^{Tissue}	cytb	ND2	ND3	FIB5
1. Mulungú, Serra de Baturité, Ceará (CE), 4°17'45"S, 38°55'W, 724 m	<i>atlanticus</i>	3	—	—	—	LGEMA10391 ^A	EU073025	EU073030	EU073035	EU073040
			—	—	—	LGEMA10392 ^B	EU073026	EU073031	EU073036	EU073041
			—	—	—	LGEMA10395 ^B	EU073027	EU073032	EU073037	EU073042

Appendix B (continued)

Locality	Sub-species	N	Cracraft (1985)	Statters-Silva et al. (2004)	Sample ID ^{TISSUE}	cytb	ND2	ND3	FIB5
2 Guaramiranga, Serra de Baturité, CE, 4°14'S, 38°56'W, 724 m	<i>atlanticus</i>	2	—	—	LGEMA10406 ^B	EU073028	EU073033	EU073038	EU073044
			—	—	LGEMA10417 ^B	EU073029	EU073034	EU073039	EU073045
3 Ibateguara, Alagoas, 8°59'S, 35°52'W, 200 m	<i>atlanticus</i>	5	SMC	AAP	Pe	FMNH399195 ^M	EF190601	EF190608	EF190645
						FMNH399196 ^M	EF190583	EF190609	EF190646
						FMNH399197 ^M	EF190584	EF190610	EF190647
						FMNH399198 ^M	EF190585	EF190611	EF190648
						FMNH399199 ^M	EF190586	EF190612	EF190649
4 Bonito, Bahia (BA), 11°54'S, 41°12'W, 945 m	<i>brevirostris</i>	1	—	DB	CB	LGEMAP2267 ^M	EF190597	EF190623	EF190660
5 Lençóis, Minas Gerais, 41°21'W, 520 m	<i>brevirostris</i>	3	—	DB	CB	LGEMAP2245 ^M	EF190594	EF190620	EF190657
						LGEMAP2249 ^M	EF190595	EF190621	EF190658
						LGEMAP2258 ^M	EF190596	EF190622	EF190659
6 Porto Seguro, BA, 17°22'S, 40°17'W, 50 m	<i>tenuirostris</i>	1	SMC	AL	CoB	LGEMAP330 ^M	AY089819 [*]	AY089851 [*]	AY089904 [*]
7 Salto Divisa, Minas Gerais (MG), 16°5'S, 40°2'W, 100 m	<i>tenuirostris</i>	2	SMC	AL	CoB	B0822 ^B	EF190577	EF190633	EF190639
						B0918 ^B	EF190582	EF190638	EF190644
8 Jequitinhonha (Mata Escura), MG, 16°20'S, 41°0'W, 900 m	<i>af. brevisrostris</i>	2	SMC	DB	CB	B0913 ^B	EF190581	EF190637	EF190643
						B0911 ^B	EF190580	EF190636	EF190642
9 Caratinga, MG, 20°50'S, 42°5'W, 55 m	<i>fuscus</i>	1	—	—	—	B0908 ^B	EF190578	EF190634	EF190640
10 Nova Lima, MG, 19°59'S, 43°49'W, 900 m	<i>fuscus</i>	1	—	—	—	B0909 ^B	EF190579	EF190635	EF190641
11 Sooretama, Espírito Santo (ES), 19°3'S, 39°55'W, 50 m	<i>fuscus</i>	2	SMC	AL	CoB	LGEMAP2876 ^M	EF190602	EF190629	EF190664
						LGEMAP2877 ^M	EF190603	EF190627	EF190665
12 Santa Teresa, ES, 19°56'S, 40°34'W, 650 m	<i>fuscus</i>	1	SMC	AL	SM	LGEMAP1644 ^B	EF190592	EF190618	EF190655
13 Cantagalo, Rio de Janeiro (RJ), 22°50'W, 42°33'W, 400 m	<i>fuscus</i>	1	SMC	AL	SM	LGEMAP2878 ^M	EF190604	EF190628	EF190666
14 Itatiaia, RJ, 22°24'S, 44°38'W, 800 m	<i>fuscus</i>	1	SMC	AM	SM	LGEMAP1321 ^B	EF190589	EF190615	EF190652
15 Bananal, São Paulo (SP), 22°41'S, 44°19'W, 500 m	<i>fuscus</i>	1	SMC	AL	SM	LGEMAP1171 ^M	EF190588	EF190614	EF190651
16 Barreirão Rico, SP, 22°38'S, 48°13'W, 550 m	<i>fuscus</i>	1	—	AL	SM	LGEMAP1544 ^M	EF190591	EF190617	EF190654
17 São Roque, SP, 23°34'S, 47°09'W, 960 m	<i>fuscus</i>	1	SMC	AM	SM	LGEMAP1024 ^B	EF190587	EF190613	EF190650
18 Morro Grande, SP, 23°42'S, 46°59'W, 800 m	<i>fuscus</i>	1	SMC	AM	SM	LGEMAP652 ^B	EF190598	EF190624	EF190661
19 Morro do Diabo State Park, SP, 22°30'S, 52°18'W, 300 m	<i>fuscus</i>	1	—	—	—	LGEMAP1379 ^B	EF190590	EF190616	EF190653
20 Wenceslau Braz, Paraná (PR), 22°51'S, 49°47'W, 800 m	<i>fuscus</i>	1	PRC	AM	SM	LGEMAP937 ^M	EF190599	EF190625	EF190662
21 Campo San Juan, Misiones, Argentina, 27°22'S, 55°39'W, 150 m	<i>fuscus</i>	1	PRC	AM	—	LGEMAP994 ^B	EF190600	EF190626	EF190663
22 Rancho Queimado, Santa Catarina, Br. 27°41'S, 49°2'W, 850 m	<i>fuscus</i>	1	PRC	AM	SM	LGEMAP1771 ^M	EF190593	EF190619	EF190656

FIB5 heterozygous individuals present two GenBank accesses, one for each chromosome.

Cytb, cytochrome b; ND2, NADH dehydrogenase subunit 2; ND3, NADH dehydrogenase subunit 3; FIB5, intron 5 of the β -fibrinogen gene.

*SMC, Serra do Mar Center; PRC, Paraná Center; AAP, Atlantic slope of Alagoas and Pernambuco; DB, deciduous forest of Bahia; AL, AF lowland; AM, AF Mountains. Pe, Pernambuco; CB, Central Bahia; CoB, Coastal Bahia; SM, Serra do Mar, Ba, Bahia; RJ, Rio de Janeiro; SC, Santa Catarina. —, not present in any area of endemism. TISSUE, tissue type, M, muscle; B, blood. 1, from Aleixo (2002). Tissue collections: LGEMA—Laboratório de Genética e Evolução Molecular de Aves, Universidade de São Paulo, São Paulo. FMNH—Field Museum of Natural History, Chicago. B—Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte.

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Anexo XVIII

The genetic effects of Late Quaternary climatic changes over a tropical latitudinal gradient: diversification of an Atlantic Forest passerine

FERNANDO M. D'HORTA,* GUSTAVO S. CABANNE,*† DIOGO MEYER* and CRISTINA Y. MIYAKI*

*Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, 05508-090 São Paulo, SP, Brazil, †División de Ornitología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Ángel Gallardo 470, Ciudad de Buenos Aires (C1405DJR), Argentina

Abstract

The increase in biodiversity from high to low latitudes is a widely recognized biogeographical pattern. According to the latitudinal gradient hypothesis (LGH), this pattern was shaped by differential effects of Late Quaternary climatic changes across a latitudinal gradient. Here, we evaluate the effects of climatic changes across a tropical latitudinal gradient and its implications to diversification of an Atlantic Forest (AF) endemic passerine. We studied the intraspecific diversification and historical demography of *Sclerurus scansor*, based on mitochondrial (ND2, ND3 and *cytb*) and nuclear (FIB7) gene sequences. Phylogenetic analyses recovered three well-supported clades associated with distinct latitudinal zones. Coalescent-based methods were applied to estimate divergence times and changes in effective population sizes. Estimates of divergence times indicate that intraspecific diversification took place during Middle–Late Pleistocene. Distinct demographic scenarios were identified, with the southern lineage exhibiting a clear signature of demographic expansion, while the central one remained more stable. The northern lineage, contrasting with LGH predictions, exhibited a clear sign of a recent bottleneck. Our results suggest that different AF regions reacted distinctly, even in opposite ways, under the same climatic period, producing simultaneously favourable scenarios for isolation and contact among populations.

Keywords: biogeography, historical demography, Neotropical Region, phylogeography, *Sclerurus scansor*

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Introduction

The marked increase in biodiversity from regions of high to low latitudes is well documented across distinct taxonomic levels and constitutes a widely recognized biogeographical pattern (Fischer 1960; Simpson 1964; Stelli *et al.* 1969; Willig *et al.* 2003). While its underlying processes have intrigued ecologists, systematists and biogeographers worldwide, few historically based hypotheses have been proposed to explain its origin (see Brown & Lomolino 1998). One such example is the notion that

the latitudinal biodiversity gradient may reflect the distinct influence of Pleistocene glacial–interglacial cycles in geographic space (Fischer 1960; Hewitt 2004).

Pleistocene climate change strongly affected environmental ranges throughout the world (Pielou 1991; Hewitt 2000). The effects of this dynamic are reflected in a diverse group of organisms, especially at higher latitudes, where postglacial expansion is usually considered primarily responsible for current genetic diversity patterns (Hewitt 1996, 1999, 2000, 2004). Like their temperate counterparts, tropical habitats also expanded to higher latitudes since the last glacial period (Behling 1995; Behling & Negrelle 2001; Bauermann 2003; Carnaval & Moritz 2008). Thus, it is expected that

Correspondence: Fernando Mendonça d'Horta,
Fax: +55 +11 30917553; E-mail: fmhorta@usp.br

within-population genetic diversity, as well as the degree of genetic structure, should decrease from low to high latitudes, along a tropical latitudinal gradient (Miller *et al.* 2010).

The genetic effects of the glacial/interglacial periods across the latitudinal gradient have been studied at multiple geographic scales. Based on analysis of a diverse group of vertebrates, Lessa *et al.* (2003) observed different demographic histories across regions at different latitudes in North America and the Amazon. Taxa from North America exhibited clear signatures of recent population expansion, while species from the Amazon forest were apparently stable since the end of the Pleistocene. A similar scenario was observed by Pinho *et al.* (2007) in a more restricted geographic scale. An analysis of the effects of climatic changes in closely related taxa (species of *Podarcis* lizards) through the Iberian Peninsula and North Africa indicated higher stability at lower latitudes, evidenced by the greater genetic structure and diversity.

Carnaval *et al.* (2009) also showed similar findings, working within the tropics. In their study with Atlantic Forest (AF) amphibians, they reported higher levels of genetic diversity and structure at low (central AF) than at high latitudes (southern AF), corroborating paleoenvironmental inferences for this region (Carnaval & Moritz 2008).

The hypothesis about the effects of climatic cycles across the latitudinal gradient (latitudinal gradient hypothesis – LGH) makes explicit predictions, also applicable to the tropics: (i) populations from higher latitudes experienced more pronounced changes in their effective population sizes and therefore exhibit signatures of recent demographic expansion and a lower genetic structure; (ii) populations from lower latitudes experienced smaller or no changes in effective sizes, thus presenting high diversity and genetic structure.

Although the LGH makes explicit and testable predictions at the population level, few studies have explicitly tested it in a strictly tropical latitudinal gradient. Here, we investigate the influences of the differential dynamics of the environment throughout a tropical latitudinal gradient on the genetic diversification and historical demography of *Sclerurus scansor* (Ménétrières 1835). *Sclerurus scansor* is a polytypic species formed by two subspecies: *Sclerurus scansor scansor* (Ménétrières 1835) that ranges from the southern limits of the AF to the southern region of the state of Bahia and *Sclerurus scansor cearensis* (Snethlage 1924) that restricts to the interior of forest relicts of northeastern Brazil. This strictly forest passerine is endemic to the AF region (Stotz *et al.* 1996). Two reasons make it a powerful model for studying AF biogeography. First, *S. scansor* is highly sensitive to environmental changes, such as those caused by forest

fragmentation (Stotz *et al.* 1996; Hansbauer *et al.* 2008a,b). Second, it is distributed throughout the AF, ranging from lat 3° to 32°S.

As stressed by Pinho *et al.* (2007), studies with closely related taxa make the inferences simpler and straightforward, because ecological requirements, dispersal ability and important quantitative information needed to obtain estimates of demographic parameters (e.g. mutational rate and generation time) can be assumed to be similar for the entire range, without adding significant noise to the analysis. Conversely, the analysis of a single biogeographical entity (the AF) to test the predictions associated with the LGH makes the inferences and interpretation of the results more robust, because its distribution is defined by the same general limits of environmental conditions (e.g. soil, temperature and precipitation).

In this study, we test the LGH predictions for AF region based on the phylogeographic and demographic history of *S. scansor* and analyse the hypothesis proposed to explain the origin of current biogeographical patterns of the AF.

Methods

Samples and markers

Tissue samples were obtained from the following collections: (i) Laboratório de Genética e Evolução Molecular de Aves (LGEMA), São Paulo, Brazil; (ii) Field Museum of Natural History (FMNH), Chicago, USA; (iii) Louisiana State University Museum of Natural Sciences (LSUMNS), Baton Rouge, USA; and (iv) Laboratório de Biodiversidade e Evolução Molecular, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil (Appendix S1, Supporting information).

Analyses were based on two data sets. *Data set A*: NADH dehydrogenase subunit 2 (ND2) sequences (1041 bp) from 85 samples, extracted from muscle and blood tissues. This data set was used to analyse the phylogeographic structure of *Sclerurus scansor*. This analysis was carried out using *Sclerurus albigularis* (the sister species of *S. scansor*) and *Sclerurus mexicanus* as outgroups, based on a *Sclerurus* molecular phylogeny (d'Horta 2009). *Data set B*: Based on the initial exploratory analysis (data set A), we sampled a subset of individuals that include all main geographic regions. For 48 individuals, we sequenced 1025 bp of cytochrome *b* (*cytb*) and 345 bp of NADH dehydrogenase subunit 3 (ND3), and for 44 individuals, we sequenced 954 bp of a nuclear locus and β -fibrinogen intron 7 (*Fib7*). Procedures of DNA extraction, purification and sequencing are described on Appendix S2 (Supporting information).

Data analyses, neutrality tests and recombination (data sets A and B)

Sequences of ND2 (data sets A and B), ND3, *cyt b* and *Fib7* (data set B) were used to perform phylogeographic and demographic analyses. Sequences were compared and edited using the program CodonCode Aligner (CodonCode Corporation), and the alignment was performed with Clustal X (Thompson *et al.* 1997) and then manually checked.

Heterozygous nucleotide positions were identified by double peaks in the electropherograms, in the case of the nuclear locus. We used a Bayesian approach implemented in the program PHASE 2.0 (Stephens *et al.* 2001; Stephens & Donnelly 2003) to identify haplotypes of *Fib7*. Heterozygous indels were not found. To test the hypothesis of neutrality in mtDNA, the MacDonald and Kreitman test (MK test – MacDonald & Kreitman 1991) was applied, considering the sister species of *S. scansor*, *S. albigularis*, as outgroup (d'Horta 2009). This test was applied to the entire species distribution and also to each lineage. To evaluate the existence of recombination on *Fib7*, we applied the *phi* test, performed in the SPLITSTREE4.10 program (Huson & Bryant 2006). We chose this test because of its sensitivity to recombination events and its power to distinguish recombination events from homoplasies (Breen *et al.* 2006).

Haplotype identification and phylogenetic analyses (data sets A and B)

We used the NETWORK 4.5.0.0. software (<http://www.fluxus-technology.com>) to identify ND2 and *FIB7* haplotypes and to obtain a network with the *median joining* method (Bandelt *et al.* 1999).

The phylogenetic analyses were performed for mtDNA (data sets A and B), using two methods, maximum-likelihood estimation (ML) and Bayesian analysis (BA). To select the simplest model of molecular evolution with the highest likelihood for the data, we applied a likelihood-ratio test (LRT) performed by the MODELTEST program (Posada & Crandall 1998). The selected model parameters were used for the ML analysis implemented on PHYML program 2.4.4 (Guindon & Gascuel 2003). The robustness of the ML tree nodes was determined by 500 bootstrap replications.

The BA, with Markov chain Monte Carlo (MCMC) sampling, was implemented with MrBayes 3.1 (Ronquist & Huelsenbeck 2003), considering the partitions of the data set applying the partitioned likelihood method (one partition per gene). Parameters were independently estimated for each data partition (*nst* = 6; *rates* = *invgamma*). Two independent analyses were performed, each with 10^6 generations, sampling every

1000 generations and with a burn-in of 20%, determined by the convergence time of the likelihood values. The posterior probability values of each node were calculated by combining all sampled trees in a consensus.

Population level analyses (data set B)

Broader population groups used for analysis of divergence times (IMa) and historical demography (LAM-ARC and neutrality tests) were defined based on geographic criteria and corroborated by the analysis of data set A (see Results).

Divergence times. Estimation of divergence times (*t*) between pairs of *S. scansor* lineages (southern, central and northern lineages; Fig. 3) were obtained applying the *isolation-migration* model (Nielsen & Wakeley 2001; Hey & Nielsen 2004), implemented by IMa program (ver. 17 December 2009). Based on a coalescent and MCMC method, IMa calculates the distribution of posterior probability values of parameters considered in the model (Nielsen & Wakeley 2001; Hey & Nielsen 2004). The effective population size was estimated for current populations (θ_1 and θ_2) and the ancestral population (θ_a). To obtain the divergence time estimates between lineages, we set migration rates (m_1 and m_2) to 0. To define the priors, initial runs were carried out, followed by five posterior runs, for each pair of populations, using distinct seed values, to verify the convergence of parameter estimates. For each analysis, a burn-in of 500 000 steps was applied, and they were finished with at least 50 000 000 steps in the chain after the burn-in stage and a minimum effective sample size value of 150. To obtain the divergence time in years, we considered $t = t/u$ (Hey & Nielsen 2004), where *t* is estimated by IMa and *u* is the geometric mean of mutational rates (per year) of mitochondrial genes (*cyt b*, ND2 and ND3) and *Fib7*. To calculate the *u* value, we assumed a divergence of 2.1% per million of years for mtDNA genes (Wier & Schluter 2007) and 0.72% for *Fib7* (Axelsson *et al.* 2004). All analyses were performed using Hasegawa-Kishino-Yano (HKY – Hasegawa *et al.* 1985) evolutionary model.

Population expansion/decline. Tajima's *D* (Tajima 1989), Fu's *F_s* test (Fu 1997) and *R₂* (Ramos-Onsins & Rozas 2002) were used to test the scenarios of population expansion. The significance of the tests was determined based on 10 000 coalescent simulations, which assumed neutrality and equilibrium conditions. These analyses were performed with DnaSP 4.10.9 program (Rozas *et al.* 2003). These three test statistics were selected because of their power to detect population expansion scenarios in

specific sampling conditions and population expansion rate, time since the expansion, sample size and number of segregation sites (Ramos-Onsins & Rozas 2002).

In addition to the tests with summary statistics, we estimated genetic diversity levels (θ) and exponential population growth rates (g) using the LAMARC program (Kuhner 2006). LAMARC calculates the likelihood surface for θ and g , allowing one to find values that maximize the probability of originating data. These parameters (θ and g) were estimated for the three main phylogeographic lineages identified in *S. scansor*, fixing the migration rate to 0. For the LAMARC analyses, five replicates of 10 short chains each (500 genealogies sampled each 50 interactions and a *burn-in* of 1000 genealogies) and two long chains (20 000 genealogies sampled each 50 interactions and a *burn-in* of 1000 genealogies) were performed. Because of the failure of the *phi* test in rejecting the null hypothesis of nonrecombination, the parameter r (recombination) was not considered for the LAMARC analyses.

Results

Sequence variability

In data set A (1041 bp of ND2; $n = 85$), we identified 53 variable and 41 parsimony-informative sites. In data set B for the mtDNA (total of 2432 bp, been 345 bp of ND3, 1041 bp of ND2 and 1046 bp of *cytb*; $n = 48$), we identified 93 (five for ND3, 49 for ND2 and 39 for *cytb*) variable and 69 (three for ND3, 39 for ND2 and 27 for *cytb*) parsimony-informative sites, and for the nuclear locus (Fib7) in 954 bp ($n = 44$), we found 10 (1.05%) variable sites, of which 9 (0.94%) were parsimony informative. The results of MK test were nonsignificant ($P > 0.1$) for

all analyses (both treating *Sclerurus scansor* as a single taxon and treating each phylogeographic lineage separately). The *phi* test did not reject the null hypothesis of nonrecombination for Fib7 ($P > 0.1$).

Twenty-five haplotypes were identified for the ND2 gene (data set A: 1041 bp, $n = 85$), while 30 were diagnosed for Fib7 (data set B: 954 bp, $n = 44$). The ND2 network showed a strong geographic association of haplotype groups (Figs 1a and 2). Three main groups were identified, representing the northern (SN), central (SC) and southern (SS) lineages. On the other hand, the Fib7 network showed extensive sharing of haplotypes between SC and SS. The SN lineage was the only one which did not share haplotypes with other groups (Fig. 1b).

Nucleotide diversity (π) and haplotype diversity (H) were low for all phylogeographic lineages (Table 1). Lineage SC showed the highest values of π and H for mtDNA (data set A and data set B; 0.0043 and 0.731 for ND2; 0.0035 and 0.908 for concatenated mtDNA, respectively). SS exhibited the lowest π and H values for mtDNA (0.00116 and 0.706 for ND2; and 0.00118 and 0.808 for mtDNA concatenated). SN (*cearensis*) exhibited intermediate values of π and H (0.002 and 0.630 for ND2; 0.0021 and 0.0846 for mtDNA concatenated). Among the three groups analysed for Fib7, SN exhibited the lowest π and H values (0.00061 and 0.499, respectively), while the SC the highest ones (0.0049 and 0.948), which can be explained by the wide sharing of haplotypes observed between the SS and SC lineages (Fig. 1b).

Evolutionary relationships, distribution and contact among phylogeographic lineages

Three well-supported clades were identified for *S. scansor*, based on mtDNA (data set B): (i) SS, which is

Fig. 1 Median joining networks based on (a) 1041 bp of ND2 (data set A) and (b) 884 bp of Fib7 (data set B) of *Sclerurus scansor*. Lineage distribution, represented in black (SS), gray (SC) and light gray (SN) circles.

distributed from the southern limit of the species' occurrence to the central portion of São Paulo state; (ii) SC, which ranges from the central portion of São Paulo state, where it occurs in sympatry with SS, until southern Bahia; (iii) SN, which occurs associated with forest enclaves from the interior of the Caatinga, apparently totally isolated from the SS and SC (Fig. 2). The relationships among populations suggest a first split between SN (*cearensis*) and the two southernmost clades (SS and SC) followed by the split between SS and SC (bootstrap of 77 and posterior probability of 100) (Fig. 3).

For two lineages, SC and SN, two phylogeographic subgroups were identified. In SN, we found two mitochondrial lineages that correspond to the two haplotype clusters in the network (Fig. 1a), which comprise the samples from the western SN range (Serra de Ibiapaba, Serra das Almas and Chapada do Araripe) and the samples from eastern SN range (Serra de Maranguape, Serra de Baturité and Serra do Machado) (Figs 2 and 4). These results suggest that the contact within the eastern as well as within western forest relicts was more frequent and recent than between these two groups. In SC, two lineages were also identified, one comprising the samples lying north and the other, those lying south of the Doce river region (Figs 2 and 4).

Among the main phylogeographic lineages of *S. scansor*, contact is observed only between the SS and SC. Of the total of 18 localities sampled SC and SS, only three

(those in the region of upper Tietê and Paraíba do Sul rivers, see discussion) showed evidence of sympatry between these lineages. The current distribution of *S. scansor scansor* (SS and SC), as well as *Sclerurus scansor cearensis* (SN), suggests a complete genetic isolation.

Population analyses

Our analysis of data set A showed a strong congruence between geographic regions and clade membership. The only exception to this pattern was found for three localities in the region of contact between SS and SC, where haplotypes from different lineages are found in sympatry. We argue that this contact zone arises as a consequence of secondary contact (see Discussion) and therefore that treating lineages as populations is justified. Our estimates of population expansion and divergence times were thus applied on these lineages and assumed zero migration rates.

Divergence times. The analysis of divergence times between intraspecific lineages resulted in values that point to a relatively recent process of intraspecific diversification, with events of cladogenesis concentrated on Middle and Late Pleistocene (Fig. 4 and Table 2). Divergence times estimated by IMa (data set B – mitochondrial and nuclear markers), as well as the topology based on mitochondrial genes, suggest a first event of cladogenesis originating SN (*cearensis*) and the SC + SS

Fig. 2 Sample localities of *Sclerurus scansor*. Different circle colours represent the three main mitochondrial lineages. In black SS, gray SC and bright gray SN. The inset map depicts the zone of sympatry between SS and SC. Black/gray circles show the localities where SS and SC lineages are reported. Dotted lines mark the position of the brakes among internal phylogeographic units in SC and SN.

Table 1 Diversity values and the results of statistical tests to ND2; concatenated mtDNA (cytb, ND2 and ND3); and Fib 7

Lineages	Taxa	Gene	N	Diversity			Tests	
				π	H	D	F_s	R_2
Southern	<i>Sclerurus scansor scansor</i>	ND2	30	0.00116	0.706	-1.6441 ns	-7.300 **	0.0571**
		Mit	15	0.00118	0.808	-0.7652 ns	-1.267 ns	0.1120 ns
		Fib7	14	0.00350	0.791	-0.3216 ns	-6.528*	0.1159 ns
Central	<i>S. scansor scansor</i>	ND2	19	0.00433	0.731	-0.5047 ns	-0.588 ns	0.1090 ns
		Mit	16	0.00350	0.908	-0.3645 ns	-1.350 ns	0.1149 ns
		Fib7	13	0.00490	0.948	-0.5230 ns	-13.366 **	0.1002 ns
Northern	<i>Sclerurus scansor cearensis</i>	ND2	36	0.00205	0.630	0.6128 ns	1.198 ns	0.1456 ns
		Mit	17	0.00210	0.846	0.4534 ns	0.016 ns	0.1464 ns
		Fib7	17	0.00061	0.499	1.5592 ns	1.711 ns	0.2496 ns
Total	<i>Sclerurus scansor</i>	ND2	85	0.01241	0.871	0.5944 ns	-0.543 ns	0.1154 ns
		Mit	48	0.01042	0.941	1.2173 ns	0.904 ns	0.1472 ns
		Fib7	44	0.00403	0.875	-0.4057 ns	-22.043**	0.0836 ns

N, number of sequences; π , nucleotide diversity; H, haplotypic diversity; D, Tajima's D (1989); F_s , Fu (1997); R_2 , (Ramos-Onsins & Rozas 2002).

ns, nonsignificant ($P > 0.05$); *Significant ($P < 0.05$); **Significant ($P < 0.01$).

(*scansor*) lineages. A second event, near the end of the Pleistocene, originated the SS and SC (Table 2). The divergence time between SS and SC was estimated to be approximately 0.06 Ma (CI 90%: 0.04–0.09 Ma). Divergence times between SS and SN, and SC and SN were estimated to be approximately 0.54 (CI 90%: 0.32–0.76 Ma) and 0.24 Ma (CI 90%: 0.06–0.39 Ma), respectively.

Historical demography. The results of Tajima's D test were nonsignificant for all populations, thus not rejecting the hypothesis that they are in equilibrium. F_s , on the other hand, showed that for SS, both ND2 (30 individuals) and Fib7 (12 individuals) had negative and significant values, supporting the hypothesis of population expansion. The R_2 values for this same lineage also point to the same scenario (when ND2 was used). The data for the SC lineage showed that only the F_s for Fib7 resulted in significant values. Conversely, none of the tests applied to the SN lineage resulted in significant values, indicating demographic stability (Table 1). Although Tajima's D is relatively sensitive to demographic expansion, the simulations performed by Ramos-Onsins & Rozas (2002) showed that it tends to have less power than F_s (Fu 1997) and R_2 (Ramos-Onsins & Rozas 2002) in scenarios where time since expansion is high and/or the sample size and/or the growth rate are low.

The demographic analyses for the three main lineages of *S. scansor*, based on genealogy sampling using LAMARC, are partially congruent with the results obtained using the tests based on summary statistics. Concerning diversity levels, SS presented the lowest

values, much lower than those observed for SC and SN, compatible with the scenario of deep changes in forest distribution at higher latitude regions. On the other hand, the estimates of exponential population expansion rate (g) showed markedly different results for the three lineages. For SS, the values indicate recent population expansion, with a point value of $g = 4314.370$ and the lower and upper limits of the confidence interval indicating positive values (CI 95%: 3711.105–4698.091). Although the SC lineage exhibits positive g values ($g = 613.0905$; CI 95%: 180.2021–951.3234), they are much closer to 0 than those obtained for SS (Fig. 5). Because of the tendency of g to be shifted towards positive values, especially when one or few markers are used (Kuhner *et al.* 1998; Kuhner 2006), it is not possible to completely discard the scenario of stability for SC. On the other hand, SN (*S. s. cearensis*) exhibits g values that indicate a completely different demographic condition than the observed for the other lineages. The values for the SN ($g = -1793.549$, CI 95%: -4472.767 to -184.0114) are compatible with a scenario of a population bottleneck (Fig. 5), mainly if we consider the tendency for LAM-ARC to overestimate g values.

Discussion

Hypothesis of vicariant events

Many vicariant hypotheses have been proposed to explain the origin of the recurrent patterns of distribution of lineages/taxa in the Neotropical forests. These hypothesis are based on the role of physiographic (e.g.

Fig. 3 Maximum-likelihood (ML) tree based on mitochondrial genes (2432 bp of ND2, ND3 and cytb – data set B). Numbers at each node represent the posterior probability from the Bayesian analysis and the bootstrap values (based on 500 replicates) from ML analysis. Number of locality sampled on the terminals (see Appendix S1, Supporting information).

Wallace 1852; Sick 1967; Ayres & Clutton-Brock 1992; Marroig & Cerqueira 1997; Nores 1999; Patton *et al.* 2000) or ecological barriers (e.g. Haffer 1969; Vanzolini & Williams 1970; Brown & Ab'Saber 1979; Colinvaux 1998) in reducing gene flow among populations and promoting divergence in allopatry. Some of these hypotheses have been considered to explain the origin of the AF biodiversity patterns (rivers as barriers – Pellegrino *et al.* 2005; refuges – Brown 1987; Cabanne *et al.* 2007; Lara *et al.* 2005; and neotectonism – Silva & Straube 1996; Pessoa 2007).

The geographic pattern of intrapopulation genetic variation observed for *Sclerurus scansor* is compatible with that proposed by the refuge hypothesis (Haffer 1969; Vanzolini & Williams 1970). First, phylogeographic lineages within *S. scansor* likely originated via vicariant events. The SN lineage is represented by an allopathic population. Available evidence strongly indicates that the origin of contact zone between SS and SC is secondary, suggesting that the lineages that occupy these two regions also originated via a vicariant event. The contact zone between SC and SS is spatially congruent with

Fig. 4 Probability distributions of divergence times (million years ago), estimated by IMa, between lineages pairs of *Sclerurus scansor*, based on mitochondrial genes and Fib7 (data set B).

those documented between phylogeographic lineages of amphibians (Carnaval *et al.* 2009), birds (*Xiphorhynchus fuscus*, Cabanne *et al.* 2007; and *Conopophaga lineata*, Pessoa 2007), as well as between morphologically differentiated populations of birds (*Lepidocolaptes squamatus*, Silva & Straube 1996; and *Heliobletus contaminatus*, Silva & Stoltz 1992). This pattern of a common contact region can be interpreted as evidence of a common vicariant event (Cracraft 1985; Cracraft & Prum 1988).

Second, the *S. scansor* phylogeographic lineages presented, in different degrees, signatures of changes in their effective population size, which is expected in a refuge scenario. Finally, the estimates of the divergence time between phylogeographic lineages point to events during Middle and Late Pleistocene, a period for which there are extensive records documenting changes in forest distribution associated with climatic cycles

(Riccomini 1989; Behling 1995, 1997, 2002; Ledru *et al.* 1996, 2005; Behling & Lichte 1997; Behling & Negrelle 2001; Cruz *et al.* 2005; Carnaval & Moritz 2008). We also noticed that the geographic distribution of the lineages is congruent with what is expected according to the mountain refuges hypothesis (Moreau 1966; Lara *et al.* 2005). Based on the mountain refuge hypothesis, during drier periods, forest formations were more likely to occur in mountain areas, because of the higher pluviometric level resulting from the orographic effect. Such phenomenon is currently observed in the north-eastern region of Brazil, where the occurrence of humid forests is strictly associated with areas of mountain ridges.

The hypothesis of rivers as primary barriers (Pellegrino *et al.* 2005) is not supported owing to the very recent origin of the phylogeographic lineages observed for *S. scansor*, as well as for other avian species (Pessoa 2007; Cabanne *et al.* 2008). However, these rivers can play an important role as a secondary barrier to dispersion, decreasing the migration between populations, or even completely isolating them. Silva & Straube (1996) propose that the tectonic activity associated with the Paraíba valley would have caused the isolation of the populations distributed in the north and south of this region. Although the location of the phylogeographic rupture between the SS and SC is relatively congruent with this notion, the divergence time between them is much more recent (Middle/Late Pleistocene, Table 2) than the periods of higher tectonic activity in the region, dated between the Miocene–Pliocene (about 15 Ma) and the Lower Pleistocene (about 1 Ma) (Petri & Fúlfaro 1983). Although there is evidence of recent tectonic movements during the Pleistocene and Holocene (Riccomini 1989), it would be necessary to admit that these movements caused landscape changes capable of imposing restrictions to gene flow between lineages SS and SC for a long period of time. On the other hand, analysing the deposition of Cenozoic sediments that fill the basins of the Continental Rift of southeastern Brazil, Riccomini (1989) recognized the effects of climatic cycles on erosion dynamics: increased erosion happened during drier climates, when vegetation cover was less dense (savannas), and decreased erosion during wetter climates, when vegetation covering was more dense (forest), which is compatible with refugia predictions.

Latitudinal gradient and demographic history

The importance of the differential effects of glaciations across latitudinal gradients, as well as their influence on the determination of current biodiversity patterns, is among the major subjects of Biogeography (Hewitt 1996, 2001; Brown & Lomolino 1998; Willig *et al.* 2003;

Table 2 Estimates of divergence times (million years ago) between pairs of phylogeographic lineages of *Sclerurus scansor*

Lineages	Divergence time (Ma)		
	Minimum (CI 90%)	Point estimates	Maximum (CI 90%)
SS vs. SC	0.04	0.06	0.09
SS vs. SN	0.32	0.54	0.76
SC vs. SN	0.06	0.24	0.39

CI, confidence interval.

Wier & Schluter 2007; Marshall *et al.* 2008). The latitudinal gradient has an important influence on the intensity of vicariant events, genetic diversity and demography, as demonstrated by Pinho *et al.* (2007) and Lessa *et al.* (2003). The description of patterns of genetic diversity and demographic history of populations across a tropical latitudinal gradient helps us to understand the differential effects of glacial cycles over the landscape and its importance in the origin of biodiversity patterns observed in these regions.

The geographic pattern of genetic diversity exhibited by lineages of *S. scansor* contrasts with the expectations of the LGH for the tropics. Although SS presents the lowest values of nucleotide and haplotype diversity for mtDNA, the values for SC, and not for SN, were the highest (Table 1). In the same way, the diversity values

of Fib7 were distinct from the expected for the SN. The values of nucleotide and haplotype diversity for SN were lower than those of the other lineages. SS showed intermediate values for Fib7, while the SC exhibits the highest (Table 1).

The results from demographic analyses support the same scenario. First of all, estimates of exponential growth rate (g) show a clear pattern of nonoverlapping confidence intervals (95%) among lineages (Fig. 5), which refutes the hypothesis of a similar demographic history related to the different latitudinal zones of AF.

The SS lineage, distributed between latitudes 23° and 32°S, shows a clear positive value of g (Fig. 5). The results from the present study, as well as from amphibians (Carnaval *et al.* 2009) and other birds (Cabanne *et al.* 2007; Pessoa 2007), support a model of recent population expansion associated with strictly forest taxa in higher AF latitude zones.

For the SC, which occurs between 24° and 14°S, a positive g was also found. Although the confidence interval does not include 0, owing to the tendency of overestimation of g values by LAMARC (Kuhner *et al.* 1998; Kuhner 2006), it is not possible to completely refute the hypothesis of recent stability of the effective size for this population. The g values as well as the highest genetic diversity for the SC lineages are in accordance with the results of Carnaval *et al.* (2009), showing a higher stability of forest distribution in the central portion of AF. Either way, the results obtained

Fig. 5 Estimated values of population growth rate (g) (CI 95%) of the three main lineages of *Sclerurus scansor* (SS, SC and SN), based on mitochondrial genes and Fib7 (Data set B). Over the latitudinal axis, the amplitude of lineage ranges is shown. Horizontal bars represent the distribution of g values estimated for each lineage. Lineages are represented in black (SS), gray (SC) and bright gray (SN).

for the SS and SC lineages corroborate the general predictions of the LGH, that is, of higher demographic instability associated with higher latitude regions, where forest distribution suffered deep changes in function of glacial cycles.

In contrast, the SN lineage, associated with the lowest latitude zone (between 3° and 10°S), exhibits a signature of a population bottleneck. The recent changes in the effective size of the SN seem to have occurred in a markedly distinct way from that observed for the other populations. As the g values tend to be overestimated by LAMARC (Kuhner *et al.* 1998; Kuhner 2006), the scenario of population bottleneck seems to be reliable. These results contrast with the expected by the LGH to the tropics, where the highest stability on the effective size of the populations would be associated with the lower latitude regions. The observed scenario is congruent with the strict forest dependency of this species, and with its fragmented distribution, associated with humid forests from the interior of the northeastern Brazilian region ('brejos de altitude'). The 'brejos de altitude' are forest relicts associated with mountain ridges, amidst the semi-arid area of the Caatinga, where the precipitation levels are significantly higher (over 1200 mm/year; Andrade-Lima 1966, 1982) than the reported for the adjacent areas (240–900 mm, IBGE 1985, Lins 1989). The pluviometric regime of this region seems to respond in a distinct way to the observed for other regions of the AF (Auler & Smart 2001; Auler *et al.* 2004; Wang *et al.* 2004), which is compatible with the results for the SN (*Sclerurus scansor cearensis*) that suggest a contraction of forest distribution in a period during which southern forest experienced a wide expansion. A similar phenomenon is documented by the demographic history of other forest organisms (Carnaval & Bates 2007).

The general results of demographic analyses are congruent with the scenario inferred by Carnaval & Moritz (2008) based on modelling of AF paleo-distribution during the Quaternary, where southern AF was the most unstable area and the central region the most stable. Our results are also congruent with the general results obtained by Miller *et al.* (2010), who observed that central populations of tropical bird are more diverse than those from the edge of their ranges, indicating a history of higher instability in the edge than in the centre of their distributions.

The recent history of the *S. scansor* lineages indicates that the AF region, and the organisms strictly dependent on it, suffered deep changes in their distribution at higher as well as at lower latitudes, differing from the expected by the LGH for this region. Additionally, forests from lower and higher latitudes could have reacted even in opposite ways under the same climatic period.

While the AF at higher latitudes expanded, its distribution on lower latitudes became smaller and fragmented.

Conclusions

Because of the complexity of the physiographic and ecological features of the AF, and the intrinsic complexity of the ecophysiology of the AF organisms, it seems too simplistic to imagine that a single diversification mechanism can explain the origin of the current biogeographical patterns exhibited by AF species. However, this study of phylogeography and historical demography of *S. scansor* strongly supports the important role that Late Quaternary climatic changes had to the origin of the current biogeographical patterns.

The expected effects of the climatic cycles over the AF latitudinal gradient, on the other hand, were not confirmed by our results. The intraspecific evolutionary history of *S. scansor* indicates that different regions of AF can react in opposite ways under the same climatic period, generating asynchronous pulses of expansion (and coalescence) and retraction (and fragmentation) of habitats, producing at the same time favourable scenarios for isolation and contact between populations.

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works with phylogeography and conservation of South American birds. Diogo Meyer is an evolutionary geneticist whose research is focused in phylogeography, population genetics, speciation and evolution of HLA genes. The geneticist Cristina Y. Miyaki is interested on systematics, biogeography and conservation of neotropical birds.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Localities of analyzed samples, tissue number, vouchers, and GenBank accession numbers.

Appendix S2 Methods.

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Anexo XIX

Phylogeny and comparative phylogeography of *Sclerurus* (Aves: Furnariidae) reveal constant and cryptic diversification in an old radiation of rain forest understorey specialists

Fernando M. d'Horta^{1*}, Andrés M. Cuervo², Camila C. Ribas³, Robb T. Brumfield² and Cristina Y. Miyaki¹

¹Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Brazil,

²Department of Biological Sciences and Museum of Natural Science, Louisiana State University, Baton Rouge, USA, ³PCAC Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil

ABSTRACT

Aim To evaluate the role of historical processes in the evolution of *Sclerurus* leaf-tossers by integrating phylogenetic and phylogeographical approaches.

Location Humid forests of the Neotropical region.

Methods We reconstructed the evolutionary history of *Sclerurus* based on DNA sequences representing all species and 20 of the 26 recognized subspecies using one autosomal nuclear locus and three protein-coding mitochondrial gene sequences. Phylogenetic relationships were inferred using Bayesian and maximum-likelihood methods. We used Bayesian coalescent-based approaches to evaluate demographic changes through time, and to estimate the timing of diversification events. Based on these results, we examined the temporal accumulation of divergence events using lineage-through-time plots.

Results The monophyly of all *Sclerurus* species was strongly supported except for *Sclerurus mexicanus*, which was paraphyletic in relation to *Sclerurus rufigularis*, and for the sister pair *Sclerurus scansor*–*Sclerurus albigularis*, which were not reciprocally monophyletic in the nuclear tree. We found remarkably deep phylogeographical structure within all *Sclerurus* species, and overall this structure was congruent with currently recognized subspecies and Neotropical areas of endemism. Diversification within *Sclerurus* has occurred at a relatively constant rate since the Middle Miocene.

Main conclusions Our results strongly support the relevance of physiographical (e.g. Nicaragua Depression, Isthmus of Panama, Andean Cordillera, great rivers of Amazonia) and ecological barriers (open vegetation corridor) and ecological gradients (elevational zonation) to the diversification of Neotropical forest-dwelling organisms. Despite the high congruence among the spatial patterns identified, the variance in divergence times suggests multiple speciation events occurring independently across the same barrier, and a role for dispersal. The phylogenetic patterns and cryptic diversity uncovered in this study demonstrate that the current taxonomy of *Sclerurus* underestimates the number of species.

Keywords

Amazonia, Andes, areas of endemism, biogeographical barriers, cryptic diversity, elevational zonation, Great American Interchange, molecular clock, Neotropics, Sclerurinae.

*Correspondence: Fernando Mendonça d'Horta, Rua do Matão 277, 05508-090, São Paulo, SP, Brazil.
E-mail: fmhorta@usp.br

INTRODUCTION

The origin of the high biodiversity that characterizes the Neotropics has long been of interest to biogeographers (Wallace, 1853). Many historical and ecological mechanisms have been hypothesized to explain the origin of the biogeographical patterns within this region (e.g. Chapman, 1917; Haffer, 1969; Ayres & Clutton-Brock, 1992; Bush, 1994; Fjeldså *et al.*, 1999; Brumfield, 2012). In the Neotropical region different taxa often show similar distributions, which are used to define areas of endemism (Cracraft, 1985). Based on the conceptual framework of vicariance biogeography (Platnick & Nelson, 1978) some studies on the diversification of Neotropical birds used congruence among area relationships to infer the biogeographical processes (e.g. Cracraft & Prum, 1988; Marks *et al.*, 2002; Aleixo & Rossetti, 2007). However, ignoring temporal information obscures the connection between biogeographical patterns and their underlying causes (Donoghue & Moore, 2003) because a single pattern of area relationships can be achieved through different processes.

In the last decade temporal inferences have often been incorporated in studies of diversification of Neotropical bird lineages (e.g. Aleixo, 2004; Brumfield *et al.*, 2007; Derryberry *et al.*, 2011). Although many of these taxa have experienced the same history of landscape evolution, the results of these studies revealed differences among spatio-temporal patterns of diversification. The explanation of this may involve stochastic processes (e.g. dispersal and extinction) and ecological differences among taxa, such as dispersal ability and ecophysiological constraints (Burney & Brumfield, 2009).

Here we present a comprehensive study of the historical diversification of the *Sclerurus* leaf-tossers (Aves: Furnariidae). *Sclerurus* is an old, monophyletic group (Derryberry *et al.*, 2011) that comprises six species of strictly understorey, leaf-litter specialists, highly sensitive to habitat disturbance (Stotz *et al.*, 1996). The geographical ranges of *Sclerurus* species are dissected by the major biogeographical barriers of the Neotropics and encompass the main Neotropical forest biomes from southern Brazil to central Mexico.

Herein we analyse the evolutionary history, divergence time and diversification patterns of *Sclerurus* leaf-tossers across the Neotropical region. We maximized the geographical sampling of all taxa to minimize problems with potential cryptic diversity. Thus, we provide a comprehensive temporal and spatial analysis of the history of this group.

MATERIALS AND METHODS

Sampling

We reconstructed the phylogeny of *Sclerurus* based on 119 ingroup samples representing all six species in the genus and 20 of the 26 subspecies (Ramsen, 2003) (see Appendix S1 in Supporting Information). The outgroup taxa were defined based on previous phylogenies (Irestedt *et al.*, 2009; Derryberry *et al.*, 2011). We included two species of *Geositta* miners

(*Geositta poeciloptera* and *Geositta tenuirostris*), that is the sister-group of *Sclerurus*, and representatives of the closely related subfamilies, e.g. *Xenops minutus* (Furnariinae) and *Lepidocolaptes angustirostris* (Dendrocolaptinae). For most samples, we sequenced a total of 2962 bp from fragments of four loci: one autosomal nuclear locus, β -fibrinogen intron 7 (*Fib7*), and three protein-coding mitochondrial DNA (mtDNA) genes: cytochrome *b* (*cyt b*) and NADH dehydrogenase subunits 2 (*ND2*) and 3 (*ND3*). DNA extraction, amplification, sequencing and alignment procedures are described in Appendix S2.

Phylogenetic analyses

The phylogenetic analyses were performed separately for mtDNA (2408 bp) and for *Fib7* (914 bp). To estimate gene trees we used maximum-likelihood (ML) and Bayesian inference (BI) approaches. To select the simplest model of molecular evolution with the highest likelihood for the data, we applied a likelihood-ratio test (LRT) performed using MODELTEST 3.7 (Posada & Crandall, 1998). The ML searches were performed using RAXML 7.0.4 (Stamatakis, 2006), assuming a general time-reversible (GTR) model of evolution with distributed rate heterogeneity, four rate categories, and estimation of the proportion of invariable sites. The ML analyses of mtDNA were carried out considering three partitions (one partition per gene: *cyt b*, *ND2* and *ND3*). To determine if analyses had become trapped in local optima, we conducted 10 independent ML searches. The robustness of the nodes was determined by 1000 bootstrap replicates, using the 'fast bootstrap' algorithm of RAXML. The BI with Markov chain Monte Carlo (MCMC) sampling was implemented in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). As in the ML analyses, a partitioned analysis was conducted with three locus-specific models, estimating parameters independently for each partition (*nst* = 6; *rates* = *invgamma*). We conducted two independent, parallel analyses with four simultaneous chains each for 10 million generations, sampling parameters and trees every 1000 generations. We evaluated the convergence between analyses by comparing the posterior probabilities of clades using AWTY (Nylander *et al.*, 2008). The first 2.5 million generations were discarded as burn-in and the posterior probabilities were estimated from the remaining trees.

Temporal patterns of lineage diversification

The timing of diversification events within *Sclerurus* was estimated using a subset of the mtDNA sequence matrix containing all species and one exemplar of each intra-specific clade that represented divergent populations (Appendix S1). Divergence times were estimated using the Bayesian approach implemented in BEAST 1.5.1 (Drummond & Rambaut, 2007) with the uncorrelated relaxed clock model (uncorrelated lognormal). The calibration used was based on the results of dating of the Furnariidae radiation obtained by Derryberry *et al.* (2011) in a very comprehensive study that included 97%

of furnariid species and used similar genetic markers (*ND2*, *ND3*, *CO2* and *Fib7*). We used the confidence intervals obtained by Derryberry *et al.* (2011) to set lower and upper limits of uniform prior distributions of ages for the most recent common ancestor for five clades within the *Sclerurus* phylogeny. Based on the resulting time-calibrated tree from BEAST, the temporal accumulation of divergence events was examined using lineage-through-time (LTT) plots to test for changes in lineage-splitting events along the evolutionary history of *Sclerurus*. Given the deep phylogeographical structure within *Sclerurus* species (see Results), we used the least inclusive terminal clades for this analysis. We used phylogenetic and likelihood-based statistical methods implemented in R 2.13.0 (R Development Core Team, 2011): packages APE 2.7 (Paradis *et al.*, 2004), LASER 2.2 (Rabosky, 2006) and GEIGER (Harmon *et al.*, 2008).

We compared diversification events of *Sclerurus* among 10 major biogeographical areas to examine the spatial and temporal congruence of divergence in co-occurring lineages. The 10 biogeographical areas considered are: Central America – north (CAN; Mexico to Nicaragua), Central America – south (CAS; Costa Rica to Darién, north-western Colombia), Chocó (CHO; from Darién to western Ecuador), base of the Andes (BAN; eastern Andean foothill forests from southern Colombia to Bolivia), western Amazonia – south (WAS; lowland forests from the right bank of the Amazon/Ucayali rivers to the left bank of the Madeira River), western Amazonia – north (WAN; lowland forests from the left bank of the Amazon/Ucayali rivers to the right bank of the Negro River), Brazilian shields – west (BSW; the right bank of the Madeira River to the left bank of the Xingu River), Brazilian shields – eastern (BSE; the right bank of the Xingu River to the eastern limits of Amazonia), Guiana Shield (GUY; northern Amazonia from the left bank of the Negro River to the eastern limit of Amazonia) and Atlantic forest (ATF; southern Brazil, eastern Paraguay and north-eastern Argentina to north-eastern Brazil). These areas were designated based on their geological, geographical and palaeoecological similarities and distinctions (Bates, 2001; Campbell *et al.*, 2006; Aleixo & Rossetti, 2007) and based on patterns of distributions for terrestrial vertebrates (Haffer, 1974; Cracraft, 1985).

Comparative demography

We examined the molecular signature of past demographic events in Amazonian populations of each species using the mtDNA and *Fib7* data. We used a Bayesian approach implemented in PHASE 2.0 (Stephens *et al.*, 2001; Stephens & Donnelly, 2003) to identify heterozygous haplotypes of *Fib7*. Heterozygous indel positions were not found. To evaluate the existence of significant evidence of recombination in *Fib7*, the pairwise homoplasy index (PHI) test (Bruen *et al.*, 2006) was performed for each lineage using SPLITSTREE 4.10 (Huson & Bryant, 2006). We calculated population genetic summary statistics and estimated parameters such as genetic diversity (Θ) and population growth rate (g) using the LAMARC package (Kuhner, 2006). LAMARC implements a MCMC method for

sampling genealogies and calculates a likelihood curve used to determine the values of Θ and g that maximize the probability of originating the empirical data. LAMARC analyses were performed with five replicates of 10 short chains each (500 genealogies sampled each 50 interactions and a burn-in of 1000 genealogies), and two long chains (20,000 genealogies sampled each 50 interactions and a burn-in of 1000 genealogies). For these analyses we did not consider the migration effect among populations. Because the PHI test indicated that the null hypothesis of no recombination could not be rejected, the parameter r (recombination) was not considered in the LAMARC analyses. In addition, we used Tajima's D (Tajima, 1989), Fu's F_S test (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) to test the scenarios of population expansion. The significance of the tests was determined based on 10,000 coalescent simulations, which assumed neutrality and equilibrium conditions. These analyses were performed using DNASP 4.10.9 (Rozas *et al.*, 2003).

RESULTS

Data characteristics

An alignment of 2048 bp of mtDNA (119 individuals) contained *cyt b* (1022 bp; 117 individuals), *ND2* (1041 bp; 118 individuals) and *ND3* (345 bp; 119 individuals). We obtained 914 bp of the nuclear intron *Fib7* from 107 individuals. No indels were present in the mitochondrial alignment, but some were identified in the *Fib7* dataset. All sequences were deposited in GenBank under the accession numbers JQ903619–JQ904023.

The mtDNA dataset was characterized by 826 (40.3%) variables and 785 (38.3%) parsimony-informative sites, and the *Fib7* dataset by 93 (10.2%) variables and 73 (8.0%) parsimony-informative sites. The GTR + I + Γ evolutionary model (pinv = 0.5353 and α = 1.3229) was selected for all partitions of mtDNA, while the HKY + Γ model (Γ = 0.2708) was selected for *Fib7*.

The uncorrected mtDNA distance between *Sclerurus* and its sister genus *Geositta* (Irestedt *et al.*, 2009; Derryberry *et al.*, 2011) was 18.4%. Within *Sclerurus*, mitochondrial genetic distances ranged from 16.1% (South American *Sclerurus mexicanus* versus *Sclerurus scansor*) to 2.7% (*S. scansor* versus *Sclerurus albigularis*). Although pronounced genetic structure was found within all species, the mean genetic distances among populations within species were quite variable, ranging from 12.1% between the two major clades of the broadly defined *S. mexicanus* (Central versus South American populations) to 0.16% among *S. scansor* populations (Appendix S3).

Phylogeny of *Sclerurus* species

The phylogenetic ML and BI analyses of the mitochondrial and nuclear data sets strongly support the monophyly of the genus *Sclerurus*. Two main clades were identified within *Sclerurus* (Fig. 1). The first includes four light-throated species segre-

Figure 1 Phylogenetic relationships of *Sclerurus* leaftossers. Maximum likelihood tree inferred from (a) 2408 bp of mitochondrial DNA (mtDNA; cyt b, ND2 and ND3) and (b) from 914 bp of *Fib7*. Maximum likelihood bootstrap (1000 replicates) and Bayesian inference posterior probability values are shown at the nodes. Illustrations are from nominate subspecies, except for *Sclerurus mexicanus* from South America, which is represented by subspecies *S. m. obscurior* (courtesy of Lynx Edicions; *Handbook of the birds of the world*, Vol. 8, 2003).

gated into two clades: (1) a sister pair of cross-Andes lowland species, *Sclerurus caudacutus* and *Sclerurus guatemalensis*; and (2) a sister pair of a circum-Amazonian species of foothill montane forests, *S. albigularis* and an Atlantic forest species, *S. scansor* (Fig. 1). The second main clade in *Sclerurus* includes the rufous-throated species: the Amazonian lowland *Sclerurus ruficularis* that forms a polytomy with two deeply divergent *S. mexicanus* clades, one from Central America (Mexico to Darién) and the other from South America (Fig. 1). Except for *S. mexicanus*, all currently recognized species of *Sclerurus* were found to be monophyletic according to the mtDNA gene tree with high statistical support (Fig. 1a). Although the *Fib7* gene tree largely reflects the same phylogenetic structure of mtDNA, the reciprocal monophyly between *S. scansor* and *S. albigularis* was not recovered. However, because the geographical sampling for *S. albigularis* was the least comprehensive of all, our current results do not allow us to draw strong conclusions about the phylogeography of that species (Fig. 1b).

Intra-specific differentiation in *Sclerurus* leaftossers

A striking pattern recovered by the wide geographical sampling is the pervasive population genetic structure within all *Sclerurus* species (Fig. 2). We found congruence between c. 80% of the subspecies sampled and the intra-specific lineages in the mtDNA tree, suggesting an overall good correspondence between phenotypic and phylogeographical breaks in *Sclerurus*. Exceptions were the subspecies of *S. mexicanus* from north-western South America and Darién (see below). However, the observed phylogeographical patterns are even more complicated than those suggested by the currently recognized subspecies. These patterns are largely consistent with the known Neotropical areas of endemism (Figs 2 & 3) and are bounded by either known physiographic barriers such as the Nicaragua Depression, the Andes, the Madeira, Negro and Amazon/Solimões rivers, open diagonal (Caatinga, Cerrado and Chaco), or by ecological gradients such as different elevational zones along the Andean foothills (Figs 2 & 3).

Temporal patterns of divergence

Divergence time estimates were obtained for 27 nodes distributed throughout the *Sclerurus* phylogeny (Fig. 3). Diversification within the genus was estimated to have occurred over the last 12 million years and at a relatively constant rate. A pure-birth model of diversification provided the best fit to the data [diversification rate = 0.28, Akaike information criterion (AIC) = -11.41, Δ AIC = 0, log-likelihood = 6.71], although the LTT curve tended to border the lower end of the expected diversification rate under that model (Fig. 4). Likelihood ratio tests did not reject the pure-birth model as the best fit to the data in comparison with birth-death and two-rate models. Intra-specific lineages of different species separated by a common geographical feature were compared to test for spatial and temporal congruence. Few

patterns were common in both spatial and temporal aspects within *Sclerurus* (Fig. 3, Table 1). The divergence between lineages from Andean foothills and Amazonian lowlands (red line) occurred at similar times both in *S. albigularis* (Fig. 3, node 19) and in South American *S. mexicanus* (Fig. 3, node 6). Also, the split associated with the Amazon River (orange line) occurred at similar times in both *S. ruficularis* (Fig. 3, node 10) and *S. caudacutus* (Fig. 3, node 23).

Historical demography in Amazonia

Tests based on summary statistics suggest distinct demographic scenarios for different populations of *Sclerurus* leaftossers (Table 2). The F_S results for both mtDNA and *Fib7* datasets, as well as the R_2 result for the mtDNA dataset, indicate recent population expansion within the *S. ruficularis* population from WAN. Significant values were also obtained for the *S. caudacutus* and *S. ruficularis* populations from BSW. For *S. caudacutus* the F_S test was significant for *Fib7*, and the R_2 test was significant for *Fib7* and mtDNA. The F_S and R_2 tests within *S. ruficularis* were only significant for the mtDNA dataset. Tajima's D -test results were not significant for any of the studied populations (Table 2). A similar scenario emerged from LAMARC analyses (Fig. 5). Populations of *S. ruficularis* and *S. peruvianus* from GUY and BAN, respectively, exhibited 95% confidence intervals (CI) of the population growth rate (g) that overlapped with zero (Fig. 5); hence, the null hypothesis of constant effective population size was not rejected. On the other hand, positive g values were obtained for populations associated with WAN, WAS and BSW, suggesting scenarios of population expansion (Fig. 5).

DISCUSSION

Origin, diversification and systematics

Sclerurus is one of the oldest genus-level lineages in the Furnariidae radiation (mean estimated age of 22 Ma; c. 26 Ma in Derryberry *et al.*, 2011), yet the genus is relatively homogeneous in morphology and coloration, and has comparatively few species. Our phylogeographical results demonstrated deep genetic divergence within *Sclerurus* species, suggesting that the low phenotypic diversity in *Sclerurus* may obscure cryptic species-level diversity. Our results provide an opportunity for a better taxonomic interpretation of *Sclerurus* diversity, particularly in *S. mexicanus*.

In some cases, intra-specific divergence times, as observed in *S. mexicanus* (3.09–5.49 Ma) and *S. caudacutus* (2.43–3.97 Ma), surpassed the divergence times between distinct species, as in *S. scansor* and *S. albigularis* (1.74–2.81 Ma), and between many sister species pairs in the Furnariidae (Derryberry *et al.*, 2011) and other passerine birds (Roy *et al.*, 1997; García-Moreno *et al.*, 1999; Chesser, 2004). The high genetic diversity within *Sclerurus* species, the long time of isolation of differentiated populations and the potential geographical overlap in some of these forms highlight the need for an

Figure 2 Geographical distribution of genetic variation in *Sclerurus* species in the Neotropics. Phylogeographies are depicted as maximum likelihood trees based on a mitochondrial DNA concatenated dataset with bootstrap and posterior probability values at the node. Individuals are labelled as in Appendix S1 and coloured according to geography and lineage. Each map depicts the overall geographical range of each species and the location of sampled localities coloured and labelled as in each tree and Appendix S1: (a) *Sclerurus albicularis* and *S. scansor*; (b) *S. caudacutus* and *S. guatemalensis*; (c) *S. mexicanus* of Central America (upper right), and *S. obscurior*, *S. andinus*, *S. peruvianus* and *S. macconnelli* of South America (lower left); and (d) *S. ruficularis*.

integrative systematic revision of this group to properly reflect this diversity in its taxonomic classification.

The non-monophyly of *S. mexicanus* given the phylogenetic uncertainty of its two major clades in relation to *S. ruficularis*, as well as the relatively ancient time of isolation among major clades, and the elevational parapatry of divergent lineages along the Andes slopes (see below) support a redefinition of species boundaries in this complex. First, the Central American lineages that include samples referable to the subspecies *Sclerurus mexicanus mexicanus* and *Sclerurus mexicanus pullus* (M1 to M8, Fig. 2c), should be regarded minimally as one full species. Second, divergent South American populations of *S. mexicanus* exhibit elevational parapatry on both sides of the Andes, which is a strong indication of evolutionary isolation and supports the elevation of these populations to species rank.

In particular, along the elevational gradient of the western Andes two deeply divergent clades were identified: one including the birds from the lowlands of Esmeraldas, Ecuador, near the type locality of the taxon *Sclerurus mexicanus obscurior* (M9 and M10, Fig. 2c); and other from the highlands (c. 1100–2000 m) of Ecuador, Colombia and Venezuela (M11–M14, Fig. 2c) corresponding to the range of *Sclerurus andinus*. In the eastern Andean slope we also identified two parapatric lineages along the elevational gradient: one referable to the taxon *Sclerurus macconnelli* is widespread throughout the Amazonian lowlands (M15–M24, Fig. 2c), while *Sclerurus peruvianus* includes populations from the Andean foothills and outlying ridges (M25–M33, Fig. 2c).

Therefore, we suggest the recognition of five species within the current *S. mexicanus*: (1) *Sclerurus mexicanus* Sclater, 1856

Figure 3 Chronogram of *Sclerurus* diversification in the Neotropics from BEAST analysis based on the concatenated mitochondrial DNA dataset. The bars on nodes represent the 95% confidence intervals around the mean divergence times, and are coloured according to the biogeographical break separating sister clades. Individuals analysed are identified as in Appendix S1. Biogeographical areas are: CAN (Central America – north), CAS (Central America – south), CHO (Chocó), NAN (northern Andes), BAN (base of the Andes), WAS (western Amazonia – south), WAN (western Amazonia – north), BSW (Brazilian shields – west), BSE (Brazilian shields – eastern), GUY (Guiana Shield), and ATF (Atlantic forest).

Figure 4 Lineage-through-time plot (logarithmic scale) and 95% confidence intervals (according to the inset legend) under the pure-birth model of *Sclerurus* lineage diversification in the Neotropics.

(type locality: Veracruz, Mexico) of Central America, comprising the ranges of *S. m. mexicanus* Sclater, 1856 and *S. m. pullus* Bangs, 1902; (2) *Sclerurus obscurior* Hartert, 1901 (type locality: Lita, Esmeraldas, Ecuador) of the Chocó lowlands of Ecuador and Colombia; (3) *Sclerurus andinus* Chapman, 1914 (type locality: Buenavista, above Villavicencio, Colombia) of the humid Andean slopes of western Ecuador, Colombia and western Venezuela; (4) *Sclerurus macconnelli* Chubb, 1919

(type locality: Ituribisci River, Guyana), comprising the Amazonian lowlands and, probably, the central Atlantic forest, corresponding to the ranges of subspecies *Sclerurus mexicanus macconnelli* and *Sclerurus mexicanus bahiae*, respectively; (5) *Sclerurus peruvianus* Chubb, 1919 (type locality: Yurimaguas, Loreto, Peru), comprising the populations of eastern Andean foothills from Bolivia to eastern Colombia, also reaching the lowlands and outlying ridges in north-western Amazonia. Hereafter we will follow this classification and refer to *mexicanus* taxa of Central American as *S. mexicanus*, and South American taxa as *S. obscurior*, *S. andinus*, *S. macconnelli* and *S. peruvianus*.

Biogeography

Central–South America disjunction

Despite the uncertainty of evolutionary relationships in the rufous-throated group, our estimates of divergence times indicate that the split between the Central (i.e. *S. mexicanus*) and all the South American lineages (i.e. *S. obscurior*, *S. andinus*, *S. macconnelli*, *S. peruvianus* and *S. rufularis*) occurred during the Middle Miocene, between 6.5 and 10.5 Ma (nodes 2 and 3; Fig. 3, Table 1), and divergence between *S. guatemalensis* clades occurred during Late Pliocene–Early Pleistocene (from 1.0 to 1.9 Ma; node 22; Fig. 3, Table 1). Two hypotheses invoke specific timings of the origin of the Panamanian land bridge between Central and South America. It is generally held that the final closure of the

Isthmus of Panama occurred around 3.5 Ma (Coates *et al.*, 1992). An emerging alternative hypothesis based on recent fossil dating suggests that the final closure occurred between 12

and 20 Ma (Farris *et al.*, 2011; Montes *et al.*, 2012). Considering the ecological characteristics of *Sclerurus* (e.g. presumed low dispersal ability), this new temporal hypothesis is attractive because the dispersion between Central and South America, followed by the differentiation (node 2; Fig. 3, Table 1), would have occurred after the final closure of the Isthmus of Panama, and not before, as under the traditional geological dating. Differences in divergence times across the isthmus have also been observed in other terrestrial organisms (Koepfli *et al.*, 2007; Cody *et al.*, 2010; Smith & Klicka, 2010; Johnson & Weckstein, 2011).

Table 1 Ages and 95% confidence intervals (CI) for nodes of the time-calibrated phylogeny of *Sclerurus* leaftoissers (Fig. 3). Minimum and maximum ages were determined for five nodes following Derryberry *et al.* (2011); see text for details.

Node	Age (Ma)	CI (Ma)	Calibration
1	12.89	11.26–15.11	11.26–16.55
2	8.74	6.99–10.51	
3	7.98	6.47–9.77	6.56–11.82
4	4.73	3.61–5.95	
5	2.55	1.92–3.31	
6	1.42	1.06–1.83	
7	1.19	0.85–1.54	
8	0.92	0.62–1.24	
9	0.66	0.40–0.94	
10	2.98	2.28–3.80	
11	1.78	1.24–2.33	
12	1.41	0.89–1.94	
13	2.17	1.53–2.84	
14	4.23	3.09–5.49	
15	6.76	5.52–8.01	5.40–8.60
16	2.27	1.74–2.81	1.72–3.43
17	0.90	0.61–1.23	
18	0.59	0.36–0.83	
19	1.74	1.30–2.24	
20	1.34	0.95–1.77	
21	5.73	4.80–6.68	3.69–6.68
22	1.47	1.00–1.98	
23	3.20	2.43–3.97	
24	2.02	1.52–2.56	
25	0.92	0.61–1.23	
26	1.08	0.75–1.43	
27	0.65	0.42–0.92	

Central America disjunction

The main phylogeographical break identified within *S. mexicanus sensu stricto* is associated with the Nicaragua Depression, the biogeographical barrier that defines the limits between the CAN and CAS. This barrier is important for a number of unrelated taxa (e.g. Duellman, 1999; Marshall & Liebherr, 2000; Pérez-Emán, 2005). The estimated divergence time between CAN and CAS lineages of *S. mexicanus* was substantial (from 3.09 to 5.49 Ma; node 14; Fig. 3, Table 1), placing the differentiation of *S. m. mexicanus* (CAN) and *S. m. pullus* (CAS) in the Late Miocene–Early Pliocene. Narrow landmasses (Kirby & MacFadden, 2005) and marine gaps occurred in this region during most of the Miocene (Coates & Obando, 1996). The estimated divergence time between the two Central American *S. mexicanus* lineages show a great overlap with that estimated for *Cerropidion* snakes (from 3.1 to 6.0 Ma; Castoe *et al.*, 2009), but is more recent than estimates for other snakes, *Bothriechis* and *Atropoides* (5.7–9.9 and 6.8–10.6, respectively; Castoe *et al.*, 2009), suggesting more than one vicariance event across the Nicaraguan Depression.

Table 2 Results of summary statistical tests applied to populations of *Sclerurus* leaftoissers associated with distinct regions of Neotropics, for mitochondrial DNA (mtDNA; cyt *b*, *ND2* and *ND3*), and to *Fib7*.

Region	Species	Marker	<i>n</i>	Tests		
				<i>D</i>	<i>F_s</i>	<i>R₂</i>
Base of the Andes (BAN)	<i>S. peruvianus</i>	mtDNA	5	−1.16 ns	1.21 ns	0.33 ns
		<i>Fib7</i>	10	1.32 ns	−0.63 ns	0.22 ns
Guiana shields (GUY)	<i>S. rufigularis</i>	mtDNA	6	−0.53 ns	1.10 ns	0.20 ns
		<i>Fib7</i>	10	0.05 ns	−0.91 ns	0.16 ns
Western Amazonia – north (WAN)	<i>S. rufigularis</i>	mtDNA	8	−1.60 ns	−8.19**	0.06**
		<i>Fib7</i>	8	−0.43 ns	−11.67**	0.11 ns
Western Amazonia – south (WAS)	<i>S. caudacutus</i>	mtDNA	11	−0.45 ns	−0.48 ns	0.19 ns
		<i>Fib7</i>	16	−0.41 ns	−1.75 ns	0.16 ns
Brazilian shields – west (BSW)	<i>S. caudacutus</i>	mtDNA	5	−0.68 ns	−0.75 ns	0.14*
		<i>Fib7</i>	10	−0.85 ns	−5.99**	0.11*
	<i>S. rufigularis</i>	mtDNA	7	−1.18 ns	−2.94*	0.12*
		<i>Fib7</i>	14	0.36 ns	−1.12 ns	0.17 ns

n, number of sequences; *D*, Tajima's (1989) *D*-test; *F_s*, Fu's (1997) *F_s*-test; *R₂*, Ramos-Onsins & Rozas' (2002) *R₂*-test. ns, not significant; *P < 0.05; **P < 0.01.

Figure 5 Values of exponential population growth rate (g) of *Sclerurus* lineages from distinct Amazonian biogeographical regions. Light grey bars represent populations for which 95% confidence intervals of g include zero, hence demographic stability is inferred; dark grey bars represent populations that have experienced demographic expansion. GUY: Guiana Shield (*S. rufigularis*); WAN: western Amazonia – north (*S. rufigularis*); WAS: western Amazonia – south (*S. caudacutus*); BAN: base of the Andes (*S. peruvianus*); BSW: Brazilian shields – west (1, *S. rufigularis*; 2, *S. caudacutus*).

Disjunctions across the Andes

The importance of the Andes in the differentiation of the Neotropical biotas is unquestionable, but their function as a primary (Chapman, 1917) or secondary (Haffer, 1967) barrier has been the subject of debate (e.g. Brumfield & Capparella, 1996; Ribas *et al.*, 2007).

The sister species pair *S. caudacutus*–*S. guatemalensis*, as well as *S. obscurior*–*S. macconnelli/peruvianus*, are isolated by the Andes (Fig. 2b,c, respectively). Divergence time estimates between these two cross-Andes pairs are largely similar, overlapping between 4.8 and 6.0 Ma (Fig. 3; nodes 21 and 4). The estimates of divergence time between cis- and trans-Andean lineages of lowland species in these *Sclerurus* taxa (Fig. 3, Table 1) and *Pyrilia* (6.8–8.8 Ma; Ribas *et al.*, 2005), suggest that those lineages diverged between the Late Miocene and Early Pliocene. On the other hand, pairs of montane lineages, i.e. *S. andinus* and *S. peruvianus* (up to 2200 m) diverged during the Late Pliocene (1.9–3.3 Ma; node 5; Fig. 3, Table 1) or, in the case of the *Pionus corallinus*–*Pionus mindoensis* group (up to 3000 m; Ribas *et al.*, 2007) during the Pleistocene. These results are congruent with the expected differential effect that the Andean uplift may have had in species with distinct elevational ranges. Additionally, the divergence times are compatible with the temporal evolution of the Andes (Gregory-Wodzicki, 2000).

In contrast, Ribas *et al.* (2007) estimated the divergence time between cis- and trans-Andean lineages of lowland lineages of *Pionus* as being between 0.34 and 1.41 Ma, indicating dispersal of populations after the Andes had already reached high elevations.

Large variation among divergence times across barriers could arise for a number of reasons, such as the stochasticity of

the coalescent processes, selection, demographic processes and ecological traits. Also, differential rates of molecular evolution between lowland and montane lineages (Gillman *et al.*, 2009) could potentially affect our interpretation of the influence of the Andean uplift on the diversification of *Sclerurus*. Such a pattern is suggested in our data, especially in the South American clade formed by *S. obscurior* (lowlands), *S. andinus* (montane), *S. macconnelli* (lowlands) and *S. peruvianus* (montane), where there appears to be a gradient of branch lengths that decrease with increasing elevation (see Fig. 2c).

Elevational zonation in the trans-Andean foothills

Along the continuum of humid forests from the Chocó lowlands to the slopes of the western Andes, two lineages seem to be segregated elevationally: *S. obscurior* restricted to the Chocó lowlands (M09 and M10, Fig. 2c), and *S. andinus* found locally from about 1000 m (often up to 2000 m) along the western slopes of the Andes from Ecuador to Venezuela (M11–M14, Fig. 2c). The two lineages are potentially syntopic at an intermediate point of the elevational and ecological gradient, where no obvious physical barrier is in place. Furthermore, our results indicate that the lowland Chocó (i.e. *S. obscurior*) and the Andean foothill species (i.e. *S. andinus*) have been evolutionarily isolated for a substantial length of time; they last shared a common ancestor in the Early Pliocene, between 3.6 and 6.0 Ma (node 4; Fig. 3, Table 1). The Andean foothill lineage originated during the Late Pliocene–Early Pleistocene, between 1.9 and 3.3 Ma (node 5; Fig. 3, Table 1). In addition, another case of elevational parapatry is observed between two divergent, non-sister lineages of the South American *mexicanus* group: *S. macconnelli* and *S. peruvianus*. These two species are in close geographical proximity in southern Peru and Bolivia but seem to occupy different elevations along the cis-Andean foothills (M25–M33 versus M15–M24; Fig. 2c). Although elevational zonation is a prominent characteristic of the Andean avifauna (Terborgh, 1977; Graves, 1985), so far only a few studies have documented ecological replacement of closely related, ecologically equivalent species along elevational gradients (Dingle *et al.*, 2006; Cadena, 2007).

Cis-Andean South America disjunctions

Several birds, including *Sclerurus*, exhibit high congruence in the geographical range boundaries of divergent populations across cis-Andean forests. However, our results and the available data strongly suggest that these biogeographical patterns emerged more than once as a result of distinct sequences of cladogenetic events (e.g. Marks *et al.*, 2002; Aleixo, 2004; Ribas *et al.*, 2011).

Recent studies have shed new light on the processes of landscape change in Amazonia since the Miocene (Hoorn *et al.*, 1995, 2010; Rossetti *et al.*, 2005; Campbell *et al.*, 2006). The available data support the idea that different areas of Amazonia experienced distinct rates of landscape change, with WA (see Fig. 3) being the most dynamic (Hoorn *et al.*, 1995;

Rossetti *et al.*, 2005; Campbell *et al.*, 2006). Marine incursions during the Miocene (Räsänen *et al.*, 1995), as well as the development of a mega-wetland system in western Amazonia during the Early to Middle Miocene, caused by the Andean uplift (Hoorn *et al.*, 2010), would have isolated three regions of Amazonia (Bates, 2001; Aleixo & Rossetti, 2007): GUY, BS and BAN (Fig. 3). With the increase in the rate of Andean uplift the sedimentary wedge progressed to the east, promoting the disappearance of the mega-wetland system in western Amazonia and allowing the expansion of *terra firme* environments through this region.

The evolutionary relationships among *Sclerurus* lineages support the geographical predictions that emerge from this scenario. Three species have lineages associated with WA (*S. macconnelli*, *S. caudacutus*, *S. rufigularis*), but no WA lineage occupies basal positions regarding BA, BS or GUY lineages, whereas in *S. macconnelli* and *S. caudacutus*, GUY and BA lineages are basal in relation to WA lineages. In addition, demographic expansion was inferred for populations associated with WA (WAS, *S. caudacutus*; WAN, *S. rufigularis*), whereas the hypothesis of demographic stability was not rejected for populations from BA (*S. peruvianus*) and GUY (*S. rufigularis*) (Fig. 5). The two populations from BSW also exhibit clear signs of population expansion (Fig. 5), unlike that observed for *Xiphorhynchus* (Aleixo, 2004) and *Psophia* (Ribas *et al.*, 2011). These contrasting patterns in BSW are expected considering that this region was partially submerged by the wetland system (Bates, 2001).

Some authors support the hypothesis that the end of the WA mega-wetlands took place during the Miocene (Hoorn *et al.*, 1995; Figueiredo *et al.*, 2009), whereas recent studies indicate that the wetland system may have persisted until the Pliocene or Pleistocene (Rossetti *et al.*, 2005; Campbell *et al.*, 2006; Latrubesse *et al.*, 2010). The estimates of divergence time among WA lineages and the historical demographic results are congruent with the scenario of recent evolution of western Amazonian drainage: the divergence between WA and GUY/BS lineages was estimated at 2.2 Ma (95% CI, 1.5–2.8; *S. rufigularis*, Fig. 3, node 13, Table 1) and 2.02 Ma (1.5–2.6; *S. caudacutus*, Fig. 3, node 24, Table 1); and the divergence time between WAN and WAS lineages of *S. caudacutus* was estimated at 0.9 Ma (0.6–1.2; Fig. 3, node 25, Table 1).

The demographic results obtained for cis-Andean *Sclerurus* could be interpreted as temporally congruent with the mechanism proposed by Haffer (1969), but are highly contrasting with the palaeoenvironmental scenarios inferred for this region (Anhuf *et al.*, 2006), which suggest that forests of GUY and parts of the BS region would have been strongly reduced during glacial periods, while those of WA would have remained stable.

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BIOSKETCH

Fernando M. d'Horta received his PhD from Universidade de São Paulo. His scientific interests include evolution and biogeography of Neotropical birds, based on molecular and morphological data.

Author contributions: F.M.H. and A.M.C. conceived the ideas and conducted the laboratory analysis; F.M.H., A.M.C. and C.C.R. analysed the data; F.M.H. and A.M.C. led the writing; and C.C.R., R.T.B. and C.Y.M. provided samples and participated in the discussions and writing.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Tissue samples analysed, taxa, collection number and localities.

Appendix S2 Procedures of DNA extraction, amplification, sequencing and alignment.

Appendix S3 Mean pairwise (*p*) distances between and within species of *Sclerurus* leaf-tossers.

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Anexo XX

*Eight anonymous nuclear loci for the
squamate antbird (Myrmeciza squamosa),
cross-amplifiable in other species of typical
antbirds (Aves, Thamnophilidae)*

**Fábio Raposo do Amaral, Scott
V. Edwards & Cristina Y. Miyaki**

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Eight anonymous nuclear loci for the squamate antbird (*Myrmeciza squamosa*), cross-amplifiable in other species of typical antbirds (Aves, *Thamnophilidae*)

Fábio Raposo do Amaral · Scott V. Edwards ·
Cristina Y. Miyaki

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Abstract Typical antbirds (~209 species) represent a diverse radiation of Neotropical birds that includes many species of conservation concern. Here we present eight anonymous nuclear loci designed for the squamate antbird *Myrmeciza squamosa*, a species endemic to the Atlantic Forest of Brazil. We also show that those anonymous nuclear loci are amplifiable in a number of other typical antbird species from related genera (*Myrmeciza*, *Pernostola*, *Gymnocichla*, *Myrmoborus*, *Pyriglena* and *Formicivora*), including three threatened species (*Myrmeciza ruficauda*, *Formicivora littoralis*, and *Pyriglena atra*). Those markers will be useful not only to help management of threatened species of typical antbirds, but also to explore their evolutionary histories, both at intra and interspecific levels.

Keywords Anonymous nuclear loci · Typical antbirds · *Thamnophilidae* · *Myrmeciza*

The Neotropical region is home of the typical antbirds, family *Thamnophilidae*, which contains approximately 209 species (Zimmer and Isler 2003). The Atlantic Forest (AF) harbours several endemic species of typical antbirds, including species of conservation concern (IUCN 2011).

Anonymous nuclear loci (ANL) are single-copy nuclear markers, randomly obtained throughout the genome (Balakrishnan et al. 2010). Compared to commonly used introns, these markers may present higher variation (Lee and Edwards 2008), be less affected by selection, gene hitchhiking, and the negative impact of ascertainment bias in marker choice (Balakrishnan et al. 2010), what make them ideal sources of neutral variation.

Eight ANLs were obtained from the squamate antbird following published protocols (Jennings and Edwards 2005; Lee and Edwards 2008). Genomic DNA from one vouchered specimen (LSUMZ B-16940) was extracted using the DNeasy kit (Qiagen, Inc) according to the manufacturer's protocol. The resulting DNA was fragmented using a HydroShear DNA shearing device (Digilab Inc., Holliston, MA), using 20 fragmentation cycles and speed code 3. Blunt-end cloning was performed using a TOPO Shotgun Subcloning Kit (Invitrogen, Carlsbad, CA) using pUC19 vector and chemically competent *Escherichia coli*.

Sixty-four positive colonies were randomly picked using sterile toothpicks, and heated to 95°C for 5 min in 10 µl of sterile water. 3 µl from the latter mixture were used as template in 15 µl PCR using standard M13 primers, consisting of 1.5 µl of BSA 10×, 1.5 µl of buffer 10×, 0.375 µl of each primer (10 µM), 0.9 µl of dNTP mix (10 mM), 7.25 µl of MilliQ water, and 0.5U of *Taq* polymerase (GE Healthcare, Waukesha, WI). Thermal conditions were: 95°C for 5 min, followed by 35 cycles of 95°C (30 s), 50°C (30 s) and 72°C (40 s), and 72°C for 10 min. Weak amplifications and fragments smaller than 500 bp were excluded from further

F. R. do Amaral (✉) · C. Y. Miyaki
Departamento de Genética e Biologia Evolutiva,
Universidade de São Paulo, Rua do Matão, 277,
Cidade Universitária, São Paulo, SP 05508-090, Brazil
e-mail: fabioraposo@gmail.com

Present Address:
F. R. do Amaral
Departamento de Ciências Biológicas, Universidade
Federal de São Paulo, Campus Diadema, Rua Prof. Artur Riedel,
275, Jardim Eldorado, Diadema, SP 09972-270, Brazil

S. V. Edwards
Department of Organismic and Evolutionary Biology, Harvard
University, 26 Oxford Street, Cambridge, MA 02138, USA

steps. A total of 48 amplifications had both strands cycle sequenced using M13 primers and Big Dye Terminator v3.1 (Life Technologies, Gaithersburg, MD), and were run using an ABI 3730 automated sequencer (Life Technologies, Gaithersburg, MD).

After exclusion of 35 low quality eletropherograms, 61 sequences were screened for known coding and non-coding regions using NCBI MegaBLAST and NCBI BLASTn searches, as well as Genome Explorer BLAT based on the Zebra Finch genome. Sequences with matches higher than 20% in coverage plus maximum identity higher than 80% in BLAST searches, or matching mRNAs in BLAT searches were not considered for primer design. Further, in order to exclude loci that could be affected by paralogy, we adopted reasoning similar to that in Lee and Edwards (2008). All database searches were performed in June/2009. Only one strand was used per clone to design primers.

Seventeen sequences were used to design primers in Primer3 (Rozen and Skaletsky 2000) using default parameters. We performed PCR reactions using an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C (30 s), 60°C (30 s) and 72°C (40 s) and a final extension step of 72°C of 10 min. Total volume was 25 µl,

containing 2.5 µl of buffer 10×, 1 µl of dNTP mix (2 µM each), 0.5 U of *Taq*-polymerase (GE Healthcare, Waukesha, WI), 1 µl of each primer (10 µM, Table 1) and 25–50 ng of DNA. From 17 primer pairs, eight primer pairs produced clean, single banded amplifications (Table 1, GenBank JQ321607–JQ321614).

As part of a phylogeography project (Amaral et al. in prep), we successfully sequenced all anonymous loci for 41 individuals of *M. squamosa* and 48 more of its sister species, *Myrmeciza loricata* (Table 1). The only monomorphic marker was Mysq-AL23, which in turn was polymorphic in *M. loricata*. No marker deviated significantly from the null hypothesis of neutral evolution and constant population size according to Tajima's D tests. We also submitted those sequences to NCBI's ORF Finder tool. All markers presented ORFs smaller than 60 aminoacids with exception of Mysq-AL23, which presented potential ORFs ranging from 34 to 126 aminoacids. However, the latter presented allele length polymorphisms (as well as four other markers, see Table 1), potentially interrupting that predicted ORF. Thus, it is possible that our makers contain short coding regions. Finally, we also successfully cross-amplified all markers in 11 of 14 selected antbird species

Table 1 Anonymous nuclear loci forward and reverse primer sequences, and descriptive statistics for *M. squamosa* and *M. loricata*

Loci and primer seq.	Species	N	Approx. amp. length (bp)	Seq. length (bp)	π	Hd	S	h	Tajima' D	GC (%)
Mysq-AL2										
F: GGGAGCCTCAAGATACCACA	<i>M. squamosa</i>	82	457	343	0.00320	0.541	6	7	−0.206	0.369
R: CCATGCTCTTGGCAACACTA	<i>M. loricata</i>	96		342–344	0.00698	0.824	18	19	−0.920	0.373
Mysq-AL8										
F: AGTCCAAGCCTGGCACTTTA	<i>M. squamosa</i>	82	501	368–382	0.00362	0.701	8	8	−0.430	0.421
R: TCAGCCTGTCTGGTGCTATG	<i>M. loricata</i>	94		382	0.00151	0.490	7	8	−1.369	0.416
Mysq-AL16										
F: TTCTTCCATCCCAGCCTAGA	<i>M. squamosa</i>	82	496	422	0.00727	0.846	12	11	0.484	0.381
R: GCTGTGGTTGCCAATATCCT	<i>M. loricata</i>	96		422	0.00464	0.903	16	21	−1.159	0.380
Mysq-AL17										
F: TGCTGCAATAGTCGTACCAGA	<i>M. squamosa</i>	82	453	412	0.00141	0.529	5	6	−0.932	0.344
R: CCAGCAAAGAGAGATTGCAC	<i>M. loricata</i>	94		412	0.00440	0.744	12	13	−0.607	0.341
Mysq-AL18										
F: GAACGGAAGATTGCTTGTGC	<i>M. squamosa</i>	82	400	351	0.00060	0.205	3	4	−1.208	0.310
R: CCTAATAGGCTTCATTTAAATTTTGT	<i>M. loricata</i>	96		350	0.00234	0.604	11	11	−1.622	0.311
Mysq-AL22										
F: TCCACCTATCACCTTCAGC	<i>M. squamosa</i>	80	354	267–268	0.00393	0.518	4	5	0.621	0.300
R: GCTTGGCTTAGCTCTTAATAACATTC	<i>M. loricata</i>	92		267–268	0.00587	0.768	8	8	−0.014	0.302
Mysq-AL23										
F: CTAGGGCCATCCATTCTTCA	<i>M. squamosa</i>	82	393	299–302	0.00000	0.000	0	1	–	0.503
R: CATCCCAAGATCCAACCATC	<i>M. loricata</i>	92		302	0.00483	0.822	10	12	−0.667	0.505
Mysq-AL25										
F: AGTGTCTCGATGGACCAAG	<i>M. squamosa</i>	82	464	311	0.00328	0.590	10	9	−1.296	0.387
R: AAACCTCCTCTTTCCCAAA	<i>M. loricata</i>	94		309–311	0.01610	0.942	24	28	0.045	0.380

The following acronyms were used: N number of gene copies, π nucleotide diversity, Hd haplotype diversity, S number of segregating sites, h number of haplotypes. All Tajima's D tests non-significant

Table 2 Results from cross amplification tests of other typical antbird species

Locus	Myrmeciza			Pyriglena		Pernastola		Gymnocichla		Myrmoborus		Formicivora	
	<i>M. loricata</i>	<i>M. ruficauda</i>	<i>M. ferruginea</i>	<i>M. goeldi</i>	<i>M. melanocephala</i>	<i>P. leucoptera</i>	<i>P. atrata</i>	<i>P. leucomata</i>	<i>P. rufifrons</i>	<i>G. nudiceps</i>	<i>M. myiophobus</i>	<i>F. serrana</i>	<i>F. littoralis</i>
Msq-AL2	X	X*	X	X	X	X	X	X	X	X	X	X**	X**
Msq-AL8	X	X	X	X	X	X	X	X	X	X	X	X	X
Msq-AL16	X	X	X	X	X	X	X	X	X	X	X	-	-
Msq-AL17	X	X	X	X	X	X	X	X	X	X	X	-	-
Msq-AL18	X	X	X	X	X	X	X	X	X	X	X	X	X
Msq-AL22	X	X	X	X	X	X	X	X	X	X	X	X	X
Msq-AL23	X	X	X	X	X	X	X	X	X	X	X	-	-
Msq-AL25	X	X	X	X	X	X	X	X	X	X	X	X	X

"X" stands for successful amplification, "X*" stands for very weak amplification, "X**" stands for products more than 150 bp larger than the expected in *M. squamosa*, and "-" stands for no amplification

(Table 2). Those markers will be useful both to conservation genetics as well as multi-loci phylogenetics and phylogeography of typical antbirds.

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Anexo XXI

Multilocus tests of Pleistocene refugia and ancient divergence in a pair of Atlantic Forest antbirds (*Myrmeciza*)

FÁBIO RAPOSO DO AMARAL,*¹ PATRICK K. ALBERS,†² SCOTT V. EDWARDS† and CRISTINA Y. MIYAKI*

*Departamento de Genética e Biologia Evolutiva, Universidade de São Paulo, Rua do Matão, 277, Cidade Universitária, São Paulo, SP CEP 05508-090, Brazil, †Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 26 Oxford Street, Cambridge, MA 02138, USA

Abstract

The Atlantic Forest (AF) harbours one of the most diverse vertebrate faunas of the world, including 199 endemic species of birds. Understanding the evolutionary processes behind such diversity has become the focus of many recent, primarily single locus, phylogeographic studies. These studies suggest that isolation in forest refugia may have been a major mechanism promoting diversification, although there is also support for a role of riverine and geotectonic barriers, two sets of hypotheses that can best be tested with multilocus data. Here we combined multilocus data (one mtDNA marker and eight anonymous nuclear loci) from two species of parapatric antbirds, *Myrmeciza loricata* and *M. squamosa*, and Approximate Bayesian Computation to determine whether isolation in refugia explains current patterns of genetic variation and their status as independent evolutionary units. Patterns of population structure, differences in intraspecific levels of divergence and coalescent estimates of historical demography fit the predictions of a recently proposed model of refuge isolation in which climatic stability in the northern AF sustains higher diversity and demographic stability than in the southern AF. However, a pre-Pleistocene divergence associated with their abutting range limits in a region of past tectonic activity also suggests a role for rivers or geotectonic barriers. Little or no gene flow between these species suggests the development of reproductive barriers or competitive exclusion. Our results suggests that limited marker sampling in recent AF studies may compromise estimates of divergence times and historical demography, and we discuss the effects of such sampling on this and other studies.

Keywords: anonymous loci, Atlantic Forest, multilocus phylogeography, refugia theory

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Introduction

Neotropical forests are well known for their astonishing diversity, and defining which evolutionary processes

originated such diversity has been the goal of evolutionary biologists for more than a century. Several early naturalists and modern biologists have sought to identify patterns and processes, such as the presence of rivers, cycles of climate variation or geologic events, that may have contributed to the build-up of Neotropical diversity (e.g. Wallace 1852; Haffer 1969, 1993, 1997; Bush 1994; Moritz *et al.* 2000; Bates *et al.* 2008; Rull 2008; Carnaval *et al.* 2009; Hoorn *et al.* 2010; Thomé *et al.* 2010). However, complexity seems to be the rule in the Neotropics, and it has become clear that a single model alone or a specific geological period cannot

Correspondence: Fábio Raposo do Amaral, Fax: +55 11 33196428; E-mail: fabioraposo@gmail.com

¹ Current address: Departamento de Ciências Biológicas, Universidade Federal de São Paulo, Campus Diadema. Rua Prof. Artur Riedel, 275, Jardim Eldorado, Diadema, SP CEP 09972-270, Brazil

² Current address: Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford, OX3 7BN, UK

explain the origin of the entire Neotropical biota (Bush 1994; Bates *et al.* 2008; Rull 2008; Thomé *et al.* 2010; D'Horta *et al.* 2011; Brumfield 2012).

One of the most diverse—but least studied—areas of the Neotropics is the Atlantic Forest (henceforth referred to as AF). Originally distributed mostly throughout eastern Brazil, Paraguay and Argentina (Rizzini 1997; Silva *et al.* 2004), the AF harbours between two and three per cent of all known vertebrate species and stands as one of the five most diverse areas in the world (Myers *et al.* 2000). Currently reduced to 11.4–16% of its preclearing distribution (Ribeiro *et al.* 2009), the AF historically (i.e. 500 years ago) stretched across 25° of latitude and from sea level to 1700 m of altitude and was isolated from other forest biomes in South America by open vegetation (Silva *et al.* 2004). The evolutionary processes involved in the generation of such diversity, however, are still poorly known. Avian phylogenies suggest a complex evolutionary history of species endemic to the AF. Both local diversification (e.g. *Tangara* tanagers, Burns & Naoki 2004) and diversification involving faunal connections with other Neotropical areas have been identified (e.g. Amazon forest, *Xiphorhynchus* woodcreepers, Cabanne *et al.* 2008; Andes, *Amazon* parrots, Russello & Amato 2004; South American savannahs, buteonine hawks, Amaral *et al.* 2009). Even a single avian radiation may present contrasting histories of AF endemics in space and time (ovenbirds, Furnariidae, Derryberry *et al.* 2011). At the micro-evolutionary level, avian phylogeography in the AF has just begun to be explored (e.g. Cabanne *et al.* 2007, 2008, 2011; Mata *et al.* 2009; D'Horta *et al.* 2011; Batalha-Filho *et al.* 2012; Maldonado-Coelho 2012). The high diversity (199) of endemic avian species (Stotz *et al.* 1996) makes birds an excellent group to explore the recent history of the AF.

Refuge isolation has been recently proposed as one of the most powerful forces shaping intraspecific diversity in the AF (Carnaval *et al.* 2009; Carnaval & Moritz 2008; henceforth referred to as the stability-extinction model). Classical refuge theory hypothesizes that forest organisms are isolated in forest patches during periods of pronounced climatic changes that affected life on Earth since the onset of the Quaternary (Haffer 1969; Vanzolini & Williams 1970) and perhaps even earlier (Haffer 1993; Hewitt 1999). Isolation in forest patches surrounded by unsuitable habitat would lead to differentiation, and in some cases, speciation. The stability-extinction model uses present and past models of the distribution of the AF to suggest that current patterns of intraspecific genetic variation are the result of periodic isolation in areas of forest stability (i.e. refuges) and extinction in areas outside refuges during glacial periods, in particular the Last Glacial Maximum (LGM) around 21 000 years

ago. Two main refuges are predicted to have existed in the AF during the LGM (Fig. 1); one between the rivers Doce and São Francisco (i.e. the Bahia refuge) and a second one north of the São Francisco River (the Pernambuco refuge). Areas south of the Doce River would have been environmentally less stable during glaciations. Thus, organisms widespread in the AF should have higher genetic diversity, more population structure and increasingly stable historical demographies as one moves north of the Doce River, while populations south of that river should present lower levels of diversity, less population structure and traces of recent recolonization events from northern populations (Carnaval & Moritz 2008; Carnaval *et al.* 2009).

In addition to refuges, it has been suggested that the development of rivers (Pellegrino *et al.* 2005) and other physiographic barriers (Silva & Straube 1996; Brunes *et al.* 2010; Thomé *et al.* 2010) have been important factors shaping diversity in the AF. Those barrier hypotheses differ in expectations from the refuge hypothesis both spatially (breaks should coincide with barriers, not refuges) and temporally (splits could be much older than those caused by refuges). More importantly, in contrast to the expectations under the refuge hypothesis (Moritz *et al.* 2000), major demographic fluctuations should not occur under the barrier hypotheses, because cycles of extinction and expansion are not expected.

The foundations of AF phylogeography were laid by recent studies on AF endemics from several taxonomic groups. Those studies were mostly based on mitochondrial DNA alone or up to two unlinked markers (e.g. Pellegrino *et al.* 2005; Graziotin *et al.* 2006; Cabanne *et al.* 2007, 2008; Carnaval *et al.* 2009; Fitzpatrick *et al.* 2009; D'Horta *et al.* 2011; Batalha-Filho *et al.* 2012; Maldonado-Coelho 2012), with a few exceptions using three or four independent loci (Tchaicka *et al.* 2007; Martins *et al.* 2009; Brunes *et al.* 2010; Thomé *et al.* 2010; Amaro *et al.* 2012). Theory predicts that inferences based on one or few independent markers may be subject to several locus-specific shortcomings, especially locus-specific stochastic error (see Discussion), which in turn may influence estimates of demographic history, divergence times and population structure (Edwards & Beerli 2000; Hickerson *et al.* 2005, 2006, 2010; Carling & Brumfield 2007; Heled & Drummond 2008; Brito & Edwards 2009). The extent to which those issues affect our current knowledge of AF phylogeography, however, is unknown.

Study system

The Squamate Antbird (*Myrmeciza squamosa*, henceforth referred to as SA) and the White-bibbed Antbird

Fig. 1 Localities sampled in the present study (see also Fig. S2, Supporting information). Squares represent White-bibbed Antbird (WBA) and circles Squamate Antbird (SA). Coloured records represent genetic samples used, while historical (grey) show species distributions. The original, preclearing distribution of the Atlantic Forest (AF) is indicated in light grey, state borders in dark grey and major rivers in black. The main rivers in the area (Paraguaçu, de Contas, Pardo, Jequitinhonha, Doce, Paraíba do Sul, Tietê, Paranapanema, and Iguaçu) are indicated. The upper inset shows the forest modelled after a recent stability-extinction model for the AF (Fig. 3b from Carnaval & Moritz 2008; AF broader definition—see original reference for additional details and also a more specific definition of the AF), indicating both Central Atlantic Forest (CAF) and Southern Atlantic Forest (SAF). Areas in black and orange show regions of most probable predicted stability, while areas in yellow and green show regions of lesser and least probable predicted stability. Acronyms of states originally covered by the AF are indicated in red. Dashed lines indicate areas of putative geographic barriers contained in WBA/SA distribution, according to Thomé *et al.* (2010). The lower inset indicates the area of the mutual distribution limit of WBA and SA, as well as the River of Paraíba do Sul Valley. Light blue squares represent WBA localities identified in the STRUCTURE runs as the 'CD cluster' (see text).

(*Myrmeciza loricata*, WBA) are sister species (F. R. Amaral & C. Y. Miyaki, in preparation, G. Bravo & R. T. Brumfield, in preparation) of suboscine birds endemic to the Brazilian AF, where they inhabit both lowland and montane humid forests from sea level to 1000 m or higher (Ridgely & Tudor 2009). They occur in parapatry, and their combined distributions cover the central and southern portions of the AF, including most of the humid forest south of the São Francisco River (Fig. 1). Under the stability-extinction model, WBA's range includes both predicted areas of stability in the AF (the Bahia refugium, Fig. 1) as well as unstable areas south of the Doce River. However, SA's range lies entirely outside major refuges, in putatively unstable areas (Fig. 1). Thus, these two species are excellent models to test the effects of refuge isolation in the AF predicted

by the stability-extinction model, as contrasting demographic histories would be expected for each species. In addition, many geologic faults and rivers not only dissect both species' distributions, but also roughly coincide with the area of their abutting ranges (Fig. 1), thus offering an opportunity to evaluate alternative processes of diversification (i.e. barrier hypotheses).

In addition to their utility as models for diversification processes in the AF, the WBA/SA species pair represents an interesting opportunity to study speciation of suboscine birds. Vocalizations are thought to be innate and very important in maintaining species integrity among antbirds (Isler *et al.* 1998), and for this reason, vocalizations have been used to help establish species limits—including in *Myrmeciza* antbirds (e.g. Chaves *et al.* 2010). However, WBA and SA are not only similar

in behaviour and plumage (males being especially alike, with females differing in throat and mask colour), but more remarkably, in vocalizations (Zimmer & Isler 2003). Their very similar songs associated with their parapatric distributions may raise questions concerning the strength of their reproductive isolation (see Fig. S1, Supporting information for sonograms).

Here we explore patterns of intra- and interspecific variation of WBA and SA using one of the largest sequence-based multilocus data sets used so far in a study of Neotropical phylogeography (nine putatively unlinked regions). We address the question whether patterns of divergence and historical demography fit the expectations of refuge isolation and/or the barrier hypotheses in the case of WBA and SA. In addition, due to their phenotypic similarities—vocalization in particular—and parapatry, we further ask whether multilocus data support recognition of WBA and SA as distinct evolutionary units.

Methods

Population and marker sampling

We obtained 89 antbird samples (48 WBA and 41 SA) from 26 localities (13 for each species), comprising one to nine individuals per locality (Fig. 1 and Table S1, Supporting information). Fieldwork in Brazilian National Parks and private land (2008–2010) provided most of the specimens, but additional samples were obtained from tissue collections. Birds were collected using the method described in Amaral *et al.* (2012a). Most individuals were represented by vouchered specimens (86 of 89). Species identification followed Ridgely & Tudor (2009).

We obtained sequences from nine markers, including eight anonymous nuclear loci designed specifically for SA (Amaral *et al.* 2012b, 268–422 bp), as well as complete sequences of the mitochondrial gene NADH subunit 2 (ND2, 1041 bp) using primers described elsewhere (for mitochondrial primer sequences and references see Table S2, Supporting information). Sequences were deposited in GenBank under the accession numbers KC714092–KC715586.

DNA extraction and sequencing

DNA was extracted using a modified version of the phenol–chloroform method of Bruford *et al.* (1992) as described by Tavares *et al.* (2006), or the DNeasy kit (Qiagen Inc.) according to the manufacturer's protocol. PCR was performed in 25 μ L reactions, containing 2.5 μ L of buffer 10 $\times$ (Pharmacia), 1 μ L of dNTP mix (2 μ M each), 0.5 U of Taq-polymerase (Pharmacia), 1 μ L

of each primer (10 μ M, Table 1) and 25–50 ng of DNA. Thermal cycling conditions for all markers were an initial denaturation step of 95 $^{\circ}$ C for 5 min, followed by 40 cycles of 95 $^{\circ}$ C (30 s), 60 $^{\circ}$ C (30 s) and 72 $^{\circ}$ C (40 s), and a final extension step of 72 $^{\circ}$ C of 10 min. Single-band products were purified using polyethylene glycol (PEG) precipitation or EXO-SAP enzymatic purification and sequenced using Big Dye terminator 3.1 cycle sequencing kit (Life Technologies), according to the manufacturer's protocol. The primers used for initial PCR and occasional allele-specific PCR (AS-PCR) were used for sequencing (Tables S2 and S3, Supporting information). Sequences were obtained using an ABI 3100, 3130 or 3730 automated sequencer (Life Technologies).

Both strands were assembled in contigs, and sequences were inspected and corrected by eye using Codoncode Aligner 3.7.1 (Codoncode Inc.). Heterozygous positions were first coded according to the IUPAC code. Individuals exhibiting alleles with indels had their heterozygous positions resolved with the method of Flot *et al.* (2006) as implemented in Champuru (Flot 2007) or using AS-PCR. Sequences were aligned in Clustal X 2.1 (Larkin *et al.* 2007) using default parameters and had their edges trimmed to equal sequence lengths in Bioedit 7.0.8 (Hall 1999).

Haplotype estimation, recombination, descriptive statistics and neutrality tests

Phased haplotypes were initially estimated computationally using Bayesian methods as implemented in PHASE (Stephens & Donnelly 2003). Gametic phases inferred with posterior probabilities equal or higher than 0.6 were considered resolved (Harrigan *et al.* 2008), and gametic phases with lower posterior probabilities were resolved using AS-PCR.

We tested for signs of past recombination using a PHI Test (Bruen *et al.* 2006) as implemented in SplitsTree4 (Huson & Bryant 2006). In case of a significant signal of recombination, the marker was reduced to the largest nonrecombining block based on the four-gamete test (Hudson & Kaplan 1985) performed in DNAsp 5.1 (Librado & Rozas 2009).

Nucleotide diversity (π), haplotype diversity (H_d), number of haplotypes (h) and number of segregating sites (S) were calculated for each marker and species. We tested for statistically significant differences in diversity estimators between species for each estimator using Mann–Whitney two-tailed tests using R 2.13.1 (R Development Core Team 2013). To detect significant deviations from the null hypothesis of neutral evolution and constant population size, we performed Tajima's D tests (Tajima 1989), Fu's F_s (Fu 1997) and R^2 tests

Table 1 Descriptive statistics and neutrality tests for each marker separated by species and STRUCTURE cluster. Bold numbers with asterisks indicate statistically significant results based on coalescent simulations. WBA non-CD does not include locality 9 (see STRUCTURE results for explanation)

	N	Length (bp)	π	Hd	S	h	Tajima' D	Fu's Fs	Ramos & Rozas's R ²
<i>ND2</i>									
WBA	47	1041	0.00317	0.946	26	21	-1.455	-10.829*	0.058*
WBA/CD cluster	6	1041	0.00211	0.867	6	4	-0.932	-0.326	0.248
WBA/non-CD cluster	36	1041	0.00302	0.927	21	16	-1.282	-6.467*	0.070
SA	40	1041	0.00072	0.571	7	9	-1.509	-6.181*	0.057*
<i>Mysq-AL2</i>									
WBA	96	72	0.01387	0.687	6	8	-0.328	-2.111	0.083
WBA/CD cluster	12	72	0.01178	0.667	2	3	0.822	0.360	0.212
WBA/non-CD cluster	74	72	0.01409	0.673	6	8	-0.415	-2.373	0.085
SA	82	72	0.00161	0.116	1	2	-0.505	-0.271	0.058
<i>Mysq-AL8</i>									
WBA	94	382	0.00151	0.490	7	8	-1.369	-4.523*	0.043
WBA/CD cluster	12	382	0.00206	0.667	2	3	0.554	0.217	0.197
WBA/non-CD cluster	74	382	0.00127	0.416	6	7	-1.435	-4.284*	0.047
SA	82	368-382	0.00362	0.701	8	8	-0.430	-1.213	0.082
<i>Mysq-AL16</i>									
WBA	96	68	0.00092	0.041	3	3	-1.614	-3.211	0.075
WBA/CD cluster	12	68	0.00000	0	0	1	—	—	—
WBA/non-CD cluster	74	68	0.00119	0.054	3	3	-1.650	-2.836	0.086
SA	82	68	0.00202	0.137	1	2	-0.378	-0.049	0.069
<i>Mysq-AL17</i>									
WBA	94	412	0.00440	0.744	12	13	-0.607	-3.619	0.074
WBA/CD cluster	12	412	0.00338	0.439	4	3	0.184	1.403	0.168
WBA/non-CD cluster	72	412	0.00374	0.691	12	13	-1.054	-5.370*	0.063
SA	82	412	0.00141	0.529	5	6	-0.932	-2.258	0.065
<i>Mysq-AL18</i>									
WBA	96	350	0.00234	0.604	11	11	-1.622	-6.527*	0.038*
WBA/CD cluster	12	350	0.00429	0.742	4	5	0.466	-0.903	0.180
WBA/non-CD cluster	74	350	0.00209	0.575	8	8	-1.409	-3.752*	0.046
SA	82	351	0.00060	0.205	3	4	-1.208	-2.543*	0.048
<i>Mysq-AL22</i>									
WBA	92	267-268	0.00587	0.768	8	8	-0.014	-0.495	0.097
WBA/CD cluster	12	268	0.00384	0.667	2	3	1.624	0.747	0.258
WBA/non-CD cluster	70	267-268	0.00631	0.777	7	7	0.385	0.167	0.119
SA	80	267-268	0.00393	0.518	4	5	0.621	0.447	0.131
<i>Mysq-AL23</i>									
WBA	92	302	0.00483	0.822	10	12	-0.667	-4.061*	0.072
WBA/CD cluster	12	302	0.00492	0.788	5	5	-0.380	-0.927	0.137
WBA/non-CD cluster	70	302	0.00468	0.815	9	11	-0.643	-3.837*	0.078
SA	82	299-302	0.00000	0.000	0	1	—	—	—
<i>Mysq-AL25</i>									
WBA	94	309-311	0.01610	0.942	24	28	0.045	-8.502*	0.101
WBA/CD cluster	12	311	0.01540	0.879	13	7	0.475	-0.130	0.171
WBA/non-CD cluster	72	309-311	0.01592	0.937	21	24	0.246	-6.605*	0.114
SA	82	311	0.00328	0.590	10	9	-1.296	-3.183	0.058
Mean polymorphism									
WBA			0.00589	0.672	12	12			
WBA/CD cluster			0.00531	0.635	4	4			
WBA/non-CD cluster			0.00581	0.652	10	11			
SA			0.00191	0.374	4	5			
Mean nuclear polymorphism									
WBA			0.00623	0.637	10	11			
WBA/CD cluster			0.00571	0.606	4	4			

Table 1 Continued

	N	Length (bp)	π	Hd	S	h	Tajima' D	Fu's F_s	Ramos & Rozas's R^2
WBA/non-CD cluster			0.00616	0.617	9	10			
SA			0.00206	0.350	4	5			

CD, Chapada Diamantina; h, number of haplotypes; Hd, haplotype diversity; π , nucleotide diversity; S, number of segregating sites; SA, Squamate Antbird; WBA, White-bibbed Antbird.

(Ramos-Onsins & Rozas 2002). Significance was obtained based on 1000 coalescent simulations. All tests and simulations were conducted with DNAsp 5.1.

Population structure

Median-joining networks were inferred using the software NETWORK (Fluxus-Engineering) to explore general patterns of variation for each marker. Population structure was assessed with STRUCTURE 2.3.1 (Pritchard *et al.* 2000) using nuclear markers only, with alleles used instead of individual SNPs. Three data sets were examined: one with all samples from both species, and each species separately. We adopted the admixture model, using both models of independent and correlated allele frequencies. Twenty independent runs were performed for 10 values of K , using 500 000 generations burn-in and 5 000 000 total generations. To detect subtle substructure, the single species runs were also analysed using the LOCPRIOR model (Hubisz *et al.* 2009) and locality information (Table S1, Supporting information). A posteriori choice of the best K was performed with the ad hoc method proposed by Pritchard *et al.* (2000). Each set of runs per K was averaged using CLUMPP (Jakobsson & Rosenberg 2007). In order to evaluate concordance between the population structure found in STRUCTURE and mtDNA variation, we performed analyses of molecular variance—AMOVA (Excoffier *et al.* 1992), as implemented in Arlequin 3.5 (Excoffier *et al.* 2005), with the groupings found in STRUCTURE assumed as populations. Significance was estimated based on 50 000 permutations. Localities represented by only one individual were excluded from AMOVA tests.

Inferences of demographic history

We explored the speciation history of both species by fitting the data set to a model of isolation with migration as implemented in IMA2 (Nielsen & Wakeley 2001; Hey & Nielsen 2007; Hey 2010). The HKY (Hasegawa *et al.* 1985) model was applied for all markers. Several runs were performed in order to establish the best priors for effective population size, time of divergence and migra-

tion parameters. The final run was performed using 2 000 000 generations of burn-in, 100 000 trees sampled during 1 000 000 generations, and use of 20 chains (command line: -q10.0 -t8.0 -m1.0 -b2000000 -l100000 -d10 -p234567 -hfg -hn20 -ha0.96 -hb0.9). The estimated parameters were converted to demographic units assuming substitution rate ranges for ND2 from 0.8×10^{-8} substitutions/site/year (Fleischer *et al.* 1998), and for anonymous loci from the mean of 0.135×10^{-8} substitutions/site/year (Ellegren 2007). Conversions were also performed using several additional substitution rates, in order to evaluate the impact of rate choice on estimates (Table S4, Supporting information). We assumed a generation time of 1 year. Sensitivity of results to marker sampling was evaluated by performing runs using the total data set, as well as mtDNA-only and nDNA-only data sets.

Increasing effective population size through time is one of the strongest expectations of the refuge model, but not of the barrier hypothesis. We performed GMRF Skyride Plots (GSP, Minin *et al.* 2008) for mtDNA-only data sets and Extended Bayesian Skyline Plots (EBSP) for the nuclear multilocus data (Heled & Drummond 2008), which provide historical estimates of population size fluctuations. We fitted a HKY model for all markers and performed runs based on groups found in STRUCTURE. Invariant markers were excluded. We adopted a strict molecular clock and uniform priors on substitution rates ranging from 0.8 to 1×10^{-8} substitutions/site/year for ND2 (Fleischer *et al.* 1998; Lovette 2004) and $0.12\text{--}0.256 \times 10^{-8}$ substitutions/site/year for nuclear markers (Ellegren 2007; Lee & Edwards 2008). Chains were run for 500 million generations, with sampling performed at each of 25 000 steps.

Approximate Bayesian Computation

Because some results from the IMA2 analyses were equivocal (i.e. we were not able to estimate divergence times in WBA-only runs, and very small migration rates were inferred between WBA and SA), we also used Approximate Bayesian Computation (ABC) as a method for demographic inference to estimate the age of divergence within WBA and to test potential gene flow

between SA and WBA (for review, see Beaumont *et al.* 2002; Beaumont 2010; Csilléry *et al.* 2010; Tsai & Carstens 2013). We focused on nuclear variation in the ABC analyses to avoid analytical difficulties related to the divergent rates of evolution of mitochondrial and nuclear sequences. Two demographic scenarios were considered: a reproductive isolation model and a post-divergence migration model, both representing potential outcomes of vicariance followed by secondary contact. In both models, the times of divergence within the WBA complex were estimated under a uniform prior distribution (0–2 million years ago). Scenarios only differed in an additional parameter in the migration model, included as postdivergence, bidirectional migration between SA and the adjacent WBA cluster under a uniform prior (0–1 diploid individuals per generation), occurring after divergence within the WBA complex. Between-species gene flow was excluded in the isolation model. Remaining demographic parameters were set to values inferred using IMA2 for nuclear data. Four diversity estimators (nucleotide diversity, haplotype diversity, number of haplotypes and number of segregating sites) served as summary statistics for the ABC analysis. These measures were obtained for SA and each WBA cluster as well as the combined population total. In addition, we calculated pairwise F_{ST} (Hudson *et al.* 2002) between each pair of SA and WBA clusters. For each summary statistic, we used the mean of single-marker measures obtained for the nuclear data set. Both speciation scenarios were simulated under the coalescent process using the programme Bayesian Serial SimCoal (BayeSSC; Anderson *et al.* 2005), which is a modification of SimCoal 1.0 (Excoffier *et al.* 2000), and the analysis was performed using the abc package in R (Csilléry *et al.* 2012).

Before estimating demographic parameters, we used ABC for model selection, using half a million simulations per model. Posterior probabilities for each model were obtained using the logistic regression method (Beaumont 2008) as well as the simpler rejection-sampling method (Tavaré *et al.* 1997), whereas support for each model was compared by calculating Bayes factors from obtained probabilities (Kass & Raftery 1995; Robert 2007). The subsequent ABC parameter estimation step was carried out for the better supported demographic model (the isolation model), for which we obtained one million simulations. We used the weighted local-linear regression algorithm (Beaumont *et al.* 2002) and applied a correction for heteroscedasticity as implemented in the abc package. Because the regression adjustment can potentially result in posterior distributions that exceed the bounds of the corresponding prior distributions, it is sometimes recommended to transform parameters (Beaumont *et al.* 2002; Blum &

François 2009). Therefore, following Hamilton *et al.* (2005), we applied a log-tangent transformation of parameters before regression. The resulting posterior distribution was back-transformed to record point estimates and the corresponding 95% highest posterior density (HPD) interval. Inferred demographic parameters were eventually confirmed by conducting back simulations.

Results

Genetic diversity

From a potential total of 801 sequences (89 individuals $\times$ 9 markers), all but six were successfully obtained and, if nuclear, had gametic phases resolved. Statistically significant PHI tests indicated signs of recombination in two markers, Mysq-AL2 and Mysq-AL16, which were reduced to 72 and 68 bp from alignments of 346 and 422 bp, respectively.

Descriptive statistics (Table 1) indicated that ND2 variation was higher than the mean nuclear polymorphism in most measures, except for nucleotide diversity. Variation of two nuclear markers (SA: Mysq-AL8 and Mysq-AL25) was also higher than that of ND2 in number of segregating sites and haplotype diversity. No marker showed significant deviation from the null hypothesis of neutrality and constant population size according to Tajima's D test. Significant values of R^2 and/or Fu's F_s tests, however, were obtained in some cases (ND2: WBA and SA; Mysq-AL8: WBA; Mysq-AL17: WBA; Mysq-AL18: WBA and SA; Mysq-AL23: WBA; and Mysq-AL25: WBA; see Table. 1). Levels of genetic diversity were significantly higher in WBA than in SA in all variables measured, namely nucleotide diversity ($W = 64$, $P = 0.04$), haplotype diversity ($W = 66$, $P = 0.02$), number of haplotypes ($W = 67.5$, $P = 0.02$) and number of segregating sites ($W = 68$, $P = 0.02$).

Population structure

Median-joining networks suggest very limited shared variation between WBA and SA (Fig. 2). In five markers, the species share no variation (ND2, Mysq-AL8, Mysq-AL18, Mysq-AL22 and Mysq-AL23), while in the remaining markers (Mysq-AL2, Mysq-AL16, Mysq-AL17 and Mysq-AL25), they share a maximum of two alleles. ND2 haplotypes of WBA and SA are separated by 46 substitutions (or a Kimura 2-Parameter net divergence of 4.8%). A minimum of one to four substitutions separate WBA from SA alleles of nuclear markers lacking shared variation. The reciprocal monophyly of most of the markers (6 of 9) underscores the substantial divergence of this species pair.

Fig. 2 Median-joining networks inferred for data sets of combined White-bibbed Antbird and Squamate Antbird samples. Squares represent inferred haplotypes/alleles.

STRUCTURE analyses based on all individuals recovered two clusters that match allocation of individuals to WBA and SA, irrespective of number of K or model used (Fig. 3 and Fig. S2, Supporting information). Admixture appears to be very limited or nonexistent, and no individual presented ancestry coefficients of less than 96.2% to its respective cluster when $K = 2$ is considered, independently of the model adopted. Based on the ad hoc method of Pritchard *et al.* (2000), $K = 2$ is the best choice under the model of admixture with independent allele frequencies, while $K = 5$ is favoured under the model of admixture with correlated allele frequencies. When species were analysed separately, all runs had $K = 1$ as best choice for SA. The best number of clusters for WBA varied among from $K = 1$ (admixture with correlated allele frequencies), $K = 2$ (LOCPRIOR + admixture with independent or correlated allele frequencies) and $K = 3$ (admixture with independent allele frequencies). Runs for WBA based on LOCPRIOR and independent allele frequen-

cies identified a cluster composed of individuals 43–48, corresponding to the region of Chapada Diamantina (henceforth referred to as CD), that is, localities 12 and 13 (Figs 1 and 3 and Fig. S2, Supporting information). The same clustering appeared when LOCPRIOR with a model of correlated allele frequencies was used, but in this case, the cluster also included individuals 34–38 (from locality 9). Given the discordance across models, which could result from introgression between CD and non-CD clusters or simply lack of statistical power to establish the boundaries between those two clusters, we focused on the cluster of individuals that was present in most STRUCTURE runs, namely the CD cluster (individuals 43–48; Fig. 3 and Fig. S2, Supporting information) and excluded WBA locality 9 from most downstream analyses (except in ABC). AMOVA combining SA and WBA indicate that 95.3% of mtDNA variation is explained by interspecific differences ($P < 0.01$). Intraspecific AMOVA performed between WBA's CD and non-CD clusters (excluding locality 9) indicates that

Fig. 3 STRUCTURE plots depicting population structure between samples of White-bibbed Antbird (WBA) and Squamate Antbird (a and b), and substructure within WBA (c and d). Plots shown represent the best K according to the ad hoc method after Pritchard *et al.* (2000). Panels (a) and (b) result from runs under the models of admixture with independent allele frequencies and admixture with correlated allele frequencies, respectively. Panels (c) and (d) result from runs under the models of LOCPRIOR plus admixture with independent allele frequencies and LOCPRIOR plus admixture with correlated allele frequencies, respectively. The red dashed line in (c) and (d) marks WBA's Chapada Diamantina cluster. See Fig. S2 (Supporting information) for additional plots.

24.0% of mtDNA variation is due to among-cluster differences ($P > 0.05$).

Divergence population genetics

Isolation-with-migration (IMa2) analyses using the total data set (Fig. 4 and Table S4, Supporting information) suggest that divergence between species occurred at 3.8 million years ago (mya) and a 95% Highest Posterior Density (HPD) interval ranging from 2.2 to 5.4 mya. Estimates of effective population size of WBA (1.9×10^6 individuals, 95% HPD: 1.4×10^6 – 2.4×10^6) are more than four times larger than for SA (0.4×10^6

individuals, 95% HPD: 0.3×10^6 – 0.6×10^6). The estimated 95% HPD interval for ancestral effective population size is large (8400 – 1.3×10^6 individuals) and encompasses the 95% HPD interval of SA, but is lower and does not overlap with the 95% HPD interval estimated for WBA. Population migration rates are nonzero in both directions, being almost 10 times larger from SA to WBA (2NM maximum-likelihood = 0.104, 95% HPD: 0.026–0.261) than from WBA to SA (2NM maximum-likelihood = 0.017, 95% HPD: 0.001–0.079). Both rates are significantly different from zero according to the likelihood ratio test of Nielsen & Wakeley (2001), implemented in IMa2. In comparison with runs based on the

Fig. 4 Isolation-with-migration plots. Solid lines represent runs based on the total data set, while dashed lines correspond to nuclear-only runs. Assumed rates were 0.008 substitutions/site/lineage/million years for mtDNA and 0.00135 substitutions/site/lineage/million years for nDNA, but see Table S4 (Supporting information) for alternative conversions.

total data set, Ima2 analyses based on nuclear markers only resulted in very similar point estimates and 95% HPD ranges of effective population sizes and Population migration rates (Fig. 4 and Table S4, Supporting information). However, divergence times were approximately 1 million years younger in the nDNA-only data set compared to results from the total data set (2.6 mya, 95% HPD: 1.5–4.4 mya). Runs based on mtDNA alone did not contain sufficient information to estimate parameters in the full model and were not considered.

Historical fluctuations of population size

Three clusters were analysed in separate runs to infer Bayesian Skyline and GMRF Skyride Plots: SA, WBA/CD and WBA/non-CD (Fig. 5). Given the requirement for panmixis to perform those inferences, individuals of WBA from locality 9 were not considered because their uncertain placement in the STRUCTURE runs could result from gene flow between CD and non-CD populations. Invariant markers were excluded for SA EBS runs (Mysq-C23) and CD cluster EBS runs (Mysq-C16). Based on medians of runs using the total data set (Fig. 5), there were no signs of historical population fluctuations in the WBA/CD cluster, while population size increase was inferred for both WBA/non-CD and SA. A gradual increase in the non-CD WBA may have begun at approximately 2 million years ago, while a sharp increase in population size for SA was inferred to have begun approximately 20 000 years ago. Nuclear-only EBSs were similar to those obtained using the total data set. The mtDNA-only GSPs for the CD cluster and SA were similar to those obtained with multilocus data, but with much shorter observation windows. The mtDNA-only estimates for non-CD WBA contrast with

the long-term stability inferred using the total and nDNA-only data sets, although 95% HPD intervals are large and could accommodate a scenario of stability (i.e. a flat line). All mtDNA-only estimates presented an observation window of less than 500 000 years, compared to millions of years in the nDNA and mtDNA + nDNA estimates.

Intraspecific divergence and interspecific gene flow

Based on the clustering inferred using STRUCTURE, we included CD and non-CD WBA as demographic units besides SA in our ABC analysis. Here, we included WBA non-CD locality 9, because it could provide insights into gene flow estimates. With the estimates of diversity for each marker, the observed vector of summary statistics contained 19 mean values of single-locus statistics (four measures for each of the three groups and the population total, and one between each group). We evaluated two scenarios that differed in the level of cross-species gene flow as demographic parameter, that is, presence or absence of migration in a reproductive isolation model and postdivergence migration model, respectively. In the latter, bidirectional migration was included between SA and WBA/non-CD as adjacent cluster, after it diverged from the CD cluster. Under the logistic regression method for model selection in ABC, Bayes factors (BF) substantially supported the reproductive isolation model (BF = 4.92) over the postdivergence migration model (BF = 0.20, see Fig. 6a). This result was further emphasized when the analysis was carried out using the rejection-sampling method (BF = 8.86 and 0.11, for isolation and migration, respectively). Under the isolation model, the time of divergence of CD and non-CD WBA was esti-

Fig. 5 Extended Bayesian Skyline and GMRF Skyride Plots. Color lines represent EBSs based on mtDNA + nDNA data, light grey lines represent nuclear-only EBSs and black lines represent mtDNA GSPs. The same runs are presented side by side using different scales for a detailed view of the last 100 000 years. The map colours represent White-bibbed Antbird (WBA)/Chapada Diamantina (CD) cluster (light blue), WBA/non-CD cluster (dark blue) and Squamate Antbird (red). WBA's locality 9, which has been excluded from those analyses (see text for reasons), is represented in black. Nucleotide diversity is also illustrated.

mated at around 135 000 YBP (95% HPD: 42 000–1 388 000) when taking the mode as point estimate, or even earlier when considering the median (249 000 YBP) or mean (359 000 YBP) of the posterior distribution (Fig. 6b). As the migration model was not supported, no gene flow between SA and the adjacent WBA/non-CD cluster was found to be present after the divergence of the CD/non-CD divergence.

After parameter estimation, we assessed these results in a model checking procedure, whereas the inferred divergence time was fixed in the isolation model, and replicate simulations were performed under the coalescent process to obtain posterior predictive distributions for each summary statistic. Observations falling outside or in the far end of tails (e.g., $P \leq 0.025$) of the predictive distributions suggest that parameters could not be appropriately estimated, most likely due to an insufficient choice of summary statistics and/or an inappropriate demographic model (Beaumont 2010). However, none of the observed summary statistics appeared outside of its posterior predictive distribution (Fig. S3, Supporting information), and the probabilities of observations to falling in either tail of the distribution were all $P \geq 0.1$ (mean P -value: 0.32). Thus, our results indicate that both the demographic model used and the divergence time inferred were adequate to approximate the underlying evolutionary history of the WBA/SA species pair.

Discussion

Myrmeciza antbirds: evidence of refugia, barriers or both?

In our study of two species of endemic antbirds, we tested alternative scenarios of diversification in the hyper-diverse Atlantic Forest (AF) biome based on the largest sequence-based data set of an AF endemic organism thus far. Our results fulfil expectations of the refuge theory as elaborated in the stability-extinction scenario for the recent history of the AF biota as recently proposed by Carnaval & Moritz (2008) and Carnaval *et al.* (2009). In support of this model, we found: (i) strong demographic fluctuations coinciding with the LGM in the Squamate Antbird (SA), which occurs entirely in portion of the AF with least forest stability (Southern AF); (ii) long-term population growth not coinciding with LGM or stability (CD cluster and non-CD cluster, respectively) in the White-bibbed Antbird (WBA), which has most of its distribution in a large putative refuge (Bahia); (iii) higher genetic diversity in WBA compared to the SA, reflecting expected differences in persistence in species occupying northern and southern portions of the AF; (iv) persistence in areas of predicted refuges, as indicated by population structure in WBA; and (v) evidence of secondary contact, based on parapatric

(a) Selected demographic model: reproductive isolation model

(b) Parameter estimation: divergence time, τ_{WBA}

Fig. 6 Results of the model selection and parameter estimation procedures in Approximate Bayesian Computation (ABC). (a) Schematic representation of the reproductive isolation model, which had more explanatory power than the postdivergence migration model, as revealed by model selection in ABC. The model is represented as phylogenetic tree (thick black lines), whereas each branch is labelled with the effective population size (N_{pop}), in units of a million diploid individuals, and the width of each branch (shaded area) is proportional to the indicated size. Adjacent vertical lines illustrate the time to divergence (τ_{pop}) with labels in units of a million years ago, whereas the splitting time between Chapada Diamantina (CD) and non-CD White-bibbed Antbird (WBA) (circled) was included as a prior distribution in the model, and its value was estimated in the subsequent parameter estimation step. (b) Posterior distribution found when estimating the divergence time of CD and non-CD WBA under the reproductive isolation model, with rejection sampling of the prior distribution (thin grey line) and the posterior distribution achieved by local-linear regression (thick black line). The prior distribution itself is indicated (dashed line) as reference.

distributions of WBA and SA associated with signs of population expansion. One aspect of our data, however, does not completely fit the stability-extinction scenario. According to that model, species currently inhabiting the Southern AF should represent recent offshoots from expansion of northern populations. Instead, our data suggest that SA is a divergent evolutionary unit endemic to the Southern AFs, echoing similar findings with other organisms (e.g. Grazziotin *et al.* 2006; Cabanne *et al.* 2007; Fitzpatrick *et al.* 2009; Thomé *et al.* 2010; D'Horta *et al.* 2011; Amaro *et al.* 2012; Maldonado-Coelho 2012). Because avian distributions are labile and may

change at timescales as short as a human lifespan (Brumfield 2012), we cannot rule out a scenario in which SA survived by shifting its distribution to northern latitudes during glacial periods. However, if major range shifts did not occur, it is possible that SA may have been persisting in Southern AF for millions of years since their initial divergence from WBA. Conceivably the original stability-extinction model lacks predictive power for the southern portions of the biome (Amaro *et al.* 2012), as exemplified by inference of many taxon-specific refuges in southeastern and Southern Brazil (Porto *et al.* 2013). Additionally, persistence in Southern

AF refuges has been hypothesized to be strongest in species capable of surviving in montane forest (as WBA and SA), because their tolerance to colder climates could shield them from the effects of the LGM (Amaro *et al.* 2012). Thus, although not originally predicted by the original stability-extinction model, recent evidence suggests that lineage persistence in the Southern AF—as may have happened with the SA—may also be consistent with a scenario of refuge isolation.

Our results also suggest that a small region west of the large Bahia refuge, the Chapada Diamantina uplands, harbours a population of WBA with signs of differentiation from the rest of the species' range. This area contains a small patch of AF harbouring populations of otherwise typically AF-inhabiting species as well as endemic organisms (e.g. Gonzaga *et al.* 2007), is mostly surrounded by open *Caatinga* vegetation, and still remains little explored in phylogeographic studies. A role for the Chapada Diamantina uplands as a small putative refuge in the stability-extinction model (Fig. 1) is consistent with the inferred stability and differentiation of the Chapada Diamantina (CD) cluster found in the present study. However, the localities sampled here are also separated from the rest of WBA's range by a potential riverine barrier, the Paraguaçu River (Fig. 1). Thus, the hypothesis of this river acting as a barrier cannot be rejected here, and further sampling in the CD area south of the Paraguaçu River will be necessary to discriminate between these hypotheses. Additional sampling of individuals in CD and neighbouring areas would also improve estimates of population structure, which in turn would provide more precise boundaries between CD and non-CD cluster (e.g. resolving the status of individuals of WBA locality 9).

Besides the unresolved role of the Paraguaçu River, on recent timescales our results do not support riverine or physiographic barriers as drivers of population structure, despite the many other rivers and topographic features that currently dissect the ranges of WBA and SA (Fig. 1). However, barriers may have acted on older timescales, namely during the initial divergence between SA and WBA. The antbirds studied here appear to have diverged well before the Pleistocene (2–4 mya), and their shared distribution boundary matches an area of high tectonic activity during the Miocene and Pliocene namely the River of Paraíba do Sul Valley (Petri & Fúlvaro 1983; Fig. 1). Alternatively, refuge isolation may have been responsible for both initial divergence and evolution of intraspecific patterns of variation, because climatic fluctuations may have been important even prior to the Pleistocene (Haffer 1993; Hewitt 1999). Thus, although intraspecific patterns of variation of WBA and SA strongly support the effect of refuge isolation in the

AF, we cannot yet reject a role of the barrier hypothesis entirely, particularly during the initial divergence of WBA/SA on a larger timescale.

Vicariance, dynamic history or stochastic error? Why Atlantic Forest phylogeography demands multilocus data?

Taking at face value recent studies inferring divergence times and demographic histories of AF organisms performed using mtDNA or small (2–4) numbers of independent markers, what would the multilocus estimates of WBA/SA divergence suggest about assembly wide historical processes? Their pre-Pleistocene divergence is among the oldest reported, especially when compared to other AF avian sister species or mtDNA phylogroups. It is, for example much older than most of the divergences inferred for codistributed mtDNA lineages of suboscine songbirds, which usually diverged less than 1 mya (Cabanne *et al.* 2007, 2008; D'Horta *et al.* 2011; Maldonado-Coelho 2012). Our estimates are rivalled so far only by divergences estimated for species of *Eleoscytalopus* tapaculos (Mata *et al.* 2009). Our timing is in line with divergences found among codistributed clades of other vertebrates, such as frogs (Fitzpatrick *et al.* 2009; Brunes *et al.* 2010; Amaro *et al.* 2012) and pitvipers (Grazziotin *et al.* 2006). Thus, it is possible that temporally distinct vicariant events may have shaped AF vertebrate diversity, sometimes leading to pulses of diversification that may have affected distantly related organisms in similar ways.

Besides the apparent periodicity of large-scale events, sister lineages resulting from temporally independent events appear to accumulate in selected areas of the AF. The River of Paraíba do Sul Valley is a clear example, by roughly demarcating the shared range limits of phylogroups/species pairs that appear to have diverged very recently (<1 mya, Cabanne *et al.* 2007; D'Horta *et al.* 2011) or even before the Pleistocene (e.g. WBA vs. SA). Consequently, these areas should be investigated not only for their roles as primary generators of diversity, but also as suture zones, because many of those lineages may have not have diverged, but expanded simultaneously (Moritz *et al.* 2009; Martins 2011). Finally, our results are consistent with a number of recent studies suggesting that refuge isolation may have been an important mechanism of diversification in the recent history of the AF (Grazziotin *et al.* 2006; Cabanne *et al.* 2007; Carnaval *et al.* 2009; Fitzpatrick *et al.* 2009; Martins *et al.* 2009; Brunes *et al.* 2010; D'Horta *et al.* 2011; Maldonado-Coelho 2012). However, our findings do not reject the idea that barriers may have played an important role in the AF diversification, in particular, before the Pleistocene

(Pellegrino *et al.* 2005; Brunes *et al.* 2010; Thomé *et al.* 2010; Amaro *et al.* 2012).

Biases of single and multilocus demographic estimates in AF studies

Here, we address the impact of limited marker sampling, including possibilities of bias in this study, on estimates of demographic parameters in AF studies. In particular, we ask: Do available molecular studies—mostly based on mtDNA alone or a few markers—already provide a general framework to understand processes of AF diversification?

Divergence times. Theory predicts that comparison of divergence times across different studies based on one or few markers may not be straightforward, because gene divergence may largely pre-date population divergence (Edwards & Beerli 2000). Our results, for example, match previous empirical inferences (Lee & Edwards 2008) suggesting that estimation of divergence times may be affected not only by the number of loci, but also by whether or not mtDNA is included. In addition, recent studies suggest that natural selection may reduce mutation rates with increasing effective population sizes in selected prokaryotes and unicellular eukaryotes (Sung *et al.* 2012). If this process also affects birds, comparisons between species/populations with considerably distinct effective population sizes—as in our case—may be subject to this additional source of error when constant substitution rates are assumed to estimate divergence times.

Demography. Another essential aspect to be considered in comparisons across AF studies is the key role that historical demography plays in the AF, and more generally, Neotropical phylogeography. The statistical power of methods we have used, such as Extended Bayesian Skyline Plots (EBSP; Heled & Drummond 2008), as well as their 'observation window' depends on the number of loci used (Heled & Drummond 2008). Our results are in line with those predictions: on the one hand, the longest observation windows (spanning millions of years) and the most narrow 95% HPD intervals in effective population size estimations were obtained in multilocus comparisons (Fig. 5), which were much more informative than the single-locus GMRP Skyride Plot, whose observation windows were limited to much less than 500 000 years. Further, our data suggests that the medians of the combined data sets do not necessarily reflect the patterns found in mtDNA-only GSP and nuclear EBSPs analysed alone (e.g. non-CD WBA, Fig. 5). A possible explanation is that combining fast and slow evolving markers

may be necessary to capture the greatest diversity of events, because specific demographic events may be concealed or revealed depending on which markers capture them (Eytan & Hellberg 2010). However, combining mtDNA and nuclear DNA is challenging because of their strongly divergent substitution rates, which are not well accommodated by some software. Thus, accurate reconstructions of demographic oscillations predicted by the refuge hypothesis will likely depend on the use of multilocus data sets and may possibly improve by combining mtDNA and many nDNA markers.

Divergence of WBA and SA, and implications for the speciation process in antbirds

The WBA/SA species pair comprises an interesting exception to most other closely related antbirds studied to date. Vocalizations have been considered one of the most important factors determining reproductive isolation among thamnophilids (Isler *et al.* 1998). WBA and SA are very similar in voice, and their parapatric distributions present a sharp transition of phenotypes within a distance of less than 20 km (Fig. 1). Considering that vocal differences are important to maintain species boundaries in such a geographic setting, very similar vocalizations, strong response to each other's voice and plumage similarities could lead to the prediction of little genetic divergence and/or high levels of gene flow between those two species. However, here, we found the opposite pattern, namely deep genetic divergence associated with low or nonexistent levels of gene flow. Thus, our results suggest that conspicuous vocal differences may not be always necessary to maintain species boundaries among antbirds, and other kinds of cues may be especially important in selected cases. Subtle vocal differences, which have been shown to suffice both interspecific and individual recognition in *Hypocnemis* antbirds (Seddon & Tobias 2010), or even visual cues (e.g. ochraceous-throated female WBA vs white-throated female SA) or non-vocal behaviours could be acting as strong prezygotic barriers. Alternatively, genomic incompatibilities caused by millions of years of divergence may have led to the development of mechanisms of postzygotic isolation (Price 2007).

But how does the WBA/SA pair interact at its zone of contact and what maintains their parapatry? Under a scenario of complete reproductive isolation, very similar ecological niches could be leading to competitive exclusion, even after substantial genetic divergence (Rundell & Price 2009; Weir & Price 2011). Alternatively, if reproductive isolation is not yet complete, a very narrow and still unidentified hybrid zone could exist, and strong selective forces against hybrids could be preventing substantial introgression and sympatry (Barton &

Hewitt 1985). Field and laboratory experiments, bioacoustic studies and genome-wide molecular surveys with this species pair will provide exciting findings on the development of reproductive isolation among antbirds, and more generally, suboscine passerines.

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F.R.A., P.K.A., S.V.E. and C.Y.M. conceived and drafted the manuscript. F.R.A. performed field and laboratory work. F.R.A., P.K.A. and S.V.E. performed data analyses. F.R.A., S.V.E. and C.Y.M. contributed with reagents.

Data accessibility

DNA sequences: GenBank accessions KC714092–KC715586

IMa2 and STRUCTURE input files: Dryad doi:10.5061/dryad.86h6f6

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sonograms of loudsongs and calls of SA (a and b) and WBA (c and d).

Fig. S2 Complete set of STRUCTURE runs.

Fig. S3 Model checking after model selection and parameter estimation in Approximate Bayesian Computation (ABC).

Table S1 Samples used in the present study.

Table S2 Mitochondrial PCR primers used for amplification and sequencing.

Table S3 PCR primers used for allele-specific PCR (AS-PCR).

Table S4 Conversion of Isolation-with-Migration parameters (population size and divergence times) based on different substitution rates obtained from the literature.

Table S5 Observed summary statistics used for Approximate Bayesian Computation (ABC).

Appendix S1 Details of ABC analysis.

Anexo XXII

Molecular systematics and evolution of the *Synallaxis ruficapilla* complex (Aves: Furnariidae) in the Atlantic Forest

Henrique Batalha-Filho^{a,*}, Martin Irestedt^b, Jon Fjeldså^c, Per G.P. Ericson^b, Luis F. Silveira^d, Cristina Y. Miyaki^a

^a Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil

^b Molecular Systematics Laboratory, Swedish Museum of Natural History, Box 50007, SE-104 05 Stockholm, Sweden

^c Center for Macroecology, Evolution and Climate at the Natural History Museum of Denmark, University of Copenhagen, Copenhagen, Denmark

^d Museu de Zoologia, Universidade de São Paulo, São Paulo, Brazil

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ABSTRACT

The Neotropical *Synallaxis ruficapilla* complex is endemic to the Atlantic Forest and is comprised of three species: *S. ruficapilla*, *S. whitneyi*, and *S. infusca*. This group is closely related to the *Synallaxis moesta* complex that occurs in the Andes, Tepuis, and Guianan shield. Here we used mitochondrial and nuclear gene sequences to infer the phylogeny and the time of diversification of the *S. ruficapilla* and *S. moesta* complexes. We also included samples of an undescribed population of *Synallaxis* that resembles other populations of the *S. ruficapilla* complex. Our results showed that different geographical lineages within the *S. ruficapilla* complex are reciprocally monophyletic, but the northern form (*S. infusca*) grouped with an Andean taxon. This suggests that at least two lineages of this group independently colonized the Atlantic Forest. Specimens of the undescribed population formed a monophyletic clade with deep divergence. Estimated diversification dates were within the late Pliocene to Pleistocene (2.75–0.16 million of years ago). This suggests that at this time there was a higher connectivity between habitats in the rugged landscapes of the circum-Amazonian bioregions. The observed Pleistocene diversification within the Atlantic Forest is congruent in space and time with studies of other co-distributed organisms, and may be associated with climate changes and tectonic activity during this period.

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The genus *Synallaxis* Vieillot (Spinetails) is endemic to the Neotropical region and occurs from southern Mexico to Argentina. This is the most species-rich genus in the family Furnariidae, with 33 recognized species (Renssen, 2003). Recently, an additional species of *Synallaxis* was described (Hilty and Ascanio, 2009). Overall, the systematics of the genus is rather obscure, as many species are similar in morphology (Ridgely and Tudor, 1994), and some species include two to ten subspecies that could represent full species themselves (Renssen, 2003). Most *Synallaxis* species inhabit dense understory vegetation in riparian thickets, edge habitat and secondary forest, with some exceptions occurring inside continuous forest (Ridgely and Tudor, 1994; Sick, 1997). Renssen (2003) suggests a close relationship between *Synallaxis* and the genera *Certhiopsis*, *Schoeniophylax*, *Gyalophylax*, and *Siptornopsis*. Recent

2011) confirm these relationships.

The *S. ruficapilla* complex (Fig. 1) comprises *S. ruficapilla*, *S. whitneyi*, and *S. infusca* (Pacheco and Gonzaga, 1995) and is endemic to the highly endangered and megadiverse Atlantic Forest – AF (Myers et al., 2000; Ribeiro et al., 2009). Pacheco and Gonzaga (1995) suggested that this species complex is monophyletic based on similarities in plumage and song and notably their nearly identical scolding call. In a recent near-complete species-level phylogeny of the Furnariidae (Derryberry et al., 2011) the *S. ruficapilla* and *S. moesta* complexes are placed together as a monophyletic clade, but with low node supports for the relationships within the clade. However, this phylogeny only included one species from the *S. ruficapilla* complex (*S. ruficapilla*) while the *S. moesta* complex was represented by all three species (*S. moesta*, *S. macconnelli*, and *S. cabanisi*), which occur in the Andes, Tepuis, and Guianan shield (Fig. 1; Ridgely and Tudor, 1994; Renssen, 2003).

Recently Stopiglia et al. (2012) suggested that *S. whitneyi* is a junior synonym of *S. ruficapilla* based on plumage, morphometric, and song characters. Thus, according to these authors, there are only two species in the *S. ruficapilla* complex: *S. ruficapilla* and *S. infusca*. They also argued that only the number of rectrices is

* Corresponding author. Address: Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, Cidade Universitária, 05508-090 São Paulo, SP, Brazil. Fax: +55 11 3091 7553.

E-mail addresses: henrique.batalha@usp.br, rickbio@hotmail.com (H. Batalha-Filho).

Fig. 1. Map showing localities sampled for species of the *S. ruficapilla* and *S. moesta* complexes, and mitochondrial tree obtained by Bayesian inference. Circles and triangles on the map indicate collecting sites and their colors follow the legend. Locality numbers in the map follows Table 1. Species distributions follow Ribon et al. (2002), Ridgely and Tudor (1994), and Vasconcelos and Silva (2004). The gradient of gray colour in the map represents the elevation gradient (the darker the higher is the altitude). The topology was obtained by Bayesian inference based on 2043 bp of cytb and ND2. Node supports are posterior probabilities and bootstrap values for Bayesian inference and maximum likelihood, respectively. The colors and symbols in the tree refer to the map and legend. Bird illustrations show the three species of the *S. ruficapilla* complex. Source: Pacheco and Gonzaga, 1995.

diagnostic for both species and that other phenotypic traits show great individual variation and no constant population differences. They also analyzed six specimens of *Synallaxis* sp. (vouchers in MZUSP; see Table 1) recently collected in Amazon (Vila Rica, Mato Grosso state in Brazil) and geographically disconnected from the AF. Accordingly to Stopiglia et al. (2012) these specimens overlap with *S. ruficapilla* in both morphology and song, suggesting that these birds, which do not occur in the AF, are *S. ruficapilla*. However, another possibility apparently not addressed by Stopiglia et al. (2012), is that this disjunct Amazonian population of the *S. ruficapilla* complex represents an independent lineage deserving a separate taxonomic status. Thus, the species limits within the *S. ruficapilla* complex are still uncertain.

Notwithstanding, defining limits between species it is not a trivial task, mainly due to ambiguity in the species concept in biology (de Queiroz, 2007). Because of retention of ancestral polymorphism and incomplete lineage sorting it is particularly difficult to establish the taxonomic status of recently diverged populations (Carstens and Dewey, 2010). However, the use of molecular data as an additional means to delimitate species has become a robust tool (Carstens and Dewey, 2010; Kubatko et al., 2011), especially when a species tree is inferred based on the coalescence of multiple independent loci (Liu and Pearl, 2007; Liu, 2008; Heled and Drummond, 2010).

Here, we infer the phylogenetic relationships within the *S. ruficapilla* complex, as well as its relationships with the *S. moesta* complex, using mitochondrial and nuclear sequences. Furthermore, we investigate the taxonomic status of the disjunct population of the *S. ruficapilla* complex from Vila Rica. In addition, we evaluated the species status of *S. whitneyi*, which was questioned by Stopiglia et al. (2012), and that might represent an independent evolutionary lineage. Our results will help to trace the evolutionary history

of South American forests. Yet, in the context of conservation, we add data to discuss the taxonomic status of the threatened taxa *S. whitneyi* and *S. infusata* (Roda, 2008; Silveira, 2008).

2. Materials and methods

2.1. Taxon sampling and molecular methods

We sampled 38 individuals from all taxa of the *S. ruficapilla* and *S. moesta* complexes (Ridgely and Tudor, 1994; Ribon et al., 2002; Vasconcelos and Silva, 2004; Stopiglia et al., 2012; Table 1, Fig. 1), including the disjunct population of the *S. ruficapilla* complex from Vila Rica (state of Mato Grosso, MT) in Brazil (Fig. 1). This sampling covered a wide geographical range of AF species. One specimen each from *S. frontalis* and *S. spixi* were used as outgroups, following Derryberry et al. (2011). The samples were obtained from fresh tissue (muscle or blood) and museum study skins (Table 1).

DNA was extracted from fresh samples following Bruford et al. (1992), while the laboratory procedures for museum study skin samples followed Irestedt et al. (2006). We sequenced two mitochondrial genes – cytochrome b (cytb) and NADH dehydrogenase subunit 2 (ND2); and tree nuclear introns – fibrinogen intron 5 (FIB5), myoglobin intron 2 (myo2), and glyceraldehyde-3-phosphodehydrogenase intron 11 (G3PDH). For fresh samples all loci were amplified as one product each, whereas additional internal primers were designed for the study skin samples to allow the amplification of shorter fragments (~200–250 bp each). All primers used in this study are described in Table 2.

PCR conditions were an initial denaturation step at 94 °C for 3 min and 30 s; followed by 35 or 40 cycles at 94 °C for 35 s,

Table 1
Samples of *Synallaxis* spp. used in this study: species, sampling locality, collection, tissue code, voucher number, and GenBank accession numbers for each marker.

Species	Locality	Collection	Tissue code	Voucher	cytb	ND2	FIB5	myo2	G3PDH
<i>Synallaxis cabanisi</i>	1. Depto. Pasco, Cacazú, Peru	MZUSP ^a	–	MZUSP73261	KC437438	KC437514	KC437538	KC437470	KC437587
<i>Synallaxis cabanisi</i>	2. Depto. Pasco, 41 km on Villa Rica – Puerto Bermudez highway, Peru	LSU ^b	B2013	B2013	KC437445	HM449848 ^f	KC437550	KC437480	–
<i>Synallaxis infusca</i>	3. Usina Serra Grande, São José da Lage, Alagoas, Brazil	LGEMA ^b	LGEMA13568	MZUSP88696	KC437425	KC437501	KC437537	KC437464	KC437568
<i>Synallaxis infusca</i>	3. Usina Serra Grande, São José da Lage, Alagoas, Brazil	LGEMA ^b	LGEMA13573	MZUSP88694	KC437426	KC437502	KC437546	KC437465	KC437569
<i>Synallaxis infusca</i>	3. Usina Serra Grande, São José da Lage, Alagoas, Brazil	LGEMA ^b	LGEMA13574	MZUSP88695	KC437427	KC437503	KC437547	KC437466	KC437570
<i>Synallaxis infusca</i>	4. Rebio Pedra Talhada, Quebrangulo, Alagoas, Brazil	LGEMA ^b	LGEMA13575	MZUSP88699	KC437428	KC437504	KC437548	KC437467	KC437571
<i>Synallaxis infusca</i>	4. Rebio Pedra Talhada, Quebrangulo, Alagoas, Brazil	LGEMA ^b	LGEMA13576	MZUSP88698	KC437429	KC437505	–	KC437468	KC437572
<i>Synallaxis infusca</i>	4. Rebio Pedra Talhada, Quebrangulo, Alagoas, Brazil	LGEMA ^b	LGEMA13577	MZUSP88697	KC437408	KC437485	KC437521	KC437448	KC437573
<i>Synallaxis macconnelli</i>	5. Rebio Maicuru, Almeirim, Pará, Brazil	MPEG ^b	CN855	66294	KC437439	KC437515	KC437555	KC437477	–
<i>Synallaxis macconnelli</i>	5. Rebio Maicuru, Almeirim, Pará, Brazil	MPEG ^b	CN928	66295	KC437440	KC437516	KC437556	KC437476	KC437580
<i>Synallaxis macconnelli</i>	5. Rebio Maicuru, Almeirim, Pará, Brazil	MPEG ^b	CN993	66293	KC437441	KC437517	KC437552	KC437478	KC437581
<i>Synallaxis macconnelli</i>	6. Sipaliwini, Balchuis Gebergte, ~78 km S Apura on Nickene River, Suriname	LSU ^b	B55261	B55261	KC437444	JF975204 ^f	KC437551	KC437481	–
<i>Synallaxis moesta</i>	7. Rio Napo, Ecuador	NRM ^a	–	NRM552855	KC437431	KC437507	–	–	–
<i>Synallaxis moesta</i>	8. San José, Ecuador	NRM ^a	–	NRM552854	KC437432	KC437508	–	–	–
<i>Synallaxis moesta</i>	9. Depto. San Martín; ~33 km NE Florida, Peru	LSU ^b	B44663	B44663	KC437446	JF975205 ^f	KC437549	KC437479	–
<i>Synallaxis ruficapilla</i>	10. Juquitiba, São Paulo, Brazil	LGEMA ^b	LGEMA1182	ITA211 ^d	KC437414	KC437491	KC437527	KC437454	KC437558
<i>Synallaxis ruficapilla</i>	11. Wencenslau Braz, Paraná, Brazil	LGEMA ^b	LGEMA1367	ITA288 ^d	KC437415	KC437492	KC437528	KC437455	KC437559
<i>Synallaxis ruficapilla</i>	12. Parque Nacional Serra dos Órgãos, Teresópolis, Rio de Janeiro, Brazil	LGEMA ^b	LGEMA13015	MZUSP82584	KC437416	KC437493	KC437529	KC437456	KC437560
<i>Synallaxis ruficapilla</i>	13. Estação Biológica Santa Lucia, Santa Teresa, Espírito Santo, Brazil	MNRJ ^b	229	MNA4873	KC437418	KC437495	KC437531	KC437458	KC437562
<i>Synallaxis ruficapilla</i>	14. José Gonçalves de Minas, Minas Gerais, Brazil	UFMG ^c	B2414	–	KC437419	–	KC437539	–	–
<i>Synallaxis ruficapilla</i>	15. RPPN Serra do Caraça, Minas Gerais, Brazil	MNRJ ^b	LGEMA15756	CPA365 ^e	KC437420	KC437496	KC437532	KC437459	KC437563
<i>Synallaxis ruficapilla</i>	16. Parque Nacional do Caparaó, Alto Caparaó, Minas Gerais, Brazil	LGEMA ^b	LGEMA13530	MZUSP87024	KC437421	KC437497	KC437533	KC437460	KC437564
<i>Synallaxis ruficapilla</i>	17. Poté, Minas Gerais, Brazil	LGEMA ^b	LGEMA13536	MZUSP87020	KC437422	KC437498	KC437534	KC437461	KC437565
<i>Synallaxis ruficapilla</i>	17. Poté, Minas Gerais, Brazil	LGEMA ^b	LGEMA13545	MZUSP87019	KC437423	KC437499	KC437535	KC437462	KC437566
<i>Synallaxis ruficapilla</i>	17. Poté, Minas Gerais, Brazil	LGEMA ^b	LGEMA13549	MZUSP87017	KC437424	KC437500	KC437536	KC437463	KC437567
<i>Synallaxis ruficapilla</i>	18. Nova Trento, Santa Catarina, Brazil	LGEMA ^b	LGEMA14592	MZUSP91242	KC437430	KC437506	KC437540	KC437471	KC437579
Unnamed <i>Synallaxis</i>	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	LGEMA ^b	LGEMA416	MZUSP78834	KC437407	KC437484	KC437520	KC437447	KC437557
Unnamed <i>Synallaxis</i>	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP ^a	–	MZUSP78833	KC437433	KC437509	KC437541	KC437472	KC437582
Unnamed <i>Synallaxis</i>	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP ^a	–	MZUSP78830	KC437434	KC437510	KC437542	KC437473	KC437583
Unnamed <i>Synallaxis</i>	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP ^a	–	MZUSP78835	KC437435	KC437511	KC437543	KC437474	KC437584
Unnamed <i>Synallaxis</i>	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP ^a	–	MZUSP78832	KC437436	KC437512	KC437544	KC437475	KC437585
Unnamed <i>Synallaxis</i>	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP ^a	–	MZUSP78831	KC437437	KC437513	KC437545	KC437469	KC437586
<i>Synallaxis whitneyi</i>	20. Serra da Ouricana, Boa Nova, Bahia, Brazil	LGEMA ^b	LGEMA13401	DZUFMG6009	KC437417	KC437494	KC437530	KC437457	KC437561
<i>Synallaxis whitneyi</i>	21. Rebio Mata Escura, Jequitinhonha/Almenara, Minas Gerais, Brazil	LGEMA ^b	LGEMA13711	MZUSP86006	KC437409	KC437486	KC437522	KC437449	KC437574
<i>Synallaxis whitneyi</i>	22. RPPN Serra Bonita, Camacan, Bahia, Brazil	LGEMA ^b	LGEMA13720	MZUSP86015	KC437410	KC437487	KC437523	KC437450	KC437575
<i>Synallaxis whitneyi</i>	22. RPPN Serra Bonita, Camacan, Bahia, Brazil	LGEMA ^b	LGEMA13736	MZUSP86031	KC437411	KC437488	KC437524	KC437451	KC437576
<i>Synallaxis whitneyi</i>	23. Plantações da Michelin, Ituberá/Camamu, Bahia, Brazil	LGEMA ^b	LGEMA13763	MZUSP86057	KC437412	KC437489	KC437525	KC437452	KC437577
<i>Synallaxis whitneyi</i>	23. Plantações da Michelin, Ituberá/Camamu, Bahia, Brazil	LGEMA ^b	LGEMA13764	MZUSP86058	KC437413	KC437490	KC437526	KC437453	KC437578
<i>Synallaxis frontalis</i>	Serra de São Vicente, Mato Grosso, Brazil	MNRJ ^b	32	MNA2246	KC437443	KC437519	KC437553	KC437482	KC437588

Table 1 (continued)

Species	Locality	Collection	Tissue code	Voucher	cytb	ND2	FIB5	myo2	G3PDH
<i>Synallaxis spixi</i>	Estação Biológica Santa Lucia, Santa Teresa, Espírito Santo, Brazil	MNRJ ^b	155	MNA4799	KC437442	KC437518	KC437554	KC437483	KC437589

Locality numbers follow map in Fig. 1.

^a LGEMA – Laboratório de Genética e Evolução Molecular de Aves, Instituto de Biociências, Universidade de São Paulo, Brazil; MZUSP – Museu de Zoologia, Universidade de São Paulo, Brazil; UFMG – Laboratório de Biodiversidade e Evolução Molecular, Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais, Brazil; DZUFMG – Departamento de Zoologia, Universidade Federal de Minas Gerais, Brazil; MNRJ – Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil; MPEG – Museu Paraense Emílio Goeldi, Brazil; NRM – Swedish Museum of Natural History, Sweden; and LSU – Museum of Natural Science, Louisiana State University, USA.

^b Study skin samples.

^c Chest tissue sample with specimen's voucher.

^d Blood sample without specimen's voucher.

^e Field numbers, MZUSP pending numbers.

^f Field number, MNRJ pending number.

^g Sequences from Derryberry et al. (2011).

Table 2

Primers used in this study. Primers used with fresh samples are in italics.

Primer name	Gene	Sequence 5' → 3'	Reference
Cytb-SynR1	cytb	GTATTCATGTCTCTTGAATAGGTA	This study
Cytb-SynR1b	cytb	GTCTCTTTGAATAGGTAAGAGCC	This study
Cytb-SynF2	cytb	ACATCGGACGAGGCTTCTACTA	This study
Cytb-SynR2	cytb	TGTCAACTGAAAATCCTCCTCATG	This study
Cytb-SynF3	cytb	CCTATATCGGACAAACACTCGT	This study
Cytb-SynR3	cytb	GAATGTCTTTGGTTGAGAAGTAAG	This study
Cytb-SynF4	cytb	TCTCATCAAACGCGATAAAATCC	This study
Cytb-SynR4	cytb	TTTATTTGGAATTGATCGTAGAATGG	This study
Cytb-SynF5	cytb	CCTGAATGATACCTTCTATTTCAT	This study
Cytb-SynR5	cytb	TCTACTGGTTGGCTGCCTACT	This study
Cytb-SynF6	cytb	ATATTCTGAATCCTAGTTACAAACCTT	This study
L14841	cytb	CCATCCAACATCTCAGCATGATGAAA	Lougheed et al. (2000)
H16065	cytb	CTCTTCAGTTTTCGTTTACAAGAC	Lougheed et al. (2000)
ND2-synR1	ND2	TAGAAAGTATTGGTTGTGGCTTCA	This study
ND2-synF2	ND2	ATCCTTCCCCTAATCTCAAAACC	This study
ND2-synR2	ND2	AATCAGAAGTGAATGGGACTAG	This study
ND2-synF3	ND2	TAACAACAGCCATTTCATCAAACT	This study
ND2-synR3	ND2	TTTTCGGATTGGGTTTGTTTGTAG	This study
ND2-synF4	ND2	CTGCCTTAGGAGGATGAATAGG	This study
ND2-synR4	ND2	TTAGTGAAGGGATTTCCTTCATGC	This study
ND2-synF5	ND2	ACCAAAATCTTAACTATCCTCAATAA	This study
ND2-synR5	ND2	ATCGGAGGTAGAAGAAAAGTCCT	This study
ND2-synF6	ND2	CAACAATCATTGCCCTCCTCTC	This study
Lmet	ND2	TATCGGCGCCATACCCGAAAAT	Hackett (1996)
H6312	ND2	CTTATTTAAGGCTTTGAAGGCC	Cicero and Johnson (2001)
FIB5	FIB5	CGCCATACAGAGTATACGTGACAT	Marini and Hackett (2002)
FIB6	FIB5	GCCATCTGGCGATTCTGAA	Marini and Hackett (2002)
FIB-FurnL	FIB5	AAGGAGACCTGGCTCATTCCTTA	Fjeldsø et al. (2007)
FIB-FurnH	FIB5	GAGTTGAAGGAATGCCCTGGTCT T	Fjeldsø et al. (2007)
Myo2	myo2	GCCACCAAGCACAAGATCCC	Slade et al. (1993)
Myo3F	myo2	TTCAGCAAGGACCTTGATAATGACTT	Heslewood et al. (1998)
Myo309L	myo2	CATAAGACCTGTCACTGGCTGGA	Irestedt et al. (2006)
Myo345H	myo2	TCCTCCAGGGTTTGCTTAAATTTGT	Fjeldsø et al. (2007)
G3PL890	G3PDH	ACCTTTAATGCGGGTGTGGCATTGC	Friesen et al. (1997)
G3PH950	G3PDH	CATCAAGTCCACACACGGTTGCTGTA	Friesen et al. (1997)

annealing temperature for 40 s and 72 °C for 1 min; plus a final extension step at 72 °C for 9 min. Annealing temperatures were: cytb and ND2 56 °C; FIB5 and myo2 52–58 °C (2 cycles at 58 °C, 2 cycles at 56 °C, 2 cycles at 54 °C and 34 cycles at 52 °C); G3PDH 64 °C. PCR and sequencing protocols followed Batalha-Filho et al. (2012) and Irestedt et al. (2006) for fresh and study skin samples, respectively.

2.2. Sequence edition, alignment and recombination

Electropherograms were inspected and assembled in contigs using CodonCode Aligner v. 3.7 (CodonCode Inc.). Heterozygous sites in nuclear introns were coded according to IUPAC code when double peaks were present in both strands of the same individual's electropherograms. Nuclear sequences that contained

heterozygous indels were analyzed using the algorithm Process Heterozygous Indels in CodonCode Aligner v. 3.7. Sequences were aligned using the CLUSTAL W method (Higgins et al., 1994) in MEGA5 (Tamura et al., 2011). All alignments were inspected and corrected visually. We used PHI test in SPLITSTREE4 (Bruen et al., 2006; Huson and Bryant, 2006) to check for recombination in the nuclear gene sequences. This test was used due to its power to distinguish recombination events from recurrent mutation (Bruen et al., 2006). To apply the coalescent species tree analysis we resolved the gametic phase of nuclear genes using the PHASE algorithm (Stephens et al., 2001) with default settings in DnaSP 5 (Librado and Rozas, 2009) assuming a threshold of posterior probability of 0.9. However, we kept the unphased sequences with probabilities below this threshold in the analysis.

2.3. Phylogenetic analyses

The best fit model for each gene was selected using MrModeltest 2.2 (Nylander, 2004) based on the Akaike information criterion (AIC). Two partitions (cytb + ND2) were considered in the Bayesian and maximum likelihood analyses using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and RAxML (Stamatakis et al., 2008), respectively. Both analyses were carried out at CIPRES Science Gateway (Miller et al., 2010). Bayesian runs consisted of 10 million generations for 2 independent runs with 4 chains of Markov chain Monte Carlo (MCMC) each. The first million generations were discarded as burn-in, after which trees were sampled every 500 generations. Chain convergence (Effective Sample Size – ESS values >200) was checked using the likelihood plots for each run using Tracer 1.5 (<http://beast.bio.ed.ac.uk/Tracer>). The Potential Scale Reduction Factor was also used to check chain convergence and burn-in; values close to one indicate good convergence between runs (Gelman and Rubin, 1992). We used RAxML under the GTRAC model; invariable sites and gamma distribution were estimated for each partition during the run. Node supports of the maximum likelihood analyses were estimated by 1000 bootstrap replications. We also obtained trees in RAxML for each nuclear gene.

2.4. Species tree and dating

In order to combine the information of all genes in a single tree and accommodate inconsistencies between gene and species trees (Degnan and Rosenberg, 2006; Kubatko and Degnan, 2007) we used the Bayesian coalescent method of species tree in *BEAST (Bayesian Inference of Species Trees from Multilocus Data; Heled and Drummond, 2010) in the Biportal at University of Oslo (Kumar et al., 2009). The main mitochondrial lineages (Fig. 1) were assigned as “species” in the analysis. We also estimated the divergence times using the multispecies coalescent method of *BEAST (in BEAST 1.6.2, Drummond and Rambaut, 2007), as this method seems to be more accurate than those based on gene trees (McCormack et al., 2011). We generated the input file in BEAUTi considering four genes (mitochondrial + FIB5 + myo2 + G3PDH) and the substitution models selected by MrModeltest (Table 3). We used relaxed clock with an uncorrelated lognormal distribution (Drummond et al., 2006) and Yule process for all genes. Piecewise linear and constant root were used as population size model priors. Given the very sparse fossil record of furnariids, and the general problem of correlation of furnariid splitting events to specific past events (Derryberry et al., 2011), we used the mutation rate of mitochondrial genes (under a normal distributed prior) available for birds to estimate divergence dates (Lovette, 2004; Weir and Schluter, 2008). This mutation rate of 1.05% (±0.05) per lineage per million years was estimated by Weir and Schluter (2008) based on 90 calibration points and 12 orders of birds, and included a range of 12 million years. We then used BEAST to estimate the mutation rates for the nuclear introns using a log-normal distributed prior under default settings for each partition. We did two

independent runs with 100 million generations each, with parameters sampled every 10,000 steps and a burn-in of 30%. We checked for convergence between runs and analysis performance using Tracer 1.5, and accepted the results if ESS values were >200. The resulting trees were combined in TreeAnnotator and the consensus species tree with the divergence times was visualized in FigTree 1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

3. Results

3.1. Phylogenetic inferences

3.1.1. Mitochondrial dataset

Our mitochondrial dataset comprised 2043 characters for 40 individuals, including outgroups: 1002 of cytb and 1041 of ND2. For the ingroup 120 and 125 sites were variable in cytb and ND2, respectively. No indels, unexpected stop codons, or ambiguous peaks in the electropherograms were found in these sequences, suggesting that they were of mitochondrial origin. The best fit model estimated for each gene is shown in Table 3.

Bayesian and maximum likelihood analyses indicated absence of monophyly of the *S. ruficapilla* complex, with *S. infusca* being paraphyletic with other AF species (Fig. 1). Besides, specimens of the undescribed *Synallaxis* from Vila Rica-MT (hereafter unnamed *Synallaxis*) formed a clade with a deep divergence from the other species (Fig. 1). Yet, unnamed *Synallaxis* appeared as sister to the AF species *S. ruficapilla* and *S. whitneyi*, but with moderate node support (0.89 of posterior probability and 82% of bootstrap; Fig. 1). The relationships between the *S. ruficapilla* and *S. moesta* complexes were recovered as a polytomy (Fig. 1). Interestingly, the results revealed that the AF *S. infusca* is sister to the Andean *S. moesta* with moderate support (0.92 of posterior probability and 94% of bootstrap; Fig. 1).

Our mitochondrial trees also revealed genetic structure in *S. ruficapilla*. Two clades were observed: a southern clade that included almost the entire geographical distribution of species, and an unexpected northern clade with samples from the north of state of Minas Gerais (Poté and José Gonçalves de Minas) where the AF meets the Cerrado biome (Fig. 1 and Table 1).

3.1.2. Nuclear dataset

We obtained sequences of all three nuclear introns for all species, except G3PDH for *S. moesta* (Table 1). In the FIB5 ingroup alignment (546 characters) 34 sites were polymorphic, and there were five indels in eight individuals ranging in length from 1 to 29 bp. We found 16 polymorphic sites in the myo2 ingroup alignment (587 characters) and one indel in 11 individuals of 1 bp long. For the alignment of the ingroup G3PDH (323 characters) there were 26 polymorphic sites and two indels (1 bp and 2 bp long, respectively) in six individuals. PHI test showed that there is no significant evidence for recombination events in any of the nuclear genes ($p > 0.1$).

Gene trees of nuclear introns did not recover the same genetic structure in the ingroup as found in the mitochondrial genes. However, a poorly supported clade that includes unnamed *Synallaxis*, *S. ruficapilla*, and *S. whitneyi* is observed in the G3PDH tree, but the last two taxa were not reciprocally monophyletic (Supplementary Fig. S1).

3.1.3. Species tree

The species tree generated by *BEAST was partially congruent with the mitochondrial one, but with poorly supported nodes (Fig. 2). The only exceptions were the central-southern AF clade (*S. ruficapilla* and *S. whitneyi*) that was moderately well supported, and its sister relationship with unnamed *Synallaxis* that was well supported.

Table 3
Best fit models for *S. ruficapilla* and *S. moesta* groups and outgroups estimated for each partition.

Partition (genes)	Model of evolution
cytb	HKY + I
ND2	HKY + Γ
Mitochondrial (cytb + ND2)	GTR + Γ
FIB5	HKY + I
myo2	GTR + I + Γ
G3PDH	HKY + I + Γ

Fig. 2. Chronogram of species tree with divergence times generated by 'BEAST. Number at the nodes are clade posterior probabilities. White circles with numbers at nodes are divergence times between clades according to Table 4. Bars on the nodes represent 95% of high posterior density of divergence times. Branch colors follow Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4

Divergence times and confidence intervals [95% of high posterior density (HPD)] between major mitochondrial lineages of *S. ruficapilla* and *S. moesta* groups estimated by 'BEAST. Node numbers follow Fig. 2.

Node	Time mya (95% of HPD)
1	2.24 (2.75–1.80)
2	2.02 (2.40–1.61)
3	1.91 (2.33–1.49)
4	1.85 (2.33–1.33)
5	1.56 (2.04–1.03)
6	1.17 (1.58–0.74)
7	0.53 (0.86–0.16)

3.2. Divergence times

The dated chronogram based on the coalescent multilocus analysis in *BEAST, revealed a recent diversification. The splitting events occurred from the late Pliocene to early Pleistocene (2.75–0.16 million of years ago [mya]; Fig. 2; Table 4). The oldest divergence event was the split between the clade including *S. infusata* and *S. moesta* at 2.24 mya. (95% of high posterior density [HPD]: 2.75–1.80 mya). The youngest diversification event was the separation of northern and southern clades in *S. ruficapilla* at 0.53 mya (95% of HPD: 0.86–0.16 mya).

4. Discussion

4.1. Systematics of the *S. ruficapilla* and *S. moesta* complexes

The mitochondrial phylogeny revealed eight well supported lineages including all previously described species and one unnamed *Synallaxis* (Fig. 1). Besides, it revealed a previously undetected cryptic diversity within *S. ruficapilla*, where a dichotomy separating a northern clade (comprising specimens from the northern state of Minas Gerais) and a southern clade (specimens from the remaining

localities sampled in this study) were observed. *S. whitneyi* was found to be monophyletic and appeared as sister to *S. ruficapilla*. Specimens of unnamed *Synallaxis* also formed a clade with a deep differentiation from its sister group (*S. whitneyi* and *S. ruficapilla*). This result reinforces the hypothesis that these specimens represent an undescribed taxon. *S. infusata* and *S. moesta* were reciprocally monophyletic and unexpectedly recovered as sister species with a moderate node support. This is not congruent with a previous suggestion that *S. infusata* should group with the other two AF species, *S. ruficapilla* and *S. whitneyi* (Pacheco and Gonzaga, 1995). *S. cabanisi* and *S. macconnelli* were also monophyletic, but their relationships to the other clades could not be resolved and are best regarded as belonging to an unresolved polytomy.

Our data also suggest that the specimens from Vila Rica-MT belong to an undescribed taxon of *Synallaxis*. Additional specimens possibly related to this new taxon were collected in other localities in the Amazon, in Coroatá, Maranhão (Oren, 1991, specimens in Louisiana State University, Museum of Natural Science) and São Félix do Xingu (specimens in Museu Paraense Emílio Goeldi, Brazil), previously identified as *S. infusata* and *S. macconnelli* respectively. The number of rectrices of these specimens suggests that they belong to the *S. ruficapilla* complex (Stopiglia et al., 2012). Also, Zimmer et al. (1997) mentioned specimens of *S. cabanisi* in Alta Floresta in the state of Mato Grosso (approx. 550 km west from Vila Rica-MT), but according to Whitney (1997) these birds possibly belong to an undescribed species of the *S. ruficapilla* complex. Therefore, further efforts of field surveys in these regions will be important to obtain specimens of unnamed *Synallaxis* to determine whether all these records represent the same cryptic taxon as identified in our study, as well as determining the geographic distribution and taxonomic status of this lineage.

Overall, our mitochondrial results were incongruent with the analyses by Stopiglia et al. (2012) that used song and morphological characters. This taxonomic revision of the *S. ruficapilla* group concluded that only two species should be accepted within the

complex: (i) *S. ruficapilla* that includes *S. whitneyi* and the unnamed *Synallaxis*, and; (ii) *S. infuscat*. According to Stopiglia et al. (2012), *S. whitneyi* is a synonym of *S. ruficapilla*, and, according to the same authors, the species in the complex are primarily diagnosed by their number of rectrices: *S. ruficapilla* (including *S. whitneyi* and unnamed *Synallaxis*) with eight rectrices, and *S. infuscat* with ten rectrices.

The absence of a clear phenotypic distinction between the lineages detected by our mitochondrial data set could be the result of retention of ancestral polymorphism, as the majority of the *Synallaxis* species share a very similar plumage (Remsén, 2003). This could also be due to morphological stasis and insufficient time to evolve morphological differentiation as the estimated population divergence date is relatively recent (late Pliocene to early Pleistocene). Yet, the initially described plumage differentiation (Pacheco and Gonzaga, 1995) could represent high flexibility in ecophenotypes with no bearing on the historical population structure. Another possible explanation is selection of phenotypic characters such as plumage and song in *S. ruficapilla*, *S. whitneyi*, and unnamed *Synallaxis*, which would make it difficult to find diagnosable states for each of these taxa.

The nuclear gene trees did not recover the same population structure as observed in the mitochondrial tree (Supplementary Fig. S1). Possible explanations for the lack of reciprocal monophyly in the nuclear DNA includes incomplete lineage sorting in these genes due to a recent diversification (Pinho et al., 2008), and the longer time of coalescence of nuclear DNA compared to the mitochondrial genome (approximately 4 times longer; Palumbi et al., 2001; Zink and Barrowclough, 2008). Also, there is the possibility of gene flow between some lineages observed here, as we found a possible contact zone (data not shown) between *S. ruficapilla* (northern and central to southern AF) and *S. whitneyi*. Moreover, this possible gene flow may also have contributed for the lack of diagnosability in vocal and morphological characters. Further analyses of gene flow modeling based on a larger dataset will help to depict this scenario.

Recent estimates (Degnan and Rosenberg, 2006; Liu and Edwards, 2009; Heled and Drummond, 2010) showed that multilocus phylogenies based on classical concatenation of genes are less accurate than inferences based on coalescent species tree approaches to recover the phylogenetic relationships among taxa. Moreover, estimates of coalescent species tree are efficient to accommodate inconsistencies between gene trees due to incomplete lineage sorting in recently diversified populations (Heled and Drummond, 2010). Therefore, in this study we implemented the Bayesian coalescent species tree by *BEAST to depict the systematic relationships between *S. ruficapilla* and *S. moesta* complexes. Our species tree was similar to the mitochondrial tree revealing the same clades with good node support values. Although all species showed long terminal branch-lengths, they were poorly supported for some species (*S. macconnelli*, *S. cabanisi*, *S. moesta*, and *S. infuscat*) and failed to resolve the polytomies observed in the mitochondrial tree (Fig. 2).

4.2. Biogeography

The majority of the species (~17) of genus *Synallaxis* occurs in the Andes and Amazon, and just five are endemic to the AF (the three species from this study plus *S. spixi* and *S. cinerascens*; Remsén, 2003). Thus, assuming that the Andes and the Amazon are the possible centers of origin of the genus, the AF species seem to have originated from at least three independent lineages as observed in a recent phylogeny of the Furnariidae family (Derryberry et al., 2011). Interestingly, our study showed that the *S. ruficapilla* complex endemic to the AF is not monophyletic, which suggests that there were additional colonization events and subsequently

isolation of geographical lineages in this biome. Similar patterns of non-monophyly of AF taxa have been observed in small mammals (Costa, 2003) and birds (Pessoa, 2007; Maldonado-Coelho, 2010; but see Weir and Price, 2011). Furthermore, the *S. ruficapilla* and *S. moesta* complexes together exhibit a circum-Amazonian pattern as described by Remsén et al. (1991).

The dating of our phylogeny suggests a recent diversification event (late Pliocene to early Pleistocene), with the two populations of *S. ruficapilla* separated during the climatically most unstable part of the late Pleistocene (Fig. 2). Recently, multi-taxon analysis in conjunction with fossil data have suggested an ancient biota diversification in South America (mainly before the Pleistocene) with the uplift of the Andes and marine incursions as the driving forces of diversification in the continent, instead of Pleistocene glacial cycles (Hoorn et al., 2010; Hoorn and Wesselingh, 2010). However, other studies argued that although the uplift of the Andes and marine incursions were important in the diversification of South American organisms, Pleistocene climate changes may also have played a key role in the diversification process (Rull, 2008, 2011), and also documented a continuous diversification from the Neogene until the Pleistocene (Rull, 2011). Despite the controversies about the tempo of diversification in South America, our data suggest a Plio-Pleistocene diversification for this group, supporting a higher connectivity between thicket and forest habitats in the rugged landscapes in the circum-Amazonian bioregions at this time.

The phylogenetic reconstructions suggest that at least two distinct lineages in the *S. ruficapilla* and *S. moesta* groups originated independently in the AF (Figs. 1 and 2): one was the *S. ruficapilla* and *S. whitneyi* clade in central-southern AF, and another was *S. infuscat* in northern AF. As *S. infuscat* is not the sister species of the other AF species and groups as sister of the Andean species *S. moesta* (even though with moderate node support), its origin seems to reflect a historical connection between the Andes and the AF (Silva, 1994; Percequillo et al., 2011). Yet, our observation that the unnamed *Synallaxis* is sister of the *S. ruficapilla/whitneyi* clade raises the possibility that this is a relictual population from the time when the Andean and south-east Brazilian upland habitats were connected (Silva, 1995). This lineage could then have survived in eastern Amazon after the end of historical connections between these two biomes. This result may support previously proposed historical routes during the Pleistocene connecting South American forests through the dry diagonal (Por, 1992; Willis, 1992; Oliveira et al., 1999; Behling et al., 2000; Auler and Smart, 2001; Costa, 2003; Auler et al., 2004; Wang et al., 2004; Batalha-Filho et al., 2013), specially the Amazon and the AF.

In addition, our results show a Pleistocene diversification within the AF with two vicariant events that lead to separation of three lineages (Figs. 1 and 2): an older split between *S. ruficapilla* and *S. whitneyi* at 1.17 mya (95% of HPD: 1.58–0.74 mya) and a more recent event separating the two groups of populations of *S. ruficapilla* at 0.53 mya (95% of HPD: 0.86–0.16 mya). While the AF holds lineages of ancient (mid-Tertiary) taxa (Fjeldså et al., 2012; Fouquet et al., 2012), the regional biota also underwent significant diversification during the Pleistocene, reflecting climatic-vegetational changes during the Quaternary (Cabanne et al., 2008; Carnaval and Moritz, 2008; Carnaval et al., 2009; d'Horta et al., 2011; Martins, 2011; Maldonado-Coelho, 2012). The cladogenetic events observed here show spatial and temporal congruence with previous studies of AF organisms (Cabanne et al., 2008; Carnaval et al., 2009; Ribeiro et al., 2010; Thomé et al., 2010). These studies evoked the refuge hypothesis (Haffer, 1969; Vanzolini and Williams, 1970) or tectonical activities (Silva and Straube, 1996; Thomé et al., 2010) as main causes of vicariance. Both these hypotheses may be relevant for the speciation events observed here, and phylogeographic studies are being conducted to help to outline the evolutionary history of this group within the AF.

4.3. Implication to conservation

Data from the present study provided significant arguments for the conservation of the threatened species *S. whitneyi* and *S. infusca* (Roda, 2008; Silveira, 2008). The phylogenetic results confirmed that these are independent evolutionary units, which are reciprocally monophyletic (Fig. 1). Stopiglia et al. (2012) suggested that morphology and songs provide no clear diagnostic differences between *S. ruficapilla* and *S. whitneyi*, but our results favor the hypothesis that each of them is a distinct evolutionary significant unit (ESU). At least *S. ruficapilla* and *S. whitneyi* lineages seem to represent well supported phylogenetic species, but extra efforts are needed to find out what happens in potential zones of contact, and to investigate if phenotypic or vocal differences can be detected, that can provide reliable practical diagnosis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2013.01.007>.

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Anexo XXIII

Evolution of *Dendrocolaptes platyrostris* (Aves: Furnariidae) between the South American open vegetation corridor and the Atlantic forest

GUSTAVO S. CABANNE^{1,2*}, FERNANDO M. D'HORTA¹, DIOGO MEYER¹, JOSÉ M. C. SILVA³ and CRISTINA Y. MIYAKI¹

¹*Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, 05508-090, São Paulo, SP, Brazil*

²*División de Ornitología, Museo Argentino de Ciencias Naturales 'B. Rivadavia', Ángel Gallardo 470, Ciudad de Buenos Aires (C1405DJR), Argentina*

³*Conservation International, Rua Antonio Barreto 130, 66055-050, Belém, PA, Brazil*

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The open vegetation corridor of South America is a region dominated by savanna biomes. It contains forests (i.e. riverine forests) that may act as corridors for rainforest specialists between the open vegetation corridor and its neighbouring biomes (i.e. the Amazonian and Atlantic forests). A prediction for this scenario is that populations of rainforest specialists in the open vegetation corridor and in the forested biomes show no significant genetic divergence. We addressed this hypothesis by studying plumage and genetic variation of the Planalto woodcreeper *Dendrocolaptes platyrostris* Spix (1824) (Aves: Furnariidae), a forest specialist that occurs in both open habitat and in the Atlantic forest. The study questions were: (1) is there any evidence of genetic continuity between populations of the open habitat and the Atlantic forest and (2) is plumage variation congruent with patterns of neutral genetic structure or with ecological factors related to habitat type? We used cytochrome *b* and mitochondrial DNA control region sequences to show that *D. platyrostris* is monophyletic and presents substantial intraspecific differentiation. We found two areas of plumage stability: one associated with Cerrado and the other associated with southern Atlantic Forest. Multiple Mantel tests showed that most of the plumage variation followed the transition of habitats but not phylogeographical gaps, suggesting that selection may be related to the evolution of the plumage of the species. The results were not compatible with the idea that forest specialists in the open vegetation corridor and in the Atlantic forest are linked at the population level because birds from each region were not part of the same genetic unit. Divergence in the presence of gene flow across the ecotone between both regions might explain our results. Also, our findings indicate that the southern Atlantic forest may have been significantly affected by Pleistocene climatic alteration, although such events did not cause local extinction of most taxa, as occurred in other regions of the globe where forests were significantly affected by global glaciations. Finally, our results neither support plumage stability areas, nor subspecies as full species. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **103**, 801–820.

ADDITIONAL KEYWORDS: Caatinga – Cerrado – mitochondrial DNA – plumage – population genetic structure – woodcreepers.

INTRODUCTION

The Neotropical Atlantic forest (AF) is separated from the forests of Amazonia and Andes by an open

vegetation corridor formed by the biomes: Caatinga, Cerrado, and Chaco (Olson *et al.*, 2001) (Fig. 1). Although dominated by different forms of savanna, the open vegetation corridor contains forests as humid relicts (*brejos*), as a network of gallery rainforests following water streams, and as seasonally dry forests (Veloso, 1991; Prado, 2000; Pennington, Lavin & Oliveira-Filho, 2009). Most rainforests within this

*Corresponding author. Current address: División de Ornitología, Museo Argentino de Ciencias Naturales 'B. Rivadavia', Ángel Gallardo 470, Ciudad de Buenos Aires (C1405DJR), Argentina. E-mail: gscabanne@yahoo.com

Figure 1. A, study area, distributions of the open vegetation corridor (Caatinga, Cerrado, and Chaco) and of the Atlantic forest. B, sampling localities for the plumage and genetic analysis of *Dendrocolaptes platyrostris*. For details of genetic sampling localities, see Appendix, Table A2. Distribution of biomes and of subspecies followed Olson *et al.* (2001) and Marantz (1997), respectively.

open habitat are physical extensions of the Atlantic and the Amazon rainforest (Cabrera & Willink, 1973; Rizzini, 1976), or were part of them during global glaciations, when larger tracts of rainforests existed in central South America (Ledru, 1992, 1993; Ledru *et al.*, 2006). This particular phytogeographical scenario could have affected the evolution of rainforest specialists inhabiting the open vegetation corridor (Costa, 2003). Particularly, the existence of a network of riverine forests and the historic expansion and retraction of forests may have allowed them to reach the core of the open habitat and to colonize other biomes. Thus, from the biogeographical standpoint of rainforest taxa, the open vegetation corridor might be viewed as a region that is not totally independent of forested biomes, notwithstanding the existence of

substantial landscape differences (Redford & Fonseca, 1986; Silva, 1996; Costa, 2003). Specifically, Costa (2003) found that Cerrado populations of some small no-volant mammals (i.e. species of *Philander* and *Caluromys*) are phylogenetically closely linked to either Atlantic forest or Amazonian populations. If Cerrado populations were not linked to other biogeographical regions, divergent local populations should have been found. Also, Silva (1996) showed that several rainforest birds use riverine forests and dry forests within the Cerrado, reaching the nucleus of this savanna-like biome. Because most forests within the open vegetation corridor are physical extensions of the neighbouring forested biomes, a recent historical connection between populations of the two habitats might be expected. A prediction for this scenario

Figure 2. Working hypotheses and their predictions for rainforest taxa. A, predicted haplotype network if the open vegetation corridor (OVC) is not biogeographically independent from neighbouring rainforests (F). Specifically, it is expected that F and OVC contiguous populations belonged to the same evolutionary lineage, in terms of the network and at the population level (expected fixation index $F_{ST} < 0.2$) (Hedrick, 2005). Haplotypes are represented as circles at the nodes and tips of the network. The size of each circle is proportional to the frequency of the haplotype. B, alternative hypothesis, with both regions as being independent biogeographically. Corresponding populations are expected to be reciprocally monophyletic and substantial population isolation should be found ($F_{ST} > 0.2$). Depending on the age of isolation between populations, monophyly might be partial. C, expected pattern of plumage versus neutral genetic divergences if selection is responsible for plumage variation (primary divergence). For a given level of genetic divergence in neutral markers, we expected to find greater plumage divergence in between-habitat comparisons than in within-habitat comparisons. Adapted from Moritz *et al.* (2000) and Smith *et al.* (2005b). D, expected pattern of plumage and genetic divergences if plumage variation accompanied population structure and not the type of biome of occurrence (isolation and secondary contact). Patterns should be similar in both habitats.

of biogeographical continuity is that populations of rainforest specialists in the open vegetation corridor and in the forested biomes show no significant genetic divergence, except for cases of divergence as a result of isolation by distance (Fig. 2A). Alternatively, if the

open vegetation corridor were an independent biogeographical region, we would expect substantial genetic differentiation between populations of the open and the continuous forested habitats (Fig. 2B).

There are species that occur in both the continuous forests (Atlantic forest or Amazon) and in the open vegetation corridor and present different phenotypes associated with each region (i.e. bird plumage coloration and fur coloration in mammals) (Ridgely & Tudor, 1996; Emmons & Feer, 1997). This is a pattern that might not be in accordance with a biogeographical continuity between habitats. However, it is important to note that forests in the open vegetation corridor are differentiated from forests of the neighboring continuous forests in several aspects, such as climate, humidity, seasonality, and luminosity levels (Veloso, 1991), which may create different selective regimes on adaptive traits between habitats. This selection on adaptive traits may lead to morphologically differentiated populations, despite the presence of gene flow (Moritz *et al.*, 2000; Smith *et al.*, 2005a, b; Smith *et al.*, 2011). For example, higher luminosity levels and lower levels of humidity might favour lighter plumages in birds in the open habitat (Zink & Remsen, 1986; Willis, 1992). Therefore, different phenotypes associated with each habitat do not necessarily reject a biogeographical continuity between the open vegetation corridor and the forest biomes.

In the present study, we investigated plumage and genetic variation of the Planalto woodcreeper (*Dendrocolaptes platyrostris* Spix (1824) Aves, Furnariidae), a bird found throughout the Atlantic forest and also occurring in gallery and dry forests within the open vegetation corridor (Fig. 1). This bird has two subspecies, each one associated with each habitat (Marantz *et al.*, 2003). The nominal subspecies is associated with the Atlantic forest and has a strongly streaked and olive-brown overall plumage. Subspecies *intermedius* is associated with gallery and dry forests of the open vegetation corridor and presents a paler and more cinnamon overall plumage. According to Willis & Oniki (2001), the subspecies present vocal differences suggesting that they could be considered as species. Even though there are intermediates at the transition between the open habitat and the forest (Marantz, 1997; Willis & Oniki, 2001; Marantz *et al.*, 2003), the phenotypic discontinuity between the open habitat and the Atlantic forest deserves to be studied. This phenotypic discontinuity may have been originated by isolation with posterior secondary contact, or by primary divergence in the presence of gene flow. If primary divergence is responsible for the plumage variation in *D. platyrostris* (e.g. by differential selection between habitats), it is expected that phylogeographical gaps within each habitat do not match significant changes in plumage characters.

Thus, for a given level of genetic divergence in neutral markers, we expected to find greater plumage divergence in between-habitat comparisons than in within-habitat comparisons (Fig. 2C) (Moritz *et al.*, 2000; Smith *et al.*, 2005a, b; Norman *et al.*, 2007). Alternatively, if plumage transition accompanied population secondary contact instead of habitat transitions, the pattern shown in Figure 2D should be found.

The present study aimed to investigate plumage and genetic variation of *D. platyrostris* to determine: (1) is there any evidence of genetic continuity between *D. platyrostris* populations of the open vegetation corridor and of the Atlantic forest and (2) is plumage variation congruent with patterns of neutral genetic structure or with ecological factors related to habitat type? To address these questions, we studied five plumage characters and mitochondrial (mt)DNA to evaluate the predictions shown in Figure 2. Marantz (1997) performed a qualitative analysis of plumage variation in *D. platyrostris* to check the diagnosability of subspecies, although plumage variation at the intra-population level was not described. To further understand the geographical plumage variation and to make comparisons between genetic and plumage dissimilarity, we performed a semi-quantitative analysis of plumage variation. In addition, because some studies (Willis, 1992) consider subspecies of *D. platyrostris* as possible species, we also present the systematic implications of our results.

MATERIAL AND METHODS

PLUMAGE ANALYSIS

To describe geographical regions of plumage stability and to obtain plumage divergence estimations, we examined 510 adult specimens (180 females, 302 males, and 28 undetermined) from the museums: Museu Paraense Emílio Goeldi, Brazil; Museu de Zoologia da Universidade de São Paulo, Brazil; and American Museum of Natural History, USA. These specimens were collected in 172 localities that encompassed the entire range of the species (Fig. 1).

We recorded five plumage characters from each study skin. For each character, we defined a scoring system of states, subdividing the total range of variation of colour or pattern (see Appendix, Table A1). The number of states per character varied from three to four, depending of the magnitude of variation of each character. Specimens were scored by only one observer (F.M. d'Horta) by comparison with reference specimens. Colours were described according to the Munsell Soil Color Charts (Munsell Color Company, 2000).

Because previous studies did not show sexual dimorphism in plumage (F. M. d'Horta, unpubl. data)

(Marantz, 1997), males and females were analyzed together. *Sensu* Cracraft (1989), we first analyzed the variation of plumage characters to identify and delimit the smallest cluster of populations that are diagnosably distinct from other clusters. The unit of plumage analysis was the population sample ($N = 38$ population samples), each consisting of at least five specimens from the same or nearest localities. For each population sample, and for each character, we obtained a median 'character score', which was the numerical score that represented the middle measurement of the data set, according to a definition of the statistic median (Quinn & Keough, 2002). Then, we obtained a population total score, or 'total plumage score', which is equal to the sum of median scores of each of the five characters.

The geographical variation in median scores of plumage characters was mapped without considering any a priori taxonomic arrangements (i.e. subspecies). This procedure allowed us to identify geographical areas with little or no change in states of a given character (zones of phenotypic stability) and areas with abrupt changes (transition zones). Individual character maps were then overlapped to identify populations that can be diagnosed by a unique combination of plumage character states (congruent zones of phenotypic stability). To avoid considering portions of clines as zones of phenotypic stability, we only considered regions of at least three population samples. For a similar analysis, see D'Horta, Silva & Ribas (2008).

GENETIC DATASETS

We used muscle and blood samples to obtain DNA. Sampling localities and vouchers are presented in the Appendix (Table A2). The subspecific status of samples was inferred from the distribution of subspecies, *sensu* Marantz (1997). To test the monophyly of *D. platyrostris* and its subspecies, we used 983 bp of the cytochrome *b* (cyt *b*) and 584 pb of the mtDNA control region (CR) from 33 *D. platyrostris* (one from GenBank and the others sequenced in the present study), four *D. picumnus*, one *D. certhia*, and one *D. sanctithomae*. We used *Xiphocolaptes promeropyrhynchus* as the outgroup (Aleixo, 2002). For the population genetic study, we used CR of 43 samples from 19 localities. We selected CR for the population analyses because a preliminary study indicated that it is more variable than cyt *b*. Procedures for obtaining cyt *b* sequences and CR were carried out *sensu* Cabanne *et al.* (2008) and Cabanne *et al.* (2007), respectively. Because both cyt *b* and CR are physically linked in a single molecule (mitochondrial genome), the results obtained with both markers are totally compatible because they reflect a single

genealogy and history (Avice, 2000). For an example on this strategy, see McCormack, Bowen & Smith (2008). GenBank accession numbers are JF276349–JF276385 for cyt *b* and JF276306–JF276348 for CR.

GENETIC DATA EXPLORATION

We used cyt *b* and CR to perform Neighbour-joining, maximum likelihood, and Bayesian analyses in MEGA, version 4.0 (Tamura *et al.*, 2007), PHYL, version 2.4.4 (Guindon & Gascuel, 2003) and MrBayes, version 3.1 (Ronquist & Huelsenbeck, 2003), respectively, to obtain phylogenetic trees for testing the monophyly of the species and subspecies, and to identify intraspecific clades. Molecular evolution models were selected in MODELTEST, version 3.7 (Posada & Crandall, 1998). We only sequenced CR for *D. platyrostris*; for the other taxa, we coded this region as missing data. Bayesian analyses considered each gene as a different partition. Population analyses only used CR sequences. Relationships among CR sequences were studied by constructing a median-joining network in NETWORK, version 4.1.0.8 (<http://www.fluxus-engineering.com>). The neutrality tests of Tajima (1989), Ramos-Onsins & Rozas (2002) and McDonald & Kreitman (1991) (MK test) were performed in DNASP, version 4.0 (Rozas *et al.*, 2003). *Dendrocolaptes picumnus* was used as outgroup in the MK test. Analyses of molecular variance (AMOVA) were performed using ARLEQUIN, version 3.1 (Excoffier, Laval & Schneider, 2006). For evaluation of whether sequence samples were representative of the genetic constitution of the species, we estimated $P = [(k - 1)/(k + 1)]$, which represents the probability that a sample of size k and the whole population share the most recent ancestor (Hein, Schierup & Wiuf, 2005). P can be interpreted as the probability of the sample of being representative of the genetic diversity of the population. The population diversity parameter Θ (theta) was estimated in LAMARC, version 2.1.2b (Kuhner, 2006) (see Supporting information).

GENETIC LANDSCAPE SHAPE

We used a visualization method to obtain a graphical representation of the pattern of genetic distances between individuals across the study area. The procedure was carried out *sensu* Miller *et al.* (2006) and using AIS, version 1.0 (Miller *et al.*, 2006). We performed analyses using p -distances between individuals because no other distance model was available in AIS, and using residual genetic distances derived from a regression between genetic and geographical distances. Because the results of both analyses were the same, we only present those obtained with genetic

distances. Conditions of the AIS analysis: # coordinates bins (X and Y) = 100; distance weight value = 5. The surface for the landscape shape interpolation was based on midpoint of edges derived from a Delaunay triangulation.

DIVERGENCE, GENE FLOW, AND HISTORICAL DEMOGRAPHY

We used CR sequences and the isolation–migration (IM) model (Nielsen & Wakeley, 2001; Hey & Nielsen, 2004), implemented in IM, version 21 April 2008 (Hey & Nielsen, 2004) to estimate gene flow and divergence times. We also used IM to evaluate whether a model without migration adjusted better to the observed data than a model with migration. According to Akaike (1985), the model that minimized $AIC = -2[\log(L) - d]$ is the best, where d is the number of parameters. We assumed the Hasegawa–Kishino–Yano model of evolution, similar population parameter theta values ($\Theta_1 = \Theta_2 = \Theta_a$), and $m_1 = m_2$. Analyses used a burn-in of 500 000 iterations and 30–100 million total iterations. We transformed time parameter t into time in years by $t = t/u$, where u is the mutation rate per marker, and migration parameter m into the effective number of migrant genes (= females) by $M = (\Theta m)/2$. We assumed a generation time of one year. To obtain u for CR sequences, we scaled the cyt *b*-rate of change (obtained from 2.1% divergence per Myr; Weir & Schluter, 2008) with the ratio between the population parameters of each marker $\Theta_{CR}/\Theta_{cytB}$, where Θ_{CR} is the Θ -value of the CR and Θ_{cytB} is the Θ -value of the cyt *b*. The calibration for CR obtained with this method was 14.2% of divergence per Myr (for details, see Results). A published application of this approach to obtain a calibration rate for CR sequences is provided in Toon *et al.* (2007).

We also evaluated historical demography by calculating the R_2 statistic (Ramos-Onsins & Rozas, 2002) in DNASP, version 4.0. Significant and low values of R_2 suggest demographic expansion if neutrality was not rejected.

MODEL-BASED PHYLOGEOGRAPHY

To further investigate the evolutionary history of populations, and particularly their relationships, we evaluated the goodness of fit of the CR sequences to simulated data under different demographic scenarios. Reviews on this approach are provided by Richards, Carstens & Knowles (2007) and Knowles (2009). Sequence simulations were performed in BAYESSC, a modification of software SERIAL SIMCOAL (Anderson *et al.*, 2005; Chan, Anderson & Hadly, 2006). We tested models that differed in terms of number of populations, migration rates, and time of divergence (see Supporting information). Parameters

for the simulations were introduced as intervals instead of single values. The modelled historical events, or populations splits, occurred at climatic extremes of the Late Quaternary (6000 years ago, 21 000 years ago, and before) (Carnaval & Moritz, 2008). For each model, we performed 1000 simulations and estimated, using ARLEQUIN, two independent summary statistics suitable for this application (Hickerson, Dolman & Moritz, 2006), namely nucleotide diversity and F_{ST} . For evaluating the goodness of fit of the observed data to simulated data, we used the empirical likelihood of each summary statistic. First, we estimated the proportion P of simulated values equal and higher than the observed summary statistics, and then we obtained an overall P -value for each model by combining individual P -values by the Fisher's method in the program METAP (Whitlock, 2005).

ANALYSIS OF GENETIC AND PLUMAGE DIVERGENCE

To study genetic divergence, we performed a multiple correlation analysis (multiple Mantel tests; Manly & Manly, 2001) using, as a dependent variable, average Tamura & Nei (1993) (TN 93) genetic distances between individuals from pairs of localities (variable DGEN) and two independent variables, DGEO and DHAB. DGEO was equal to the straight geographical distance between localities and variable DHAB was an indicator variable that indicated whether comparisons were made within the same habitat (within open vegetation corridor or within AF, DHAB = 0) or between habitats (open vegetation corridor versus AF, DHAB = 1). An explanation on indicator variables is provided in Quinn & Keough (2002). If genetic distances are only affected by geographical isolation, we expected DGEO to be the only predictor of DGEN. However, if habitat transition was also related to genetic divergence, we also expected a significant correlation between DGEN and DHAB. We used FSTAT (Goudet, 2002) to perform partial Mantel tests with 20 000 replicates to check significance.

Population isolation was evaluated by mtDNA neutral genetic divergence. To evaluate whether plumage variation was related to population isolation and habitat transitions, we performed two analyses. First, we used a multiple Mantel test to address whether plumage variation between pairs of localities (variable DPLUM) was related to two independent variables, namely genetic distances (DGEN) and DBIOM. DPLUM consisted of a Manhattan distance (Quinn & Keough, 2002) equal to the sum (across characters) of absolute differences in the median character scores between pair of localities with associated genetic data (Fig. 1; see also Appendix, Table A2). To obtain DPLUM plumage, character states were considered as ordered. We also performed

analyses considering plumage states as unordered but, because the results were similar to those obtained with ordered states, we only present the results with ordered states. Some localities with genetic data did not have available associated museum specimens for plumage scoring. For those localities, we assumed plumage states from the nearest locality within a radius of 50 km (see Appendix, Table A2). We used FSTAT to perform partial Mantel tests (20 000 replicates). A second plumage analysis qualitatively evaluated the relationship between DPLUM and DGEN to test the predictions of Figure 2C, D. Analyses of this section excluded localities Felixlândia and Bocaiuva, which were located in an area of transition between Cerrado and AF, and have neither museum specimens for the plumage analysis available, nor close localities to extrapolate plumage data.

RESULTS

PLUMAGE VARIATION

Two areas of plumage stability were found: populations I and II (Fig. 3). The core of population I was located at Cerrado and the diagnosis was: HEAD very dark greyish-brown with streaks pale yellow (state 2); TAIL dark reddish-brown (state 3); UPPERTAIL-coverts yellowish-red (state 2); BACK dark yellow-brown without streaks (state 0); and VENT light yellowish-brown with thin bars and very dark greyish brown (state 2). Population II was located at southern Atlantic forest and was diagnosed by: HEAD black with white streaks (state 6); TAIL dark reddish-brown (state 6); UPPERTAIL-coverts dark brown with a black patch at the subterminal portion of feathers (state 6); BACK dark brown streaked white (state 6); and VENT light olive-brown with bold bars dark greyish brown (state 6).

GENETIC ANALYSIS

We obtained a cyt *b*-CR alignment of 33 *D. platyrostris* and seven other dendrocolaptids [1567 bp, 214 variable positions, 125 (58.4%) informative for parsimony]. Neutrality of the cyt *b* from *D. platyrostris* was not rejected by the MK test (G -test $P > 0.05$), the test of Tajima ($D = -0.092$, $P > 0.1$) and by the R_2 test of Ramos-Onsins and Rozas ($R_2 = 0.1189$, $P > 0.05$). The CR alignment had 43 *D. platyrostris* (584 bp, 41 variable positions, 33 (80.5%) informative for parsimony). The test of Tajima for the CR data set was not significant ($D = -0.03438$, $P > 0.1$) and the R_2 test of Ramos-Onsins and Rozas ($R_2 = 0.1101$, $P > 0.05$). The cyt *b*-CR and the CR set of sequences have a 94% and 95.5% probability of having correctly sampled the size

Figure 3. Geographical distribution of plumage variation in *Dendrocolaptes platyrostris*. Circles represent plumage units of analysis (population samples) and different levels of grey represent character scores. A, B, C, D, E, single plumage characters. For a detailed description of character states, see Appendix (Table A1). F, areas of plumage stability obtained by overlapping A to E.

Table 1. Summary statistics of *Dendrocolaptes platyrostris* lineages based on the mitochondrial DNA control region

	All samples	Populations		
		OVC	CAF	SAF
Sample size	43	19	13	11
Population diversity parameter, Θ (95% CI)	0.0395 (0.025–0.063)	0.0278 (0.015–0.051)	0.0072 (0.003–0.017)	0.0131 (0.006–0.030)
Nucleotide diversity, π (SE)	0.0168 (0.0031)	0.014 (0.003)	0.0042 (0.0015)	0.00116 (0.0024)
Tajima's <i>D</i>	–0.03438	–0.02490	–0.94334	–0.25745
<i>R</i> ²	0.1101	0.1266	0.1206	0.1382*

*P < 0.05. Significance determined based on 1000 coalescent simulations under a model of population stability using empirical sample sizes and estimates of Θ .

95% confidence intervals (CI) are shown within parentheses. For configuration of populations, see Fig. 3.

OVC, open vegetation corridor; CAF, central Atlantic forest; SAF, southern Atlantic forest.

of the gene genealogy of the whole population, respectively. Therefore, our gene samples are suitable for the subsequent studies.

We evaluated the relative diversity of *cyt b* and of CR in a subsample of 31 individuals. Θ for CR was 0.0395 [95% confidence interval (CI) = 0.025–0.063] and for *cyt b* was 0.005843 (95% CI = 0.003–0.0105). The ratio was $\Theta_{CR}/\Theta_{cyt\ b} = 6.76$. Because we used the same individuals for this analysis, the ratio $\Theta_{CR}/\Theta_{cyt\ b}$ indicated that the mutation rate at the CR of *D. platyrostris* was approximately 6.7-fold higher than the rate for *cyt b*, in accordance with rates of change described for mtDNA control region of other birds (Ruokonen & Kvist, 2002). Therefore, we selected CR for the phylogeographical analyses because it was more variable than *cyt b*.

Even though we studied four species of a total of five (Marantz *et al.*, 2003), *D. hoffmannsi* was not sampled, our analysis supported *D. platyrostris* as being monophyletic. The phylogeny grouped *D. platyrostris* and *D. picumnus* as sister taxa (Fig. 4A), in agreement with plumage and morphology (Raikow, 1994; Marantz & Patten, 2010). Subspecies were not supported as monophyletic. The concatenated Bayesian phylogeny did not resolve well the basal node of *D. platyrostris*. This may have occurred because CR sequences were only available for *D. platyrostris*. We repeated the Bayesian phylogeny only using *cyt b* and confirmed the results obtained with the other two methods. *Dendrocolaptes platyrostris* appears to be a young species, as inferred by the moderate intraspecific differentiation (*cyt b* TN 93 + G distance of 0.47% at the basal node; approximately 0.22 Mya) and to the proximity to *D. picumnus* (TN 93 + G *cyt b* distance of 3.35%; approximately 1.6 Mya), in contrast to results obtained with other endemic passerines that are older species (Pessoa *et al.*, 2006; Cabanne *et al.*, 2008; d'Horta *et al.*, 2011). The low sequence variation within this species relative to the congeners that we

studied suggests that we sampled the existing variation in *D. platyrostris*. Some intraspecific nodes of the phylogeny are weakly supported. This result was expected because the phylogenetic hypothesis was obtained with approaches developed to reconstruct interspecies relationships and many of the assumptions of these methods are violated by intraspecific datasets, which may result in low phylogenetic resolution (Crandall & Templeton, 1996; Posada & Crandall, 2001). Networks are more suitable for intraspecific datasets because they cope with population-level phenomena (i.e. ancestral and derived sequences present in the sample) that traditional species tree methods do not (Posada & Crandall, 2001).

The CR network, which presented 26 haplotypes, in conjunction with the phylogeny, indicated the existence of three main mtDNA lineages in *D. platyrostris* (Fig. 4A, B). The AIS analysis denoted two main genetic barriers: one between the open habitat and the AF and the other one within the AF (Fig. 4C). The genealogical analysis in conjunct with the AIS study indicated the existence of three regions with genetic identity, or lineages: the open vegetation corridor, the central AF, and the southern AF. Most sequences of central AF have an interior localization and multiple connections in the network, which are both conditions that, according to predictions of the coalescent theory, suggest that this lineage is ancestral (Posada & Crandall, 2001). Summary statistics for those lineages are presented in Table 1. The existence of the three lineages was further corroborated by the analysis AMOVA (Table 2); AMOVA I showed substantial structure among the three regions. In addition, AMOVA II showed structure between habitats, in accordance of the predictions of Figure 2B. AMOVA analyses III and IV showed that structure within the AF is higher than in the open habitat.

The lineage of the open vegetation corridor was associated with the plumage stability area I and

Figure 4. A, maximum likelihood phylogenetic tree based on concatenated cytochrome *b* (cyt *b*) and mitochondrial (mt)DNA control region of *Dendrocolaptes platyrostris* and closely-related species. *init*, *Dendrocolaptes platyrostris intermedius*; *plat*, *Dendrocolaptes platyrostris platyrostris*. Model: HKY + I (I = 0.65) + G ($\alpha = 0.8421$), Tr/Tv ratio = 11.066. Estimated base frequencies: A = 0.296, C = 0.303, G = 0.130, T = 0.269. Support values at nodes: maximum likelihood $\times 500$ bootstrap/Neighbour-joining $\times 500$ Bootstrap/Bayesian probability analysis with cyt *b*/Bayesian probability analysis with cyt *b* + CR. Bootstrap > 70 and Bayesian probabilities > 0.5 . Divergence time obtained using cyt *b* corrected distances and 2.1% of divergence/Myr. Numbers within parenthesis indicate sampling locality (Fig. 1; see also Appendix, Table A2). Distribution of samples by region is also shown. B, median-joining network based on the mtDNA control region of *D. platyrostris*. Numbers indicate the sampling localities of Fig. 1 and the Appendix (Table A2). Numbers that are underlined represent localities at the open habitat-forest ecotone. C, location of plumage stability areas and the main genetic barriers inferred by analysis of mtDNA control region sequences in AIS, version 1.0 (Miller *et al.*, 2006).

Table 2. Analyses of molecular variance of *Dendrocolaptes platyrostris* based on 584 bp of the control region of the mitochondrial DNA

Analysis	Tested population structure	Φ_{ST} (95% confidence interval)
I, All study region	[OVC][CAF][SAF]	0.332 (0.23–0.41)*
II, AF versus OVC	[AF] [OVC]	0.483 (0.38–0.58)*
III, within AF	[CAF][SAF]	0.752 (0.58–0.857)*
IV, within OVC	[Northern OVC] [southern OVC]	0.329 (0.297–0.357)*

*Significant with Bonferroni correction, $P < 0.01$. See configuration of populations in Fig. 3. OVC, open vegetation corridor; CAF, central Atlantic forest; SAF, southern Atlantic forest.

subspecies *intermedius*. The other lineages were associated with the nominal subspecies and the AF. The CR network also denoted derived haplotypes that occur in the ecotone between the open habitat and the forest (e.g. localities 3 and 6; Fig. 4B). Those derived haplotypes may indicate a past range expansion of forest birds toward the open habitat, followed by a restriction of gene flow and divergence (Omeland, Baker & Peters, 2006).

COALESCENCE ANALYSIS

Studies with IM and the Akaike (1985) criterion indicated that a model of isolation with migration was the best explanation for all the studied divergences (Table 3). Migration rates were moderate to high. Point divergence estimations were in the range 80 000–148 000 years, although their posterior distributions were flat at ends and did not reach likelihood zero (data not shown). This pattern may be consequence of lack of information about a specific divergence time because gene flow rates are high and therefore divergences are very shallow.

We simulated CR sequences under 12 different demographic scenarios to explore whether populations fitted the following four situations: (1) a single panmictic population; (2) three populations connected by gene flow without a specific data of divergence; (3) three populations that diverged together from an ancestral population (politomy); and (4) three particular hierarchical relationships among the three lineages. Seven models were rejected (for details on each model, see the Supporting information, Table S1). Specifically, simulations rejected the model of a single

panmictic population, the models of politomy, one model considering central AF and the open vegetation corridor as sisters, and all models where the open habitat and southern AF were sister populations. The two simplest models not rejected suggested that the open habitat, and the central and southern AF, are connected with low to moderate gene flow (migration rate interval 0–0.0001) without a specific divergence time period (see Supporting information, Table S1, models 2a and 2b).

Simulations supported *D. platyrostris* populations as being neither panmictic, nor indicating a specific divergence date. Even though IM analyses estimated point divergence times, their posterior distributions denoted a reduced amount of information regarding divergence dates (Table 3). Thus, we consider the results of both approaches to be in complete agreement. They have indicated a lack of information about a specific divergence time, possibly because gene flow rates might have been high for a long time. Relationships among the open habitat, and the central and southern AF, suggested in Figure 4 are not very strong because populations are closely related and because the study used a single marker. However, the simulation of sequences, an approach that considers stochastic variation in genealogies (Richards, Carstens & Knowles, 2007; Knowles, 2009), did reject a sister condition between the open habitat and the southern SAF, and supported models that considered the open habitat sister of central AF and southern AF sister of central AF (see Supporting information, Table S1). Even though simulations did not support a single relationship among populations, they corroborated what was suggested by the network (i.e. that central AF sequences were not derived) (Fig. 4B).

GENETIC VERSUS PLUMAGE VARIATION

Multiple Mantel tests indicated that genetic distances are moderately correlated with geographical distances and only marginally related to habitats transition (Table 4, analysis I). Other analysis indicated that plumage divergence was not correlated with genetic divergence but was substantially related to habitat transitions (Table 4, analysis II). The historical demographics analysis indicated that only the SAF clade presented evidence of past population expansion (Table 1). Specifically, the R_2 statistic is significant and the region SAF presented a low level of genetic diversity.

The relationship between plumage and genetic divergence within and between habitats indicated that, for a specific genetic distance, comparisons between habitats in most of the cases resulted in larger plumage divergences than comparisons within habitats (Fig. 5).

Table 3. Divergence time and migration between pairs of populations of *Dendrocolaptes platyrostris* based on the mitochondrial DNA control region and in a model of isolation with migration (Hey & Nielsen, 2004)

Analysis	Model	Migration (females/generation)	Φ (absolute substitutions)	Divergence time (years)	AIC
OVC × CAF	With migration	2.41 (0.52–10.42)	26.43 (17.01–42.57)	83 590 (29 900–240 000 ^{approximately})	2047.364
	No migration	Forced to be zero	44.3852 (31.00–62.00)	11 940 (3 980–22 800)	2048.606
OVC × SAF	With migration	1.87 (0.12–8.11)	36.30 (22.77–57.24)	84 318 (22 000–280 000 ^{approximately})	2156.752
	No migration	Forced to be zero	48.60 (32.87–72.15)	35 800 (16 000–61 000)	2159.05
CAF × SAF	With migration	1.97 (0.08–18.79)	22.34 (13.37–38.36)	148 000 (> 47 000)	2152.53
	No migration	Forced to be zero	36.94 (22.65–54.88)	19 900 (7 600–36 500)	2153.718

Models with and without migration were contrasted by the Akaike information criterion (AIC). The model that minimized AIC was the best. Confidence intervals are HPD90. Maximum values of HPD90 tagged (approximately), or absent, indicate that the end of the posterior distribution was flat and did not reach likelihood zero. For properties of samples, see Fig. 3 and Table 1. OVC, open vegetation corridor; CAF, central Atlantic Forest; SAF, southern Atlantic Forest.

Table 4. Multiple Mantel tests in *Dendrocolaptes platyrostris* to analyze the correlation of genetic distances (DGEN) and plumage divergence (DPLUM) with geographical distances (DGEO) and habitat transitions (DHAB)

	Partial correlation coefficient (<i>r</i>)	<i>P</i>	<i>R</i> ²
Analysis I: DGEN in function of (DGEO, DHAB)			0.20
DGEN × (DGEO)	0.421	0.0003	
DGEN × (DHAB)	0.171	0.026	
Analysis II: DPLUM in function of (DGEN, DHAB)			0.56
DPLUM × (DGEN)	0.35	0.815	
DPLUM × (DHAB)	0.662	0.0000	

Predictor variables are shown within parenthesis. DGEN is based on 584 pb of the mitochondrial DNA control region.

DISCUSSION

PLUMAGE COLOUR IN *D. PLATYROSTRIS*

The analysis identified two areas of phenotypic stability (Fig. 3). Birds from population I were lighter, less streaked, and associated with subspecies *intermedius*. Birds from population II were the darkest and were associated with the nominal subspecies. Both populations are associated with subspecies, although their geographical ranges are smaller than the subspecies ones (Fig. 1). Birds outside the phenotypic stability regions showed different combinations of characters, grading from population I to population II (Fig. 3). As suggested by the subspecific taxonomy, our results on plumage variation of *D. platyrostris* do not support the idea of continuity between the open vegetation corridor

Figure 5. Relationships of plumage divergence versus mitochondrial DNA control region genetic distances of *Dendrocolaptes platyrostris* within and between habitats. TN 93: Tamura & Nei (1993). OVC, open vegetation corridor; AF, Atlantic Forest.

and the AF because each plumage type (populations I and II) is associated with each habitat. Plumage of *D. platyrostris* followed the rule of Gloger, which states that plumages in humid regions are darker and plumages in dryer regions are lighter (Zink & Remsen, 1986). The pattern is not only evident in the comparison Cerrado-Atlantic forest, but also the birds from the Catinga (an even dryer region) appear to be even lighter than in the Cerrado, thus making the pattern even stronger (Fig. 3F).

GENETIC PATTERN AND POSSIBLE ORIGIN

According to some studies, gallery forests in the open vegetation corridor act as corridors between the Amazonian and Atlantic forests (Costa, 2003). Under this

model, we expected to find no genetic divergence between contiguous populations in the forest habitat and in the open habitat (Fig. 2A), a prediction that was not supported for *D. platyrostris*. Even though the genetic divergence between habitats is shallow (Fig. 4), the phylogeographical gap is substantial ($\Phi_{ST} = 0.48$) (Table 2). This result was further confirmed by multiple Mantel tests, which indicated that habitat transitions, as well as geographical distances, are correlated with genetic differentiation (Table 4). Under the evaluated model of biogeographical continuity between the open habitat and the forest habitat, we expected to find no significant correlation between genetic distances and habitat transition. Also, the existence of population structure was supported by simulations that rejected models of panmixia.

Potential explanations for our findings are: (1) isolation and secondary contact along habitats transition or (2) divergence with gene flow (primary contact). We consider secondary contact unlikely because current and historical transitions between the open habitat and the forest habitat are gradual and occur over a latitudinal axis of thousand of kilometers (Fig. 1), and also because geographical landmarks (i.e. valleys, rivers or mountain ranges) that could have isolated both habitats do not exist. One scenario that would make vicariance and secondary contact plausible suggests that a small population could have been isolated in a forest relict, diverged in isolation, and then expanded to all the open habitat gallery forests. This model is known as the 'vanishing refuge' (Vanzolini & Williams, 1981; Moritz *et al.*, 2000). However, this hypothesis predicts signals of population expansion to be found in the open habitat, which were not found in the present study. Specifically, the R_2 test was not significant (Table 1) and no star-like genealogy was observed (Fig. 4B).

On the other hand, primary contact appears to be more likely not only because secondary contact was not very plausible, but also because the model was supported by the IM analysis. Under a scenario of divergence with gene flow, the habitat transition might be a partial barrier if populations acquired adaptations for their corresponding habitats. Characters favoured at the open habitat could have been negatively selected at the forests, and vice versa, which could have caused a partial barrier along the transition of habitats that, over the long term, was reflected by neutral markers such as the mtDNA (Norman *et al.*, 2007). A future study for further discrimination between vicariance and divergence with gene flow across the open and forest habitat transition may incorporate multiple independent markers aiming to analyze the distribution of migration events over time using the isolation migration model in IM. Currently, populations of the open habitat and of the

central AF meet at the transition of habitats (Fig. 4). If this pattern was generated by vicariance followed by secondary contact, intense recent gene flow should be observed (Won & Hey, 2005; Niemiller, Fitzpatrick & Miller, 2008; Nosil, 2008). Otherwise, if the pattern evolved in parapatry, migration should be distributed homogeneously during the divergence period.

The results obtained in the present study are congruent with the few studies available on population genetic of birds from the open vegetation corridor (Bates, Tello & Silva, 2003; D'Horta *et al.*, 2008; Nodari, 2008). These studies suggest that those birds have a lower genetic differentiation among populations than do birds from continuous forests (Aleixo, 2004; Cheviron, Hackett & Capparella, 2005; Pessoa *et al.*, 2006; Nyari, 2007; Cabanne *et al.*, 2008). The population genetic structure of *D. platyrostris* in the open habitat was smaller than in the AF (Table 2), which would indicate different demographical processes in each habitat. Additional studies are needed in an attempt to explain this apparent difference.

EVOLUTION OF PLUMAGE: POPULATION HISTORY VERSUS HABITAT TRANSITIONS

We investigated whether plumage variation in *D. platyrostris* accompanied population histories, as evaluated by genetic divergences, or whether it could have been affected by other factors, such as differential selection associated with each habitat. Multiple Mantel tests indicated that genetic divergence did not affect plumage and that habitat transition was correlated with plumage variation (Table 4, analysis II). Furthermore, the relationship between plumage and genetic divergence always resulted in larger plumage divergences between habitats than within habitats (Fig. 5), supporting the predictions of Figure 2C. All these results are in accordance with what is expected for primary divergence of plumage.

Dendrocolaptes platyrostris at the open vegetation corridor was lighter and less streaked than at the forest habitat, representing a morph that is suggested to be an adaptation of woodcreepers for habitats with high luminosity levels, as are forests at the open vegetation corridor (Willis, 1992; Marantz, 1997). On the other hand, in continuous rainforest, individuals are darker and more streaked, and this is considered to be an adaptation for living in low luminosity and very humid conditions (Zink & Remsen, 1986; Willis, 1992; Marantz, 1997). For example, more melanic plumages are more resistant to feather degradation by bacteria that are abundant in humid habitats. Therefore, the results of the present study support the idea that the two regions of stable plumage of *D. platyrostris* may have evolved by divergent selection regimes between habitats.

The open vegetation corridor and its network of gallery forests and dry forests are contiguous with the Atlantic and Amazon forests. Our results support the idea that the two plumages types of *D. platyrostris* may have evolved by divergent selection regimes between habitats. There are several other species that occur in both habitats and might present a similar evolutionary story. For example, the pair of sister species *Thamnophilus ruficapillus* Vieillot (1816) and *Thamnophilus torquatus* Swainson (1825) (Brumfield & Edwards, 2007), which are mainly differentiated by plumage, may have diverged by selection in different habitats. *Thamnophilus ruficapillus* is the darkest and occurs in lower growth, borders, and secondary growth at AF and tropical Andes forests, whereas *T. torquatus* occurs in scrubs and lower growth at Cerrado and Caatinga. Females of both species are very similar. Another example might be the pair *Basileuterus culicivorus* Deppe (1830) and *Basileuterus hypoleucus* Bonaparte (1850). The former is the darkest and occurs in most of forested regions of the Neotropics, whereas the latter occurs in forest borders and scrubs of the Cerrado. Both species are not reciprocally monophyletic (Vilaça & Santos, 2010); they hybridize (Robbins, Faucett & Rice, 1999) and plumage differentiation is subtle.

INTRA-ATLANTIC FOREST BIOGEOGRAPHICAL IMPLICATIONS

The results of the present study are compatible with what is known about Atlantic forest evolution during the late Pleistocene (Cabanne *et al.*, 2008; Carnaval & Moritz, 2008; Carnaval *et al.*, 2009; d'Horta *et al.*, 2011). Rainforests in the central region of the biome (i.e. Bahia, Espírito Santo, eastern Minas Gerais, and Rio de Janeiro) are proposed to be one of the most persistent during the climatic oscillations of the late Pleistocene, and apparently have acted as a refuge for forest organisms during the maximum of glaciations. Therefore, it is expected that populations of several forest specialist from that region were ancestral in relation to populations located in the periphery of the biome, such as the southern AF and the open vegetation corridor. This particular prediction is supported by the network and the simulations that positioned sequences associated with population central AF as the oldest population, and sequences from the open habitat and southern AF as the newest.

Dendrocolaptes platyrostris presented a mtDNA phylogeographical structure within the AF that resembles patterns described for other endemic taxa (Cabanne *et al.*, 2008; Batalha-Filho *et al.*, 2010; but see Mustrangi & Patton, 1997; Grazziotin *et al.*, 2006; Pessoa *et al.*, 2006; Carnaval *et al.*, 2009; Fitzpatrick *et al.*, 2009; Thomé *et al.*, 2010; d'Horta *et al.*, 2011).

The common pattern consists of the existence of two main mtDNA clades south of the Doce river that come into contact along no evident gene flow barrier in central-southern São Paulo (Fig. 4C). Some species present a third lineage, north of the Doce river. Another feature of the common pattern is that the corresponding southern populations present evidence of population expansion. In *D. platyrostris*, the southern AF population is the only one presenting evidence of past population expansion (Table 1). The existence of shared phylogeographical patterns suggests that common evolutionary events have affected an important proportion of the Atlantic forest taxa.

Several studies have proposed that the southern Atlantic forest region was dominated by grasslands during peaks of global glaciations. Palynology studies suggest that the forest southern limit shifted almost 750 km northward (Behling, 2002; but see also Prado & Gibbs, 1993). However, phylogeographical studies on AF organisms indicate the existence of endemic lineages to the southern portion of the biome (i.e. lineage southern AF of *D. platyrostris*; Mustrangi & Patton, 1997; Grazziotin *et al.*, 2006; Pessoa, 2007; Cabanne *et al.*, 2008; Carnaval *et al.*, 2009; Batalha-Filho *et al.*, 2010; Thomé *et al.*, 2010; d'Horta *et al.*, 2011), a pattern that suggests that this region maintained enough forest during the glacial maxima to avoid extinction of several forest organisms. Interestingly, many of these taxa are forest specialists, such as the passerines *Xiphorhynchus fuscus* Vieillot (1818) and *Sclerurus scansor* Ménétries (1835) (Cabanne *et al.*, 2008; d'Horta *et al.*, 2011). Thus, the results obtained in the present study, together with those of previous studies, indicate that the southern Atlantic forest may have been significantly affected by Pleistocene climatic alteration, although such events did not cause local extinction of most taxa, as occurred in other regions of the globe where forests were significantly affected by global glaciations (i.e. Europe; Hewitt, 2000).

SYSTEMATIC IMPLICATIONS

Do plumage stability areas I and II (Fig. 3), or the associated subspecies, deserve the status of species? Even though both plumage stability areas are diagnosable, a condition for considering a population as a full species according to the general lineage concept of species (de Queiroz, 1998; Aleixo, 2007), the genetic analysis sampled those areas only marginally and therefore did not demonstrate that they are reciprocally monophyletic (Fig. 4C). Reciprocal monophyly is another condition for considering populations as full species. Besides, both populations are connected by a large area of plumage transition across the open vegetation corridor–AF ecotone and along a large

track of AF (Fig. 3), indicating that they are evolutionary closely related. This close relationship is further demonstrated by the high gene flow rates that existed between regions (Table 3). The former considerations can also be made for both subspecies of *D. platyrostris*. They are neither reciprocally monophyletic, nor have stability of plumage. Thus, our results neither support plumage stability areas, nor subspecies as full species because they do not represent independent evolutionary lineages.

CONCLUSIONS

Despite the fact that the South American open vegetation corridor and the Atlantic forest are today physically contiguous and connected by gallery and dry forests, our results for *D. platyrostris* are not compatible with the idea that forest specialists in each region are linked at the population level. Different ecological conditions and selection in each biome might explain this result. Most of the plumage variation of *D. platyrostris* followed changes of habitat instead of population historical divergence, suggesting that selection might be directly related to the evolution of the plumage of this species. The results obtained in the present study do not support plumage stability areas, nor subspecies as full species because they do not represent independent evolutionary lineages. Further research with additional genetic samples and independent markers is needed to complement our findings.

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APPENDIX

Table A1. Studied plumage characters of *Dendrocolaptes platyrostris*

Characters (number of states)	Description of characters and states
HEAD (4)	Characteristics of contour feathers of the crown and nape. <i>State 0</i> , external (ext.) portion of the vane dark yellowish brown (10YR 3/3) and internal (int.) portion of the vane pale yellow (2,5Y 8/4) –ref. MZUSP 41640-. <i>State 2</i> , ext. vane very dark greyish brown (10YR 3/2) and int. vane pale yellow (10YR 8/2) –ref. MZUSP 72052-. <i>State 4</i> , ext. vane very dark grey (10YR 3/1) and int. vane white (2,5Y 8/8) –ref. MZUSP 72374-. <i>State 6</i> , ext. vane black (2,5Y 8/1) and int. vane as state 4 –ref. MZUSP 35465-. Heads with state 0 did not present strong streaks, whereas state 6 represented the most streaked heads.
TAIL (3)	Vane colour. <i>State 0</i> , yellowish–red (5YR 4/6) –ref. MZUSP 41640-. <i>State 3</i> , dark reddish–brown (5YR 3/4) –MZUSP 72374-. <i>State 6</i> , dark reddish–brown (5YR 3/2) –ref. MZUSP 35469-.
UPPERTAIL (4)	Uppertail-coverts. <i>State 0</i> , yellowish–red (5YR 5/8) –ref. MZUSP 41.640). <i>State 2</i> , yellowish–red (5YR 4/6) –ref. MZUSP 72.374-. <i>State 4</i> , background dark yellowish–brown (10YR 3/6) with three to four bars black (2,5Y 8/1) –ref. MZUSP 73767-. <i>State 6</i> , unbarred with background dark brown (7,5YR 3/4) and a black patch at the subterminal portion of feathers –ref. MZUSP 70204-.
BACK (3)	Mantle and back contours. <i>State 0</i> , no streaks, dark yellow–brown (10YR 4/4) –ref. MZUSP 41640-. <i>State 3</i> , no streaks evident, olive–brown (2,5Y 4/4) –ref. MZUSP 51859-. <i>State 6</i> , streaked, ext. vane dark brown (2,5Y 3/3) and int. vane white (2,5Y 8/8). Also, black mark at distal portion of internal vane –ref. MZUSP 35469-.
VENT (4)	Belly and flank contours. Varied from slightly barred to strongly barred. <i>State 0</i> , background olive–yellow (2,5Y 6/6) and fine bars dark olive–brown (2,5Y 3/3) –ref. MZUSP 41064-. <i>State 2</i> , background light yellowish–brown (2,5Y 6/4) and bars very dark greyish brown (2,5Y 3/2) –ref. MZUSP 72374-. <i>State 4</i> , background light olive–brown (2,5Y 5/3) and bars very dark greyish brown (2,5Y 3/2) –ref. MZUSP 35469-. <i>State 6</i> , colour as state 4 but bolder bars (thicker than 2 mm) –ref. AMNH 794072-.

Colours follow the Munsell Soil Color Charts (Munsell Color Company, 2000). Within parenthesis after each colour name are hue, value/chroma. ref., museum specimen taken as reference; MZUSP, Museu de Zoologia da Universidade de São Paulo, Brazil; AMNH, American Museum of Natural History, USA.

Table A2. Collection localities of *Dendrocolaptes platyrostris* and of other taxa for the genetic analyses, sample identification, vouchers, and locality of plumage data collection

Species	Locality (habitat)	Genetic data: sample size and tissue identification ^{TISSUE}	Vouchers for tissue samples ^{VOUCHER}	Plumage versus genetic comparison: plumage data, locality and sample size ^{PLUMAGE}
<i>Dendrocolaptes platyrostris</i>	1 – Ubajara, Ceará (CE). 3°51'S, 40°56'W (Caatinga)	N = 1. LGEMA 11443 ^B	Photo ^P	Baturité, CE. 4°17'S, 38°55'W, N = 6
	2 – National Park (NP) Serra das Confusões, Piauí (PI). 9°40'S, 44°8'W (Caatinga)	N = 7. All LGEMA: P1853 ^M , P2181 ^M , P2277 ^M , P2278 ^M , P2329 ^M , P2379 ^M , P2431 ^M	LFS 353 ^F All MZUSP: 77719, 77720, 77721, 77722, 77723.	Locality: Idem genetic data. N = 7
	3 – Bonito, Bahia (BA). 11°56'S, 41°15'W (Caatinga)	N = 1. LGEMAP2272 ^B	Photo ^P	Fazenda Mocambo e Iracema (Chapada Diamantina), BA. 12°25'S, 41°21'W. N = 3
	4 – Brasília de Minas, Minas Gerais (MG). 16°59'S, 46°0'W (Cerrado)	N = 6. B1718 ^B , B2055 ^B , B2058 ^B , B2064 ^B , B2083 ^B , B2278 ^B		Arinus, MG. 15°55'S, 46°4'W, N = 3
	5 – Felixlândia, MG. 18°44'S, 44°48'W (Cerrado)	N = 1. B1625 ^B		Excluded from the plumage versus genetic analysis
	6 – Bocaiúva, MG. 17°5'S, 43°48'W (Cerrado)	N = 2. B1790 ^B , B2215 ^B		Excluded from the plumage versus genetic analysis
	7 – Simonésia, MG. 20°7'S, 42°00'W (Atlantic forest)	N = 1. B431 ^B		NP Caparaó, Espírito Santo (ES). 20°26'S, 41°24'W. N = 3
	8 – Araponga, MG. 20°39'S, 42°32'W (Atlantic forest)	N = 1. B930 ^B		NP Caparaó, N = 3
	9 – NP Itatiaia, Rio de Janeiro. 22°25'S, 44°36'W. (Atlantic Forest)	N = 1. LGEMA P1326 ^B	Photo ^P	Locality: Idem genetic data. N = 10
	10 – Três Lagoas, Mato Grosso do Sul. 20°46'S, 51°43'W (Cerrado)	N = 1. LGEMA11444 ^M	GBN2 ^F	Locality: Idem genetic data. N = 6
	11 – Morro do Diabo State Park, São Paulo (SP). 22°30'S, 52°18'W (Atlantic forest)	N = 5. All LGEMA: P1496 ^B , P1901 ^B , P1912 ^B , P1948 ^B , P1952 ^B	Photo ^P	Locality: Idem genetic data. N = 2
	12 – Barreiro Rico, SP. 22°38'S, 48°13'W (Atlantic forest)	N = 1. P1696 ^M	FMH 050054 ^F	Victoria, SP. 22°46'S, 48°2'W. N = 3
	13 – Morro Grande State Park, SP. 23°42'S, 46°59'W (Atlantic forest)	N = 8. All LGEMA: P1033 ^B , P2066 ^B , P2480 ^B , P2520 ^B , P2544 ^B , P2553 ^B , P2668 ^B , P2699 ^B ,		Eng. Ferraz, SP. 23°58'S, 46°36'W. N = 8

<i>Dendrocolaptes picumnus</i>	14 – Buri, SP, 23°39'S, 48°32'W (Atlantic forest)	N = 1. LGEMAP862 ^M	MZUSP 75589	Locality: Idem genetic data. N = 1
	15 – Pinhalão, Paraná (PR). 23°46'S, 50°3'W (Atlantic forest)	N = 1. LGEMAP885 ^M	MZUSP 75622	Locality: Idem genetic data. N = 1
	16 – Wenceslau Braz, PR. 22°5'S, 48°47'W (Atlantic forest)	N = 1. LGEMAP957 ^M	MZUSP 75690	Locality: Idem genetic data. N = 1
	17 – Ortigueira, PR. 24°12'S, 50°55'W (Atlantic forest)	N = 1. LGEMA11429 ^M	GBN IIA092 ^F	Locality: Idem genetic data. N = 1
	18 – Rancho Queimado, Santa Catarina (SC). 27°40'S, 49°1'W (Atlantic forest)	N = 1. LGEMAPI750 ^B		Hansa, SC, 26°26'S, 49°14'W, N = 3
<i>Dendrocolaptes certhia</i>	19 – Arroio do Padre, Pelotas, Rio Grande do Sul (RS). 31°31'S, 52°23'W (Atlantic forest)	N = 2. LGEMAPI736 ^B , LGEMAPI738 ^B	Photo ^F	Sinimbu, RS, 29°32'S, 52°32'W, N = 2
	20 – San Antonio, Concepción, Paraguay. 23°33'S, 56°56'W. (Atlantic forest and Cerrado)	N = 1. Genbank AY442990 (Irestedt, Fjeldsa & Ericson, 2004)		Not considered
	Amazonas, Brazil	N = 4. All LGEMA ^F : P355 (= LSUB 35687) ^M , P360 (= LSUB 35704) ^M , P368 (= LSUB 35727) ^M , P369 (= LSUB 35728) ^M		
	Amazonas, Brazil	N = 1. LGEMA9791 ^M		
		N = 1. Genbank EF212895 (Weir & Schluter, 2007)		
<i>Xiphocolaptes promerops</i>		N = 1. Genbank AY089798 (Aleixo, 2002)		

TISSUE – tissue type. B, blood; M, muscle. Tissue samples are deposited at the Laboratório de Genética e Evolução Molecular de Aves (LGEMA) of the Universidade de São Paulo (São Paulo, Brazil), at the Laboratório de Biodiversidade e Evolução Molecular (B) of the Universidade Federal de Minas Gerais (Belo Horizonte, Brazil), or at the Louisiana State University Museum of Natural Science (LSUB), Baton Rouge (USA).
VOUCHER – P, photo available from authors; F, indicates collector and field number when specimens have not been catalogued. Collectors: LFS, Luis Fabio Silveira; GBN, Renato Gaban Lima; FMH, Fernando d'Horta. *Samples deposited in two collections.
PLUMAGE – Whenever plumage data were extrapolated from the nearest location, this locality is given. Otherwise, the locality of the plumage data is the same locality of the genetic data.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Model parameters used to simulate genetic datasets under different demographic scenarios and to evaluate the goodness of fit of the observed mitochondrial DNA control region sequences of *Dendrocolaptes platyrostris*. Variables t_1 and t_2 represent divergence times (years). For models with two historical events (models 4a to 6b), t_1 is the most recent event and t_2 is the oldest event. Migration rates in proportion of the effective population that migrates each generation. Significance of combined P -values was evaluated at the 1% level, after using the sequential Bonferroni correction (Quinn & Keough, 2002). Rejected models are denoted with an italicized combined P -value.

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Anexo XXIV

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Phylogeography of an Atlantic forest passerine reveals demographic stability through the last glacial maximum

Henrique Batalha-Filho^{a,*}, Gustavo Sebastián Cabanne^{b,c}, Cristina Yumi Miyaki^a

^a Laboratório de Genética e Evolução Molecular de Aves, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, 05508-090 São Paulo, SP, Brazil

^b CONICET, Av. Rivadavia 1917, Ciudad de Buenos Aires C1033AAJ, Argentina

^c División de Ornitología, Museo Argentino de Ciencias Naturales "B. Rivadavia", Ángel Gallardo 470, Ciudad de Buenos Aires C1405DJR, Argentina

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ABSTRACT

In this study we analyzed the phylogeographic pattern and historical demography of an endemic Atlantic forest (AF) bird, *Basileuterus leucoblepharus*, and test the influence of the last glacial maximum (LGM) on its population effective size using coalescent simulations. We address two main questions: (i) Does *B. leucoblepharus* present population genetic structure congruent with the patterns observed for other AF organisms? (ii) How did the LGM affect the effective population size of *B. leucoblepharus*? We sequenced 914 bp of the mitochondrial gene cytochrome b and 512 bp of the nuclear intron 5 of beta-fibrinogen of 62 individuals from 15 localities along the AF. Both molecular markers revealed no genetic structure in *B. leucoblepharus*. Neutrality tests based on both loci showed significant demographic expansion. The extended Bayesian skyline plot showed that the species seems to have experienced demographic expansion starting around 300,000 years ago, during the late Pleistocene. This date does not coincide with the LGM and the dynamics of population size showed stability during the LGM. To further test the effect of the LGM on this species, we simulated seven demographic scenarios to explore whether populations suffered specific bottlenecks. The scenarios most congruent with our data were population stability during the LGM with bottlenecks older than this period. This is the first example of an AF organism that does not show phylogeographic breaks caused by vicariant events associated to climate change and geotectonic activities in the Quaternary. Differential ecological, environmental tolerances and habitat requirements are possibly influencing the different evolutionary histories of these organisms. Our results show that the history of organism diversification in this megadiverse Neotropical forest is complex.

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1. Introduction

The Atlantic forest (AF) is among the top five biodiversity hotspots in the world and these areas hold more than 60% of all terrestrial species on the planet (Myers et al., 2000; Galindo-Leal and Câmara, 2005). It is distributed along the coastal regions of eastern Brazil to eastern Paraguay and northeastern Argentina (Galindo-Leal and Câmara, 2005) (Fig. 1). Also, the AF now holds only between 11.4% and 16% of its original forest cover (Ribeiro et al., 2009).

The high species diversity in the AF may be a consequence of various evolutionary processes. Some of them possibly occurred in the Quaternary, especially in the late Pleistocene (Whitmore

and Prance, 1987). During this period the planet went through many glacial and interglacial cycles, when continental glaciers advanced and retreated and forests suffered cycles of reduction, fragmentation and expansion (Sant'Anna Neto and Nery, 2005). Palynological data analyses show that the last glacial maximum in the AF occurred between 18 and 48 thousand years ago, with open areas (grasslands) in southeastern Brazil that extended 750 km northward, from latitude 28°/27°S to at least 20°S (Behling and Lichte, 1997; Behling, 2002). The final expansion of forested areas to its current distribution occurred only at the end of the Holocene (Behling, 2002; Behling and Pillar, 2007).

One of the most discussed hypotheses on the origin of the Neotropical biodiversity during the Pleistocene is the theory of forest refugia (Haffer, 1969; Vanzolini and Williams, 1970; Brown and Ab'Saber, 1979). According to this theory, refugia are islands of humid forest isolated by open vegetation, such as grasslands or dry forest. These rainforests would shrink during glacial maxima (forming refugia) and expand during warmer periods (interglacial cycles), while areas of open vegetation would behave the other

* Corresponding author. Address: Laboratório de Genética e Evolução Molecular de Aves, Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, Cidade Universitária, 05508-090 São Paulo, SP, Brazil. Fax: +55 11 3091 7553.

E-mail addresses: rickbio@hotmail.com, henrique.batalha@usp.br (H. Batalha-Filho).

Fig. 1. (a) Main phylogeographic breaks observed in Atlantic forest organisms (see Section 4). Gray area represents the limits of the Atlantic forest and dotted lines the phylogeographic breaks. Break i observed by: Grazziotin et al. (2006), Cabanne et al. (2007, 2008, 2011), Carnaval et al. (2009), Martins et al. (2009), Batalha-Filho et al. (2010), Thomé et al. (2010), and d'Horta et al. (2011). Break ii observed by: Cabanne et al. (2007, 2008), Carnaval et al. (2009), Ribeiro et al. (2010), Thomé et al. (2010), and d'Horta et al. (2011). Break iii observed by: Cabanne et al. (2008) and Carnaval et al. (2009). (b) Sampling sites of *B. leucoblepharus*. The black continuous line shows the approximate limit of the species distribution based on Ridgely and Tudor (1996). The circles indicate the sites and their colors are represented in the haplotype networks. Sampling site numbers represent operational localities used in AMOVA and BAPS analyses. The different gray tones in the map represent elevation (the darker the higher are the altitudes). (c) Haplotype network based on 914 bp of cytb. (d) Haplotype network based on 512 bp of FIB5. Colors in networks correspond to colors in the map. (e) Bayesian analysis of population structure (BAPS) of *B. leucoblepharus*. Clustering based on 914 bp of cytb, and 512 bp of FIB5. Colors indicate the best numbers of clusters for each marker. Numbers represents the operational localities according to the map.

way round. Thus, the geographic distribution of species that inhabit rainforests should have accompanied these cycles of contraction and expansion of forests during the Pleistocene. If these populations were isolated for enough time to accumulate differences between them, even if they subsequently got in contact again after the expansion of forests, no gene flow would occur. However, geological and palaeoclimatic studies questioned the existence of Pleistocene refugia (Colinvaux et al., 2000; Bush and de Oliveira, 2006).

Another possible driver of diversification is rivers as barriers to gene flow for terrestrial animals. This hypothesis was initially proposed by Alfred Russell Wallace (1852), and recent studies showed that rivers played an important role for diversification in Amazonia (Ribas et al., 2012), but not in the AF (Maldonado-Coelho, 2012). Another hypothesis is the ecological gradient theory (Smith et al., 1997, 2001) that suggests that diversification can occur in parapatry, i.e., species or lineage differentiation may occur due to differential selective pressures acting on different and adjacent habitats. This hypothesis was evaluated and supported by Cabanne et al. (2011) in the intra-specific diversification of a bird that inhabits the AF and gallery forest of Caatinga, Cerrado, and Chaco. Finally, geotectonic activity during the Quaternary (including

movement of geological faults in Brazil; Saadi et al., 2002) may have originated gene flow barriers that triggered diversification of some AF organisms (Batalha-Filho et al., 2010; Thomé et al., 2010).

Recent phylogeographic and paleoclimatic modeling studies do not reject the Pleistocene refuge theory as possible explanation of intra-specific diversification of Neotropical species (Cabanne et al., 2007, 2008; Carnaval and Moritz, 2008; Solomon et al., 2008; Carnaval et al., 2009; d'Horta et al., 2011; Martins, 2011; Maldonado-Coelho, 2012). Phylogeographic studies focused on organisms that occur in the AF are still scarce (Grazziotin et al., 2006; Cabanne et al., 2007, 2008; Carnaval et al., 2009; Fitzpatrick et al., 2009; Batalha-Filho et al., 2010; Thomé et al., 2010; Cabanne et al., 2011; d'Horta et al., 2011) compared to its high species diversity. These phylogeographic studies revealed three main phylogeographic breaks shared among taxa (Fig. 1a), but there is no clear temporal congruence between estimated dates of divergence of these populations or lineages (see Section 4 for more details).

In the present study we performed a phylogeographic analysis to study the historical demography of the white-rimmed warbler, *Basileuterus leucoblepharus*, an endemic bird of the AF (Passeriformes, Parulidae). We based the study on nuclear and mitochondrial

DNA sequences for testing the effect of the last glacial maximum on its demographic history.

This species is distributed from the states of Minas Gerais and Rio de Janeiro to Rio Grande do Sul in Brazil to Paraguay, Uruguay and the northeastern and eastern Argentina (Fig. 1b; Ridgely and Tudor, 1996; Sick, 1997). It can be fairly common and easily identified by its characteristic song. It occurs from uplands in its northern distribution (e.g. between 800 and 2200 m in latitude 22°) to sea level in the southern part of its distribution. *B. leucoblepharus* lives inside moist and shaded forests (Sick, 1997), but also occurs in secondary and mixed forests in agroecosystems (i.e. *Eucalyptus* sp. and *Araucaria angustifolia* plantations), as well as gallery forests within the humid Chaco, and Argentinean and Uruguayan Pampas (Stotz et al., 1996).

In this study we addressed two main questions: (i) Does *B. leucoblepharus* present population genetic structure concordant with patterns observed for other AF organisms (Fig. 1a), such as birds (Cabanne et al., 2007, 2008, 2011; d'Horta et al., 2011; Maldonado-Coeiro, 2012), other vertebrates (Grazziotin et al., 2006; Carnaval et al., 2009; Fitzpatrick et al., 2009; Martins et al., 2009; Thomé et al., 2010), invertebrates (Batalha-Filho et al., 2010) and plants (Ribeiro et al., 2010)?; and (ii) How did the last maximum glacial affect the effective population size of *B. leucoblepharus*?

2. Material and methods

2.1. Sampling and molecular analyses methods

Samples (muscle and blood) of 62 specimens from 15 localities in Brazil and Argentina were collected between 2000 and 2009 (Table 1, Fig. 1b). Samples are deposited in three collections in Brazil: Laboratório de Genética e Evolução Molecular de Aves (Instituto de Biociências, Universidade de São Paulo); Laboratório de Biodiversidade e Evolução Molecular (Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais); and Departamento de Genética (Universidade Federal do Rio Grande do Sul). Specimens' vouchers of all muscle samples are deposited at Museu de Zoologia da Universidade de São Paulo (MZUSP). The identification codes of samples, specimens' vouchers and GenBank accession numbers are presented in Table 1.

Total DNA was extracted following Bruford et al. (1992). The mitochondrial gene cytochrome b (cytb) was amplified with primers L-14841 and H-16065 (Lougheed et al., 2000) and the nuclear beta-fibrinogen intron 5 (FIB5) with primers FIB5 and FIB6 (Marini and Hackett, 2002). PCR (25 L) contained template DNA (50 ng), 1X of *Taq* buffer (GE Healthcare), dNTPs (0.32 M), 0.5 M of each primer and 0.5 U of *Taq* polymerase (GE Healthcare). PCR conditions were: an initial denaturation step at 94 °C for 3 min and 30 s; followed by 35 cycles at 94 °C for 35 s, 56 °C (cytb) or 51–56 °C (FIB5) for 40 s and 72 °C for 1 min; plus a final extension step at 72 °C for 9 min.

Amplicons were purified using polyethylene glycol 20% (PEG) precipitation (Sambrook et al., 1989). This DNA was then directly sequenced in both directions with the same amplification primers using Big Dye terminator 3.0 cycle sequencing kit (Applied Biosystems) following the manufacturer's protocol. Sequences were analyzed in an automated sequencer ABI PRISM 3100 (Applied Biosystems).

2.2. Sequence editing, haplotype reconstruction and recombination test

The electropherograms were edited and assembled in contigs using the software package Phred, Phrap, Consed (Ewing et al., 1998; Ewing and Green, 1998; Gordon et al., 1998). Sequences

were aligned using the CLUSTAL W method (Higgins et al., 1994) in MEGA4 (Tamura et al., 2007). All alignments were inspected and corrected visually.

Initially, we codified double peaks present in both strands in the FIB5 sequence electropherograms as ambiguous sites according to the IUPAC code. We did not find any indel heterozygote. The gametic phase of heterozygote individuals was resolved using the algorithm PHASE (Stephens et al., 2001) with the default settings in DnaSP 5 (Librado and Rozas, 2009) and 0.7 as the minimum probability. Individuals with lower probabilities were removed from further analyses. We used PHI test in SPLITSTREE4 (Bruen et al., 2006; Huson and Bryant, 2006) to check for recombination in the FIB5 sequences. This test was used due to its power to distinguish recombination events from homoplasies (Bruen et al., 2006).

2.3. Population structure tests

Median-joining networks (Bandelt et al., 1999) were obtained using NETWORK 4.5.1.0 (www.fluxus-engineering.com) in order to study the relationships between haplotypes and their geographic distribution. To check the level of population genetic structure among localities we performed an analysis of molecular variance (AMOVA, Excoffier et al., 1992) with two hierarchical levels for each gene separately using ARLEQUIN 3.11 (Excoffier et al., 2005). We also calculated an AMOVA with three hierarchical levels for the cytb. For the third level of this analysis, we separated in two groups those localities south and north of the phylogeographic break i (Fig. 1a). In order to verify if there is correlation between geographic and genetic (pair wise Φ_{ST}) distances we used Mantel test (Sokal and Rolf, 1995) using ARLEQUIN 3.11. Significances of these analyses were obtained with 1000 permutations.

In addition, we used BAPS 5.3 (Bayesian Analysis of Population Structure; Corander and Marttinen, 2006; Corander et al., 2008) to access the number of populations without prior information of the sampling location for mitochondrial and nuclear datasets. Due to the difference in ploidy, BAPS analysis was performed for each gene separately. Firstly, we ran the analysis assuming mixture model to determine the most probable number of populations (k) given the data. After that the resulting mixture clustering was used for an admixture analysis with 500 reference individuals and repeated the admixture analysis 500 times per individual. The analyses were repeated five times to check for convergence between different runs.

2.4. Demographic analyses

The nucleotide diversity per site (π) and number of haplotypes (h) were calculated in DnaSP 5. In order to test if there is any evidence of demographic expansion, the neutrality test indexes Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) and the population size change test R_2 (Ramos-Onsins and Rozas, 2002) were calculated for each gene using DnaSP 5. The significances of these tests were obtained based on 1000 coalescent simulations. In order to identify the putative direction of the demographic expansion (north to south, or vice versa) we plotted the nucleotide diversity per site (π) of each locality against its latitude in degrees, and we performed a linear regression. It is expected that higher values of π is associated with a larger and more stable historical population size (Spellman and Klicka, 2006). Thus, localities with higher values of π could be associated to past refugia.

In addition, to analyze population size dynamics through time for both loci combined, we used the Extended Bayesian Skyline Plot method (EBSP; Heled and Drummond, 2008) implemented in BEAST 1.6.1 (Drummond and Rambaut, 2007). Two independent EBSP runs were obtained using the following parameters: an initial

Table 1
Samples of *B. leucoblepharus*: localities sampled, geographic coordinates, sample size (N) per locality, sample code, voucher and GenBank accession numbers for each marker.

Locality	Coordinates	N	Sample code	Voucher ^d	GenBank cyt b	GenBank FIB5
1. Nova Lima – MG, BRA	19°58'S; 43°49'W	8	B0627 ^a	–	–	JX488594
			B0628 ^a	–	–	JX488595
			B0629 ^a	–	JX488543	JX488596
			B0630 ^a	–	JX488544	JX488597
			B0631 ^a	–	JX488545	JX488598
			B0705 ^a	–	JX488546	–
			B1279 ^a	–	–	JX488599
			B1281 ^a	–	–	JX488600
2. Itamonte – MG, BRA	22°21'S; 44°46'W	2	12017 ^b	MZUSP82630	JX488535	JX488586
			12020 ^b	MZUSP82629	JX488536	JX488587
3. Resende – RJ, BRA	22°23'S; 44°45'W	4	12014 ^b	MZUSP82617	JX488533	JX488584
			12015 ^b	MZUSP82627	JX488534	JX488585
			12023 ^b	MZUSP82628	JX488537	JX488588
			12024 ^b	MZUSP82616	JX488538	JX488589
4. Parque Nacional Serra dos Órgãos – RJ, BRA	22°26'S; 43°00'W	4	13000 ^b	MZUSP82588	JX488539	JX488590
			13006 ^b	MZUSP82589	JX488540	JX488591
			13008 ^b	MZUSP82587	JX488541	JX488592
			13019 ^b	MZUSP82586	JX488542	JX488593
5. Parque Estadual Morro Grande – SP, BRA	23°41'S; 45°51'W	10	779 ^b	–	JX488492	JX488547
			820 ^b	–	JX488493	JX488548
			825 ^b	–	JX488494	JX488549
			828 ^b	–	JX488495	JX488550
			830 ^b	–	JX488499	JX488554
			834 ^b	–	JX488497	JX488552
			835 ^b	–	JX488498	JX488553
			852 ^b	–	JX488496	JX488551
			853 ^b	–	JX488500	JX488555
			854 ^b	–	JX488501	JX488556
6. Buri – SP, BRA	23°42'S; 48°34'W	5	1240 ^b	MZUSP76135	JX488502	JX488557
			1251 ^b	MZUSP75604	JX488503	JX488558
			1254 ^b	–	JX488504	JX488559
			2962 ^b	–	JX488505	JX488570
			2966 ^b	–	JX488506	JX488571
7. Cunha – SP, BRA	23°07'S; 44°56'W	3	12003 ^b	MZUSP 81387	JX488530	JX488581
			12006 ^b	MZUSP82632	JX488531	JX488582
			12007 ^b	MZUSP82631	JX488532	JX488583
8. Ortigueira – PR, BRA	24°12'S; 50°55'W	3	11407 ^b	MZUSP75679	JX488527	JX488578
			11408 ^b	–	JX488528	JX488579
			11412 ^b	–	JX488529	JX488580
9. Pinhalão – PR, BRA	23°58'S; 50°03'W	1	1296 ^b	MZUSP75641	JX488507	JX488560
10. Wenceslau Braz – PR, BRA	23°51'S; 49°48'W	2	1384 ^b	–	JX488508	–
			1388 ^b	–	JX488509	JX488561
11. Campo Belo do Sul – SC, BRA	27°53'S; 50°45'W	3	DA29326 ^c	–	–	JX488601
			DA29328 ^c	–	–	JX488602
			DA29522 ^c	–	–	JX488603
12. Rancho Queimado – SC, BRA	27°40'S; 49°01'W	7	2150 ^b	–	JX488512	JX488565
			2153 ^b	–	JX488513	–
			2160 ^b	–	JX488514	JX488566
			2161 ^b	–	JX488515	JX488567
			2164 ^b	–	JX488516	JX488568
			2165 ^b	–	JX488517	JX488569
			2196 ^b	MZUSP82783	JX488518	–
13. Arroio do Padre – RS, BRA	31°22'S; 52°23'W	2	2135 ^b	–	JX488510	JX488563
			2138 ^b	–	JX488511	JX488564
14. Parque Uruguái, Uruzú, Misiones, ARG	25°51'S; 55°10'W	1	1409 ^b	–	JX488519	JX488562
15. Paraje Maria Soledad, Misiones, ARG	25°51'S; 53°59'W	7	10345 ^b	–	JX488520	JX488572
			10358 ^b	–	JX488521	JX488573
			10364 ^b	–	JX488522	–
			10371 ^b	–	JX488523	JX488574
			10383 ^b	–	JX488524	JX488575
			10385 ^b	–	JX488525	JX488576
			10387 ^b	–	JX488526	JX488577

BRA – Brasil; ARG – Argentina.
Tissue collections:
^a Laboratório de Biodiversidade e Evolução Molecular, Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais.
^b Laboratório de Genética e Evolução Molecular de Aves (LGEMA), Instituto de Biociências, Universidade de São Paulo.
^c Departamento de Genética, Universidade Federal do Rio Grande do Sul.
^d Samples with museum voucher correspond to muscle samples, the remaining ones are blood samples. Specimens are deposited in Museu de Zoologia da Universidade de São Paulo (MZUSP).

UPGMA tree, linear model, 50 million steps, parameters sampled every 10,000 steps and a burn-in of 10%. The hLRT test did not reject the molecular clock hypothesis for both loci. The best fit substitution model for each partition (loci) was determined by Modeltest 3.7 (Posada and Krandall, 1998). We used 1.05% (s.d. 0.05%) per lineage per million years as the mutation rate for cytb (Weir and Schluter, 2008) under a normal prior distribution. Based on the probabilities obtained, BEAST estimated the mutation rate for FIB5 under a default lognormal prior distribution. To check the convergence of parameters between runs and analysis performance (ESS values > 200) we used TRACER 1.5 (<http://beast.bio.ed.ac.uk/Tracer>).

2.5. Coalescent simulations

In order to evaluate the demographic history we simulated scenarios in BAYESSC, a modified version of SERIAL SIMCOAL (Anderson et al., 2005; Chan et al., 2006), and evaluated the goodness of fit of the observed data to the simulations. The general procedure followed Richards et al. (2007). We also followed the methodology suggested by Voight et al. (2005) that considers several summary statistics and results on a single posterior probability for each model. This procedure has been widely applied in recent studies (i.e.: Belle et al., 2006; Fabre et al., 2009; Ghirotto et al., 2010; Cabanne et al., 2011; Fischer et al., 2011; Brace et al., 2012).

The rationale of this procedure consists in testing models by simulating genealogies under specific demographic scenarios and then evaluating the fit of the empirical data to the simulated data (Knowles, 2009). If the empirical (observed) data fits well to the simulated data, this given demographic scenario would receive a high posterior probability and therefore, be supported. Alternatively, if the observed data does not fit well into the distribution of the simulated data, the posterior probability would be low and the specific scenario would not be supported.

The models that were tested differed in the timing and number of bottlenecks (Fig. 2, models A to E). Also, as genetic signals of expansion may be found in a demographically stable island system (see Nielsen and Beaumont, 2009; Peter et al., 2010), we also simulated two island models that originated before the Pleistocene (Fig. 2, models F and G). This date was estimated based on the divergence of *B. leucoblepharus* and its sister species *B. flaveolus* (Lovette et al., 2010). For the island models we grouped sampling localities by geographic proximity into five populations as follows: pop 1 – localities 1–4; pop 2 – locality 5; pop 3 – localities 6–10; pop 4 – localities 11–13; pop 5 – localities 14 and 15. Grouping by geographic proximity was the simplest way to recreate a plausible island model. Other configurations of island models could have been evaluated, but they would be more complex. Complex models should be avoided in this kind of procedure because they would get a higher posterior probability but without being plausi-

Fig. 2. Parameters of the historical demographic models of *B. leucoblepharus* evaluated by coalescent simulations in BAYESSC. For all models we simulated populations that suffered two types of historical events: bottleneck (I–III) and population fragmentation. The prior distribution of time of events (t), migration (m) and of the demographic growth rate (r), are specified. Time is expressed in thousand years (ky). Uniform prior distributions are denoted with an U. Normal prior distributions are denoted by N, the mean value and standard distribution (SD). Intensities of bottlenecks are indicated by the proportion of the current effective size (N_e) that persisted during the bottleneck. Time is expressed in kilo-years (ky). LGM: last glacial maximum.

Table 2
Summary statistics for cytochrome b (cytb) and beta-fibrinogen intron 5 (FIB5) of *B. leucoblepharus*.

Locus	N	π	<i>l</i>	<i>D</i>	<i>F_s</i>	<i>R₂</i>
cytb	55	0.00562	34	−1.7072 [*]	−27.1696 ^{***}	0.0494 ^{**}
FIB5	86	0.00536	25	−1.3053 ^{ns}	−14.1737 ^{***}	0.0554 [*]

N: sample size; π : nucleotide diversity per site; *l*: number of haplotypes; *D*: Tajima's test; *F_s*: Fu's test; *R₂*: Ramos-Onsins and Rozas' test; ^{ns} not-significant.
^{*} 0.10 > *p* > 0.05.
^{**} *p* < 0.05.
^{***} *p* < 0.01.

ble (Nielsen and Beaumont, 2009). Specifically, Nielsen and Beaumont (2009) suggested to use simple models as a baseline against which to compare more complex models.

We simulated bottlenecks that reduced the population effective size (*N_e*) to 1–10% of the current size. We estimated *N_e* from theta ($\Theta = 2\mu N_e$) that was obtained assuming the F84 model of sequence evolution (Felsenstein and Churchill, 1996), and empirical base frequencies and transition/transversion ratios with a Markov chain Monte Carlo (default settings) in LAMARC 2.1.2b (Kuhner, 2006). We assumed that *N_e* from each present population and from ancestral populations in models F and G (Fig. 2) was 1/5 of the species' *N_e*. Effective number of genes was introduced in BAYESSC infles as presenting a normal distribution with a mean equal to the maximum likelihood estimation of *N_e* and standard deviation estimated from Θ confidence interval. For mtDNA we used a mutation rate as a uniform interval 1.05×10^{-8} – 9.14×10^{-7} changes/site/generation (Weir and Schluter, 2008). The transition bias was 0.85, the mutation rate gamma distribution was 0.84 and the number of mutation categories was six. For FIB5, the mutation rate was obtained from the EBSF analysis in BEAST; the uniform rate interval was 3.86×10^{-9} – 6.99×10^{-10} changes/site/generation. The transition bias was 0.7, the mutation rate gamma distribution was 0.77 and the number of mutation categories was six. We assumed a generation time of 1 year as Cabanne et al. (2008).

For each model we ran 1000 simulations per marker (mtDNA and FIB5). Then, for each simulated data we estimated three sum-

mary statistics [nucleotide diversity – π , Tajima's *D* (Tajima, 1989), and number of haplotypes – *h*] to obtain null distributions against which we tested the observed data (Hickerson et al., 2006). For evaluating the goodness of fit of the observed data to the simulated one, we followed Voight et al. (2005) and used the two-tailed empirical likelihood *pi* of each summary statistics *i*;

$$pi = 1 - [2 \times (|0.5 - p|)]$$
 (1)

being *p* the proportion of simulated values equal or higher than the observed summary statistic. When the observed statistic fell outside the simulated distribution we attributed *pi* = 0.00001, as in Cabanne et al. (2011). Then, we combined the three *pi* values by

$$C_{obs} = -2 \sum_{i=1}^k \ln p_i$$
 (2)

and obtained its significance. The significance was assessed by comparing *C_{obs}* against a null distribution of *C* obtained following Voight et al. (2005) and Fabre et al. (2009). For each simulated dataset, each summary statistic was compared with the other values representing the empirical distribution of the statistic from simulation. Specifically, we treated the value of each summary statistic as the observed value and calculated with Eq. (1) its *p_{sim}*-value relative to the remaining 999 simulated data. Then, the null distribution of *C* was obtained by combining with Eq. (2) *p_{sim}*-values across summary statistics and the significance of *C_{obs}* was obtained as in step 1 (Eq. (1)). This procedure generated two-tailed combined *p*-values associated to each marker. Finally, the overall *P*-value for the model was obtained by combining *p*-values of both markers by the parametric Fisher's method in METAP (Whitlock, 2005), which is authored by Ge (available at <http://compute1.lsrc.duke.edu/software/MetaP/metap.php>). We rejected models when *P* ≤ 0.05.

3. Results

3.1. Diversity and genetic structure

We obtained 914 bp of cytb (*N* = 55) and 512 bp of FIB5 (*N* = 114 sampled chromosomes). No indels were observed in cytb, but FIB5

Fig. 3. Extended Bayesian Skyline Plot (EBSF) result. The dotted horizontal line shows the median estimate of the EBSF and the gray line shows the upper and lower 95% highest posterior density limits. The dotted vertical line illustrates the approximate time when demographic expansion of *B. leucoblepharus* started. The Y-axis is in ln scale.

Fig. 4. Correlation between nucleotide diversity (π) of mitochondrial (cytb) and nuclear (FIB5) genes, and degrees of latitude of localities. Dashed lines represent the fitted linear model and the solid lines represent 95% of confidence intervals calculated for the regression line.

had an indel of 6 bp in ten samples from various localities. There were 40 polymorphic sites in cytb and 41 in FIB5 after excluding indels and ambiguous sites. No unexpected stop codons or ambiguous peaks in the electropherograms were found in cytb sequences, suggesting that they were of mitochondrial origin. The haplotype reconstruction by PHASE resolved a total of 43 samples (86 sampled chromosomes) with $p > 0.7$, and the remaining 11 specimens with low PHASE probability were removed from further analyses. The PHI test ($p = 0.09$) excluded the hypothesis of recombination in FIB5. The summary statistics for both loci are shown in Table 2.

Table 3

Values of likelihood for nucleotide diversity (π), Tajima's D , and number of haplotypes (l) and overall likelihood for models of demographic scenarios for *B. leucoblepharus* based on mtDNA and FIB5 datasets.

Demographic model	mtDNA				FIB5				Overall P-value
	$p\pi$	pD	pl	Combined p-values	$p\pi$	pD	pl	Combined p-values	
A Panmixia, stability	0.00001	0.016	0.87	0.00001	0.006	0.088	0.868	0.076	0.00001146
B Panmixia, bottleneck LGM	0.364	0.136	0.00001	0.00001	0.45	0.074	0.132	0.254	0.00003526
C Panmixia, bottleneck 120 ky ago	0.304	0.294	0.342	0.61	0.384	0.264	0.26	0.584	0.7239*
D Panmixia, bottlenecks LGM and 120 ky ago	0.68	0.236	0.00001	0.006	0.594	0.18	0.054	0.32	0.0139
E Panmixia, bottleneck 300–500 ky ago**	0.614	0.6918	0.755	0.3483	0.42	0.4225	0.3159	0.8305	0.6481*
F Stable finite island, origin 2–4 m.y.a. ago, gene flow 0–0.01	0.004	0.024	0.284	0.010	0.07	0.114	0.418	0.212	0.0152
G Model F, bottleneck 300–500 ky ago	0.928	0.994	0.258	0.474	0.00001	0.106	0.052	0.00001	0.00006284

* Scenarios not rejected.

** Date in accordance with observed results from EBSF analysis.

We used different strategies to identify population genetic structure. Haplotype networks based on each of the two marker sequences showed absence of genetic structure. The most common and internal haplotypes were found in geographically distant locations (Fig. 1). AMOVA indicated that the highest percentage of genetic variation was observed within localities, 69.8% ($p < 0.00001$) for cytb and 92.3% ($p = 0.02053$) for FIB5, indicating that the genetic structure among localities is low (mtDNA) or absent (FIB5). When we applied the three hierarchical level AMOVA we did not find strong genetic structure between the hypothetical groups (variation among groups = 18.23%; variation among localities within groups = 17.53%; variation within localities = 64.23%). Mantel test indicated no correlation between genetic and geographic distances [$r_{cytb} = 0.2567$ ($p = 0.107$); $r_{FIB5} = 0.1108$ ($p = 0.201$)], also suggesting absence of isolation by distance between localities. BAPS analysis indicated presence of two and three populations for mitochondrial and nuclear genes, respectively (Fig. 1e). Notwithstanding, these clusters were not spatially coherent for either markers and therefore confirmed absence of population structure.

3.2. Historical demography

All neutrality test values were significant (except Tajima's D for FIB5), indicating sign of demographic expansion in both loci (Table 2). According to Ramos-Onsins and Rozas (2002), F_s and R_2 tests are more robust in detecting events of demographic expansion, and F_s is more suitable for larger samples while R_2 , for smaller samples.

The EBSF indicated that *B. leucoblepharus* experienced a demographic expansion starting about 300,000 years ago (Fig. 3), possibly associated to climate changes during the late Pleistocene. The demographic expansion event dated here is older than the last glacial maximum (LGM), when paleopalynological records indicate that open areas (grasslands) were present in the AF between 48,000 and 18,000 years ago (Behling and Lichte, 1997; Behling, 2002). Thus, we performed coalescent simulations to test the effect of the LGM on the effective population size of *B. leucoblepharus*.

The relationship between π and latitude indicated a possible demographic expansion from south to north for cytb ($R^2 = 31.36\%$, $p = 0.029$), but for FIB5 there is no correlation between latitude and π ($R^2 = 5.21\%$, $p = 0.4998$) (Fig. 4).

3.3. Coalescent simulations

We simulated mtDNA and FIB5 sequences under seven different demographic scenarios to explore whether populations suffered specific bottlenecks, as expected according to the history of the AF, or alternatively, followed a panmictic or finite island model. The simulated models are (Fig. 2): (A) a panmictic stable population;

(B) a panmictic population that suffered a bottleneck during the LGM; (C) a panmictic population that suffered a bottleneck during the glaciation previous to the LGM; (D) a panmictic population that suffered bottlenecks during the last two glacial maxima; (E) a panmictic population that suffered a bottleneck during the upper Pleistocene (300,000–500,000 years ago), according to the results of EBSP (Fig. 3); (F) a stable finite island system connected by gene flow and that originated before the Pleistocene (2–4 m.y.a. ago); (G) model F but including a bottleneck at the upper Pleistocene.

We initially estimated the effective number of genes to be used in simulations. Species' N_e for mtDNA was 1,476,000 (SE 200,000) genes and for FIB5 2,551,810 (SE 33,700) genes. We used one fifth of the total species' N_e as the effective size of each of the five populations used in simulated models: 295,200 genes for mtDNA and 510,362 for FIB5.

Five models were rejected (overall $P < 0.05$; Table 3), namely, a single panmictic and stable population (model A), a bottleneck during the LGM (B), two consecutive bottlenecks during the last two glaciations (D), and island systems (F and G). The last result shows that our data does not support the possibility that the observed expansion signal could be explained by an island model.

There were two models that were not rejected. The simplest scenario suggests that the observed data could have been produced after a bottleneck that occurred at the maximum glaciation before the last one (model C, ~120,000 years ago). The other scenario suggests a bottleneck at mid Pleistocene (model E, 300,000–500,000 years ago).

4. Discussion

4.1. Phylogeographic structure and demography of *B. leucoblepharus*

The results of the haplotype networks, AMOVA, and comparison between genetic and geographic distances based on both mitochondrial and nuclear loci indicate absence of a strong population genetic structure in *Basileuterus leucoblepharus* (Fig. 1). Nevertheless, BAPS analysis indicated the presence of clusters for both loci, but these groups did not present any geographic structure (Fig. 1e). However, Bayesian approaches implemented in BAPS and Structure (Pritchard et al., 2000) are very sensitive to low levels of genetic differentiation (i.e. F_{ST} around 0.03–0.05; Latch et al., 2006), thus, when F_{ST} values are very low the number of incorrect assignments is high (Latch et al., 2006). In sum, even though AMOVA also indicated that 30% of the genetic variation of cytb is allocated between localities, no clear geographic structure was observed. Yet, the three hierarchical level AMOVA showed that just 18% of variation is allocated between the hypothetical groups. Thus, our data indicated absence of genetic structure in *B. leucoblepharus*.

Interestingly, the phylogeographic pattern we found in *B. leucoblepharus* is not congruent with previous phylogeographic studies of other AF organisms (Fig. 1a) that show recurrent geographic discontinuities possibly caused by vicariant events associated to climate changes or geotectonic activities in the Quaternary (Grazziotin et al., 2006; Cabanne et al., 2007, 2008; Carnaval et al., 2009; Martins et al., 2009; Batalha-Filho et al., 2010; Ribeiro et al., 2010; Thomé et al., 2010; d'Horta et al., 2011).

The absence of a strong differentiation among localities and the significant signal of demographic expansion (Table 2 and Fig. 4) suggest that *B. leucoblepharus* suffered a bottleneck followed by demographic expansion. This expansion occurred approximately in mid Pleistocene, as suggested by the EBSP, and is likely related to climate changes that occurred locally in that period. This date estimate is not congruent with the end of the LGM, when the glaciers started to retreat between 20,000 and 14,000 years ago (Sant'Anna Neto and Nery, 2005; Anderson et al., 2007). Therefore, our

results suggest that *B. leucoblepharus* maintained its population size relatively stable during the LGM in the AF, contrarily to what would be expected according to the Pleistocene forest refuge model.

The particular pattern exhibited by *B. leucoblepharus*, in comparison to other organisms from the AF (Grazziotin et al., 2006; Cabanne et al., 2007, 2008; Carnaval et al., 2009; Martins et al., 2009; Batalha-Filho et al., 2010; Ribeiro et al., 2010; Thomé et al., 2010; d'Horta et al., 2011; Cabanne et al., 2011; Maldonado-Coelho, 2012), might be associated to different habitat requirements presented by these species. In addition, the phylogeographic barriers observed for some endemic taxa from AF (Fig. 1a) may have acted as selective ecological filters (see Toon et al., 2010), i.e., only species with higher tolerance to habitat fragmentation were able to maintain gene flow or disperse across these barriers. *B. leucoblepharus* is relatively tolerant to habitat fragmentation (Stotz et al., 1996), and thus, barriers that affected several other AF forest birds (references as above) were probably not strong enough to preclude gene flow in *B. leucoblepharus*. This would explain the absence of phylogeographic structure in this species.

According to Moritz et al. (2000), the location, size and existence of forest refugia during the glacial maximum are dependent on the ecological and environmental tolerances of each species. Thus, *B. leucoblepharus* seems to have been able to maintain a stable population through the late Pleistocene, even during peaks of glaciations when forests fragmented and the climate was temperate (Behling, 2002). Contrarily to other AF birds that present a strong population genetic structure and historical demography affected by late Pleistocene glaciations, such as *Sclerurus scansor* (d'Horta et al., 2011) and *Xiphorhynchus fuscus* (Cabanne et al., 2007, 2008), the current southeastern distribution limit of *B. leucoblepharus* reaches temperate regions with discontinuous forest; namely the riparian forest from humid Chaco and pampas grasslands in eastern Argentina and Uruguay. Also, in the core of the AF *B. leucoblepharus* is found in montane forests where winters are temperate to cool (personal observation by HBF, Stotz et al., 1996), and in forest fragments within the Cerrado (Stotz et al., 1996). Thus, *B. leucoblepharus* seems to have been more tolerant to the climatic changes of the late Pleistocene than *S. scansor* and *X. fuscus*.

We rejected the presence of a bottleneck and demographic expansion at the LGM, and apparently the ecological flexibility of *B. leucoblepharus* might have been determinant for this response. However, it is not clear why this bird seems not to have been affected by the last glaciation, but affected by the previous glaciation, as suggested by the observed data (Fig. 3) and the simulations (Fig. 2). The two last glaciation maxima seem to have been similar in duration and intensity of cooling (Petit et al., 1999; Anderson et al., 2007), therefore, the effect on forests and their organisms should have been similar. Knowledge on the detailed paleogeographic and paleo-vegetation histories of the AF during the mid Pleistocene is almost inexistent. At the global scale this period coincides with the Mid-Brunhes event, a transition period from relative cool interglacial and small amplitude of glacial/interglacial temperature variation to warmer interglacials with larger amplitude of temperature variation (Anderson et al., 2007). However, the conditions in the AF and how organisms were affected during this period are unknown. Anyway, even though simulations did not favor a single date for bottlenecks, they suggest that there was a strong one. More phylogeographic studies are needed to identify the nature of the demographic expansion suffered by *B. leucoblepharus*.

4.2. Phylogeographic patterns in the Atlantic forest: a new pattern revealed by *Basileuterus leucoblepharus*?

In general, phylogeography studies of AF organisms show three main recurrent breaks (Fig. 1a): (i) one break in the state of São

Paulo, close to the valleys of the rivers Paraíba do Sul, Tietê and Ribeira do Iguape (snake, birds, frogs, bats, and toad; Grazziotin et al., 2006; Cabanne et al., 2007, 2008, 2011; Carnaval et al., 2009; Martins et al., 2009; Batalha-Filho et al., 2010; Thomé et al., 2010; d'Horta et al., 2011); (ii) one break in the state of Minas Gerais, close to the valleys of the rivers Jequitinhonha and Doce (bird and frogs; Cabanne et al., 2007, 2008; Carnaval et al., 2009; d'Horta et al., 2011); and (iii) a break in northeastern Brazil, close to the mouth of the São Francisco river (bird and frogs; Cabanne et al., 2008; Carnaval et al., 2009).

Regarding phylogeographic breaks i and ii, the dates of the origins of the river valleys are older than the estimated intraspecific divergence dates, and therefore the geologic origin of the valleys could not have been the primary cause of lineage divergence. Phylogeographic studies of AF birds (Cabanne et al., 2007, 2008; d'Horta et al., 2011), bats (Pavan et al., 2011) and frogs (Carnaval et al., 2009) could not reject that lineage divergences were associated with habitat fragmentation due to the glacial cycles in the Pleistocene. These studies were also congruent with a model of Pleistocene forest refugia in the AF based on paleoclimatic modeling of the LGM (Carnaval and Moritz, 2008). However, it is interesting to note that a snake (Grazziotin et al., 2006) also presents a phylogeographic break in this same region (break i, Fig. 1a). Even though these barriers show congruence in space, the estimated dates were not congruent (e.g. for break i: 3.87 m.y.a. for the snake and 0.39 m.y.a. for a bird). Moreover, two other studies with bees (Batalha-Filho et al., 2010) and toads (Thomé et al., 2010) suggest that some of these phylogeographic breaks also coincide with putative barriers associated to neotectonic activities.

The ecological niche modeling of the AF during the LGM by Carnaval and Moritz (2008) indicated that the forested areas in the southern portion of this biome were more fragmented than forests at the northern portion. Some recent studies have confirmed this hypothesis (Cabanne et al., 2007, 2008; Carnaval et al., 2009; Martins et al., 2009; d'Horta et al., 2011; Martins, 2011). However, our results were not congruent with the Carnaval and Moritz (2008) hypothesis because we did not find a strong genetic structure, a bottleneck followed by a strong signal of demographic expansion at the LGM, and a tendency of expansion from north to south (Figs. 1 and 3). Moreover, the comparison of nucleotide diversity of the mtDNA versus latitude showed that the south holds higher levels of genetic diversity (Fig. 4). This result disagrees with the latitudinal gradient hypothesis, which states that higher diversity levels should be found in lower latitudes (Miller et al., 2010; d'Horta et al., 2011). Furthermore, our simulations rejected the scenarios where the effects of LGM were tested (Table 3). Thomé et al. (2010) observed toad lineages that survived in the southern portion of the AF, and this is also not congruent with the Carnaval and Moritz (2008) hypothesis.

Both traditional and model-based phylogeographic approaches allowed us to infer about the evolutionary history of *B. leucoblepharus* and the diversification dynamics within the AF. To our knowledge, the present work revealed the first example of an AF organism (*B. leucoblepharus*) without a strong population genetic structure and whose population size did not change during the LGM. Thus, our results show that more AF organisms should be investigated to help in the reconstruction of the evolutionary history of this biome. As more taxa are studied, new scenarios shall be revealed. Costa (2003) suggested that speciation in the Neotropics could not be explained by any single model of vicariance or climatic change. Thus, it is possible that no general pattern for organisms' diversification in the AF will arise, but a complex range of scenarios shall be described.

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Anexo XXV

Matrilineal evidence for demographic expansion, low diversity and lack of phylogeographic structure in the Atlantic forest endemic Greenish Schiffornis *Schiffornis virescens* (Aves: Tityridae)

G. S. Cabanne · E. H. R. Sari · D. Meyer ·
F. R. Santos · C. Y. Miyaki

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Abstract Studies of Atlantic forest (AF) organisms suggest that the historical dynamics of the forest cover produced demographically stable populations in its central region and unstable populations in the southern regions. We studied the mitochondrial phylogeographic structure of an AF passerine, the Greenish Schiffornis *Schiffornis virescens* (Tityridae), and evaluated questions related to the history of the AF. We analyzed cytochrome *b* and control region sequences of the mitochondrial genome by traditional phylogenetic and population genetic methods based on summary statistics. In addition, we used coalescent simulations to evaluate specific models of evolution of the populations of *S. virescens*. The results did not support

phylogeographic partitions of the genetic variability of *S. virescens*. The overall Φ_{st} was = 0.32 and gene flow between regions was moderate to high. The analyses suggested that the total population of *S. virescens* suffered a bottleneck followed by a demographic expansion in the late Pleistocene. The bottleneck might have contributed to the extinction of intraspecific lineages, and hence to the observed lack of a strong phylogeographic pattern and low genetic diversity. Our results suggest that some AF taxa have had all their populations similarly affected by the recent history of the biome, contrary to what has been revealed from most of the other phylogeographic studies in the region and as suggested by a model of AF refuges (the Carnaval–Moritz model). We suggest that the response of organisms to common histories may be idiosyncratic, and predictions about the history of the biome should take into account ecological characteristics and distribution of each specific taxa.

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G. S. Cabanne · D. Meyer · C. Y. Miyaki
Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, São Paulo, SP 05508-090, Brazil

G. S. Cabanne
CONICET, Av. Rivadavia 1917, C1033AAJ Ciudad de Buenos Aires, Argentina

G. S. Cabanne (✉)
División de Ornitología, Museo Argentino de Ciencias Naturales “B. Rivadavia”, Ángel Gallardo 470, C1405DJR Ciudad de Buenos Aires, Argentina
e-mail: gscabanne@yahoo.com

E. H. R. Sari · F. R. Santos
Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil

Keywords Atlantic forest · Phylogeography · mtDNA · *Schiffornis* · Historical demography

Zusammenfassung

Matrilineare Hinweise auf demografische Expansion, geringe genetische Diversität und fehlende phylogeografische Struktur bei der Olivtrauerkotinga *Schiffornis virescens* (Aves: Tityridae), einer endemischen Vogelart der Mata Atlantica

Untersuchungen an Organismen der Mata Atlantica deuten darauf hin, dass die historische Dynamik der Waldbedeckung dort demografisch stabile Populationen in den inneren Bereichen und instabile Populationen in den südlichen Regionen hervorgebracht hat. Wir betrachteten

die mitochondriale phylogeografische Struktur bei der Olivtrauerkotinga *S. virescens* (Tityridae), einem Sperlingsvogel der Mata Atlantica, und untersuchten Fragen im Zusammenhang mit der Geschichte dieses atlantischen Waldes. Wir analysierten Sequenzen von Cytochrom b und von Kontrollregionen des mitochondrialen Genoms unter Anwendung der gebräuchlichen phylogenetischen und populationsgenetischen Methoden auf der Grundlage statistischer Parameter. Zusätzlich verwendeten wir Coalescent Simulations, um spezifische Evolutionsmodelle für die Populationen von *S. virescens* zu testen. Die Ergebnisse konnten keine phylogeografischen Untereinheiten in der genetischen Variabilität von *S. virescens* belegen. Der Gesamtwert für Φ_{st} betrug 0,32 und der Genfluss zwischen den Regionen war mäßig bis hoch. Die Analysen deuten an, dass die gesamte Population von *S. virescens* zunächst durch einen genetischen Flaschenhals ging, worauf anschließend im Oberen Pleistozän eine demografische Expansion erfolgte. Dieser Flaschenhals könnte zum Aussterben intraspezifischer Abstammungslinien und somit zum beobachteten Mangel an deutlichen phylogeografischen Mustern und zu der geringen genetischen Diversität beigetragen haben. Unsere Ergebnisse legen nahe, dass bei manchen Taxa der Mata Atlantica alle Populationen durch die jüngere Geschichte dieses Bioms ähnlich beeinflusst wurden. Dies steht im Gegensatz zu den Erkenntnissen der meisten anderen phylogeografischen Studien aus dieser Region und zu einem Modell atlantischer Waldrefugien (Carnaval–Moritz-Modell). Wir vermuten, dass verschiedene Organismen selbst auf eine gemeinsame Geschichte in ihrer ganz eigenen Weise reagieren. Prognosen über die Geschichte des Bioms sollten daher immer auch die ökologischen Eigenschaften und die Verbreitung jedes einzelnen Taxons berücksichtigen.

Introduction

The Atlantic forest (AF) of Brazil, northeastern Argentina and eastern Paraguay is amongst the richest and most endangered rainforests in the world (Galindo Leal and de Gusmão Câmara 2003) (Fig. 1). Congruence among palynological studies (i.e. Behling 2002; Behling and Negrelle 2001; Ledru et al. 2005), and models of paleo-distribution of forests and of some species of frogs (Carnaval and Moritz 2008; Carnaval et al. 2009), suggest that during the late Pleistocene the southern AF was strongly fragmented by the advance of grasslands and of other savanna-like vegetation. In contrast, the central and northern AF were relatively stable. More precisely, during the last glacial maximum (between 24,000 to 18,000 before present; Anderson et al. 2007), the southern grassland–forest ecotone seemed to have shifted 750 km north from its current location, which

was possibly established in the late Holocene (Behling 2002). Under the former scenario of temporal and geographic stability–instability of the AF (Carnaval and Moritz (2008), hereafter the Carnaval–Moritz model, the majority of the forest-dependent taxa should have been similarly affected by the retraction and fragmentation of forests during the Pleistocene, and therefore the existence of shared phylogeographic patterns is expected.

The Carnaval–Moritz model has received particular support from some phylogeographic studies with forest birds (i.e. Maldonado-Coelho 2012; Cabanne et al. 2008; d'Horta et al. 2011), frogs (Carnaval et al. 2009) and bees (Batalha-Filho et al. 2010). But, on the other hand, the model has been recently challenged by a phylogeographic study of the *Rhinella crucifer* group of toads (Thomé et al. 2010) and by a study of planarians (Álvarez-Presas et al. 2011), which did not find support for a southern AF Holocene colonization and therefore suggest persistence of forested habitats in the south (a southern refuge). In addition, the existence of a southern refuge is further supported by a recent study with species distribution models of several endemic taxa (Porto et al. 2012).

In the present study, we used sequence data derived from mitochondrial DNA (mtDNA) to explore the population genetic structure of the Greenish Schiffornis *Schiffornis virescens* (Tityridae; Remsen et al. 2009) (Fig. 1a) and to address questions of the AF history. *S. virescens* is a good model to address issues related to the AF history because it is endemic to the biome and occurs with low to medium density in well-preserved forests (Stotz et al. 1996). The species does not present geographic variation in plumage, song or morphometry (Ridgely and Tudor 1996). Since it inhabits the understory of preserved forests and is sensitive to forest fragmentation (Anjos et al. 2011; Stotz et al. 1996), chances to have been affected by Pleistocene fragmentation of habitat, and therefore to present a strong population genetic structure, are high (Burney and Brumfield 2009). Assuming that most AF organisms were affected similarly by the Pleistocene forest dynamics, we expected to find in *S. virescens* results similar to those obtained by other studies of AF organisms, which apparently revealed a shared phylogeographic pattern (Maldonado-Coelho 2012; Batalha-Filho et al. 2010; Grazziotin et al. 2006; Cabanne et al. 2007, 2008; Mustrangi and Patton 1997; Carnaval et al. 2009; d'Horta et al. 2011; Pessoa 2007). This shared pattern for each species consists of the existence of two main mtDNA clades south of the Doce river that come into contact in south-central São Paulo where no barrier to gene flow is evident (Fig. 1a, b). In addition, some species present a third lineage north of the Doce river (Cabanne et al. 2008; d'Horta et al. 2011; Maldonado-Coelho 2012). Another characteristic of the shared phylogeographic pattern is that the corresponding southern clades present

Fig. 1 a Distribution of the Atlantic forest (AF), of *Schiffornis virescens* and of the sampling localities for the genetic study. See “Appendix 1” for details of sampling localities. Phylogeographic barriers observed in other AF organisms are: barrier I, the passerine *Sclerurus scansor* (d’Horta et al. 2011), the passerine *Xiphorhynchus fuscus* (Cabanne et al. 2008), the passerine *Pyriglena leucoptera* (Maldonado-Coelho 2012), frogs of the genus *Hypsiboas* (Carnaval et al. 2009), and the toad *Rhinella crucifer* (Thomé et al. 2010); barrier II, opossum *Marmosops incanus* (Muistrangi and Patton 1997), the passerines *Sclerurus scansor* (d’Horta 2009) and *Conopophaga lineata* (Pessoa et al. 2006; Pessoa 2007); barrier III, the passerines *Xiphorhynchus fuscus* (Cabanne et al. 2007, 2008) and *Sclerurus scansor* (d’Horta et al. 2011), frogs of the genus *Hypsiboas* (Carnaval et al. 2009), and monkeys of genus *Alouatta* (Martins et al. 2011); barrier IV, the passerine *Dendrocolaptes platyrostris* (Cabanne et al. 2011), the bee *Melipona quadrifasciata* (Batalha-Filho et al. 2010), the viper *Bothrops jararaca* (Grazziotin et al. 2006), the frogs *Hypsiboas faber* (Carnaval et al. 2009), *Proceratophrys boiei* (Amaro et al. 2012) and another frog of the genus *Thoropa* (Fitzpatrick et al. 2009). b Predicted phylogeographic pattern in *S. virescens* based on the genetic constitution of other AF species (see a). The predicted genetic structure among regions ($\Phi_{CT} > 0.2$) is related to a level of gene flow that is not sufficient to avoid allele fixation and strong divergence (Wang 2004)

evidence of population expansion, differently to clades north of the Doce river. These shared phylogeographic features could be the product of alterations in the forest cover that occurred during the late Pleistocene, as would be expected from the Carnaval–Moritz model.

Phylogeographic studies of AF organisms are still scant and a robust synthetic hypothesis about the evolution of AF

organisms is not yet possible (but see Carnaval et al. 2009; Martins et al. 2011). Up to now, the selection of AF species for phylogeographic studies was not only driven by biogeographic questions but also by systematic issues in polymorphic taxa (i.e. Cabanne et al. 2008). This aspect may have biased the possibility of finding complex phylogeographic patterns because variation in external

characters (i.e. plumage and voice) is frequently correlated with genetic structure. *S. virescens* has no described geographic variation in external characters, therefore an alternative working hypothesis might be to find no phylogeographic structure.

Nyari (2007) showed that *S. turdina*, which is the sister species of *S. virescens*, presents a substantial intra-specific genetic structure. Because both species are very similar in song and plumage, the evolutionary relationship between them is not yet clear. The study of Nyari (2007) included samples of *S. virescens*, but without sampling all the variation. Thus, before addressing our biogeographic questions, we evaluated whether the species is monophyletic.

The objective of this study was to describe the mtDNA genetic variation of *S. virescens* to address the following questions: (1) Is the species monophyletic? (2) Does it present the phylogeographic structure described for other AF organisms? (3) How did the species' phylogeographic pattern originate? and (4) What are the implications of results for understanding the evolution of AF organisms?

Methods

Genetic datasets

In the phylogenetic analysis, we included 28 *S. virescens*, 14 *S. turdina* representing the variation of that species (Nyari 2007), as well as 1 *S. major* and 2 *Laniocera hypopyrra* as the outgroup. We used an alignment consisting of 840 bp of the cytochrome *b* (*cytb*) and 476 bp of the first domain of the mtDNA control region (CR). Sequences of *cytb* from *S. turdina*, two *S. virescens* and the two outgroups were obtained from the study of Nyari (2007) (see GenBank accession numbers in Fig. 2). We were unable to collect CR sequences for some *S. turdina* and for the outgroups, so we coded these as missing data. Amplification and sequencing of *cytb* was done following Cabanne et al. (2008). See "Appendix 1" for details of the samples used in this study.

For the population genetic study, we used CR sequences (476 pb) from 57 *S. virescens* collected in 19 localities (Fig. 1a). Amplification and sequencing of the CR was performed by specifically designed primers: forward LSvCB GATCTATCCCCAATAAACTTGGAGG and reverse HSvCR GATTACGAACTCGAAGTTGGACGG. PCR annealing temperature for CR was 60 °C for *S. virescens* and 53 °C for *S. turdina*. In order to avoid working with possible copies of the CR translocated to the nucleus, we only used sequences obtained from clean amplification bands, presenting the expected length, single peaks, and a base composition compatible with mtDNA (Baker and Marshall 1997).

Since both *cytb* and CR are physically linked in a single molecule (mitochondrial genome), results obtained with both markers are totally compatible because they reflect a single genealogy and history (Avice 2000). Genbank accession numbers for *cytb* and for CR are JX477687–JX477770. For evaluating whether the used sequences were representative of the genetic constitution of *S. virescens*, we estimated $P = [(k - 1)/(k + 1)]$, which represents the probability that a sample of size *k* and the whole population share the most recent common ancestor (Hein et al. 2005). Hence, *P* can be interpreted as the probability of the sample being representative of the genetic diversity of the population.

Phylogenetic analyses and molecular clock

Molecular evolution parameters were selected in MODELTEST 3.7 (Posada and Crandall 1998) or directly estimated during phylogenetic reconstruction. To obtain phylogenetic trees, we used bayesian and maximum likelihood approaches as implemented in MrBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) and PHYML 2.4.4 (Guindon and Gascuel 2003), respectively. MrBAYES analysis used a MCMC of 5×10^6 generations with sampling every 1,000 generations. We discarded the first 1,750 genealogies (burn-in), and parameters of molecular evolution between genes (*cytb* and CR) were unlinked. CR sequences of some taxa were coded as missing data. As missing data might potentially mislead phylogenetic inference (Lemmon et al. 2009), we analyzed the dataset with and without CR sites. As topologies did not change, the final phylogenetic analyses used the dataset with missing data (*cytb*+CR).

We used BEAST 1.6.1 (Drummond and Rambaut 2007) to estimate node ages by using a relaxed uncorrelated lognormal molecular clock analysis based on the *cytb* partition, a molecular clock calibration of 2.1 % divergence per million years (Weir and Schluter 2008), and a tree prior of constant size. From this analysis in BEAST, we estimated a substitution rate for the CR in relation to the *cytb* rate. Node ages obtained with the *cytb*-CR dataset (with missing data) and with only the *cytb* (no missing data) were similar, and therefore we based our dating estimation in the *cytb*-CR dataset.

To study the constancy of the molecular clock we used two procedures. First, we evaluated the parameter *ucl.d.stdev* (standard deviation of the molecular rate of *cytb*), estimated in BEAST. If the parameter estimate is close to zero then the data is clock-like. If the *ucl.d.stdev* has an estimated value much greater than one, then the data exhibits substantial rate heterogeneity among lineages (Drummond and Rambaut 2007). Secondly, we used a likelihood-ratio test, as implemented in MODELTEST, by

positive selection, we used the compound test HEW and DHEW (Zeng et al. 2007), performed with *S. turdina* as outgroup in the NH software (kindly provided by K. Zeng). The tests HEW and DHEW evaluate the null hypothesis of neutrality and are significant only when positive selection has acted in the target sequence or linked regions (hitchhiking) (Zeng et al. 2007). The skyride analysis (Minin et al. 2008) for studying the change of population effective size through time was performed in BEAST 1.6.1 with 10 million generations, sampling every 1,000 generations and a burn-in of 1,000 genealogies. We used an uncorrelated log-normal clock model, time aware smoothing and a generation time of 1 year (Klicka and Zink 1999).

Estimation of gene flow assuming an island model in equilibrium was obtained using the formula $M = (1 - \Phi_{st})/2\Phi_{st}$ (Hedrick 2000). Also, we used the isolation–migration (IM) model (Nielsen and Wakeley 2001; Hey and Nielsen 2004), implemented in IM (v. April 21, 2008), to estimate non-equilibrium gene flow rates and divergence times between regions. We applied the HKY model of evolution, similar population parameter theta values ($\Theta_1 = \Theta_2 = \Theta_a$) and $m_1 = m_2$. A total of 500,000 iterations were used for burn-in and analyses were stopped when the smallest effective sample size (ESS) was higher than 50 and parameter trend lines stabilized, usually after 20 million iterations. We assumed a generation time of 1 year.

Coalescent simulations

In order to evaluate different demographic histories, we simulated demographic scenarios in the program BAY-ESSC, a modification of software SERIAL SIMCOAL (Anderson et al. 2005; Chan et al. 2006), and evaluated the goodness of fit of the observed data (CR sequences) to the simulations; see Richards et al. (2007) and Knowles (2009) for reviews on this approach. Because genetic signals of expansion may be found in a demographically stable island system (Nielsen and Beaumont 2009; Peter et al. 2010), the main objective of this section was to investigate if the phylogeographic pattern could have been generated in an island system with demographic stability or in a non-structured system that expanded demographically. The demographic models assessed, detailed in Fig. 2, differed in the timing, intensity, and numbers of bottlenecks, and also in the number of populations. The populations in model G were originated before the Pleistocene, as suggested by the divergence of *S. virescens* with its sister species (see “Results”). In order to obtain a metric of population genetic structure (Φ_{st}) for the models of single populations and to simulate the island models, we organized the sampling localities by geographic proximity into five populations (pop). This procedure is a conventional practice in this kind

of analysis (i.e. Fabre et al. 2009; Belle et al. 2006). In preliminary analyses, we tested several other population configurations and results did not change. The final organization of sampling localities was: pop 1: localities 1–5; pop 2: localities 6–8, 11; pop 3: localities 12–14; pop 4: localities 9, 10, 15–17; pop 5: localities 18 and 19. See localities in “Appendix 1”.

For each model, we performed 1,000 simulations and used ARLEQUIN to estimate five summary statistics for the complete dataset: number of segregating sites, nucleotide diversity, Tajima’s *D* (Tajima, 1989), Fu’s *F_s* (Fu 1997), and Φ_{st} amongst populations (Excoffier et al. 1992). To evaluate the plausibility of each model, we first estimated the proportion *pi* of simulated values equal and higher than the observed summary statistics, and then we reported a global *p* value for each model by combining individual *pi* values by a nonparametric procedure described in Voight et al. (2005) and Fabre et al. (2009). See electronic supplementary material for further methodological details.

Results

Genetic data, neutrality and mtDNA diversity

The *cytb* and CR concatenated alignment and the CR alignment presented probabilities to represent the complete gene genealogy equal to 93 and 96.5 %, respectively, which indicated that the samples are suitable to the study. The phylogenetic analysis showed that *S. virescens* is a well-supported monophyletic lineage (Fig. 3a) that diverged from its sister species, *S. turdina*, approximately 6.2 Mya (million years ago) (HPD95 % 4.3–8.14 Mya). Contrary to what we hypothesized, no intraspecific matrilineal genetic structure was present in *S. virescens*. The CR dataset presented 17 haplotypes and the network confirmed the lack of phylogeographic structure (Fig. 3b). The parameters *cytb*.*ucl.d*.*stdev* = 0.293 (HPD95 % 0.0000015–0.76) and *CR*.*ucl.d*.*stdev* = 0.3757 (HPD95 % 0.0000119–1.05) estimated in BEAST indicated a slight deviation from the constant molecular clock in *cytb* and CR sequences, respectively. This result was further confirmed by the likelihood ratio test for the dataset *S. virescens*/*S. turdina* and for the datasets of each species ($p < 0.00001$). The divergence rate of CR estimated in BEAST was 2.34 % divergence per million years, slightly higher than the *cytb* rate (2.1 % divergence per million years). We used this rate in the following analyses with CR.

We used *cytb* to compare the genetic diversity of *S. virescens* with other AF birds. We reviewed the available studies on AF birds and found that the nucleotide diversity of a sample of 12 species (including *S. virescens*) ranged from 0.01574 to 0.00187 (average = 0.0086, see

Fig. 3 **a** Bayesian phylogenetic tree based on 1,533 bp of the cytb and control region of the mtDNA of *Schiffornis virescens* and closely related species. Each gene partition used the model of molecular evolution GTR+I+G with parameters specifically estimated. Posterior probability obtained in MrBAYES (Bay. prob.) and maximum likelihood bootstrap (ML Boot, 500 replicates) are

presented at nodes. Divergence dates were obtained in BEAST based in the cytb molecular clock (Weir and Schluter 2008). **b** Median joining network based on 476 bp of the mtDNA control region of *S. virescens*. **c** Skyride plot showing the variation in population effective (N_e) size through time

“Appendix 2” for details). *S. virescens* presented the smallest diversity (cytb nucleotide diversity = 0.00187) among the 12 species considered.

The AMOVA results did not support any of the hypothesized genetic barriers (Table 1). All Φ_{ct} values were small and not significant, which indicates that most of the variation in the mtDNA of *S. virescens* was organized among and within populations, and not among regions separated by the tested barriers. In addition, the global Φ_{st} value ($\Phi_{st} = 0.3184$) indicated a moderate population genetic structure among localities, which corresponded to moderate equilibrium gene flow of ~ 1.1 females per generation among populations. The analysis in IM indicated moderate to high gene flow between regions separated by the putative phylogeographic barriers (Fig. 1a): $M_{CAF-SAF}$: 0.8 females (HPD95 % 0–6.5), $M_{CAF-SEAF}$: 5.5 females (HPD95 % 1.6–42.7); and $M_{SAF-SEAF}$: 10.4 females (HPD95 % 1.7–?). All divergence times correspond to the late Pleistocene: $T_{CAF-SAF}$: 0.16 MYA (HPD95 % 0.04–0.7), $T_{CAF-SEAF}$: 0.1 MYA (HPD95 % 0.0014–0.5); and $T_{SAF-SEAF}$: 0.06 MYA (>0.012). However, as all

posterior distributions of divergence estimations are very wide (results not shown), populations cannot be considered as independent, which confirms the absence of population structure in *S. virescens*.

The summary statistics tests collectively suggested a demographic expansion in *S. virescens* (Table 2). The neutrality–demographic tests suggested a demographic expansion because Fu’s tests were significant. Results of the test of Tajima were borderline but compatible with the Fu’s tests because the D statistics were negative. Results of the positive selection tests did not support the action of positive selection; however, some p values are borderline significant. The demographic analysis in LAMARC further corroborated that *S. virescens* expanded recently (positive $g = 932$, HPD95 % CI: 367–1966). The Skyride plot analysis showed the existence of a moderate expansion during the late Pleistocene, near 21 times the initial N_e (Fig. 3c). The region of origin of the demographic expansion should present the highest level of genetic diversity. However, because the three regions into which we divided the study area (Fig. 1a; CAF, SEAF and SAF) presented

Table 1 Analyses of molecular variance of *Schiffornis virescens* based on the mtDNA control region and the testing of phylogeographic barriers

Tested barrier ^a	Population structure ^b	Φ_{st} (95 % c.i.)	Φ_{st} <i>p</i> value	Φ_{ct} (95 % c.i.)	Φ_{ct} <i>p</i> value
No barrier	[(2–3), (4–5), (6–7), 10, 11, (12–13), 14, 9, (15–15), 17, 18, 19]	0.3185 (0.0710–0.4717)	<0.00001	–	–
CSP	[(2–3), (4–5), (6–7), 10, 11, (12–13), 14] [9, (15–15), 17, 18, 19]	0.3348 (0.0911–0.4825)	<0.00001	0.0528 (0.0148–0.0896)	0.0733
DR	[(2–3), (4–5)] [(6–7), 10, 11, (12–13), 14, 9, (15–15), 17, 18, 19]	0.3367 (0.0513–0.5044)	<0.00001	0.0405 (0.1253–0.2871)	0.2308
CSP and DR	[(2–3), (4–5)] [(6–7), 10, 11, (12–13), 14] [9, (15–15), 17, 18, 19]	0.3227 (0.0766–0.4720)	<0.00001	0.0220 (0.0627 –0.1598)	0.1899

^a See Fig. 1a for localities and tested phylogeographic barriers (CSP, Central-São Paulo, and DR Doce river)

^b Numbers represent localities from Table 1. Specific structures indicated by samples within square brackets. Localities within parenthesis were pooled to form a new sample. Localities 1 and 8 were excluded due to small sample sizes

Table 2 Diversity indices and test of significance of neutrality–demography in *Schiffornis virescens*

Alignment ^a	<i>n</i>	Number of segregating sites	Nucleotide diversity	Neutrality–demographic test <i>p</i> values		Positive selection test <i>p</i> values	
				Tajima's test (statistic)	Fu's test (statistic)	HEW	DHEW
cytb and CR (1533 pb)	28	13	0.00272 (SE 0.00059)	0.04 (<i>D</i> = −1.578)	0.001** (<i>F_s</i> = −8.484)	0.0706	0.0425
CR (476pb)	57	13	0.0041 (SE 0.0013)	0.038 (<i>D</i> = −1.47)	0.002** (<i>F_s</i> = −7.5)	0.027	0.0178

^a cytb cytochrome *b*, CR control region

** Significant test at 1 % level after Bonferroni correction for four tests

similar levels of genetic diversity (results not shown), the origin of the expansion was not detected.

Simulations results

Results of the evaluation of demographic models by coalescent simulations are presented in Table 3. The model A of panmixia and demographic stability, the model E of panmixia and expansion, and the models of islands and demographic stability F and G obtained low global probabilities, indicating they all have low plausibility. All the models with high global probability (*p* value >0.1: models B, C, and D) suggest that *S. virescens* suffered bottlenecks during the glacial maxima followed by posterior recovering of the population effective size. In particular, the model with highest *p* value (model C1, *p* = 0.84) suggests that *S. virescens* is conformed by a single population that suffered an intense bottleneck before the last glacial maxima.

The results of these simulations are compatible with the analysis performed in LAMARC, as well as with the demographic summary statistics and the skyride plot, collectively suggesting that the species suffered an expansion during the late Pleistocene. A genetic pattern compatible with demographic expansion could also result from an

island system with demographic stability, but our simulations did not support this situation (models F and G).

Discussion

Schiffornis virescens was found to be monophyletic, and presented low mtDNA genetic diversity and a weak population genetic structure. In addition, the genetic partitions across genetic barriers important for other organisms were not found to be influential for *S. virescens*. These results are in contrast to what was found in its sister species, *S. turdina*, and in other AF taxa dependent on preserved forests. *S. turdina* has high genetic diversity and substantial phylogeographic structure (Nyari 2007). Within the lineage *S. virescens*/*S. turdina*, the most ancient split separated birds from the AF (*S. virescens*) from birds associated primarily to the Amazon and Central America forests (*S. turdina*). Interestingly, and as observed in some other taxa (i.e. Cabanne et al. 2008; Ribas et al. 2005), the AF lineage showed shorter branches and lower genetic distances among terminals (Fig. 3a), a pattern that suggests different evolutionary dynamics in both *Schiffornis* species (Ricklefs 2007). Also, contrary to what was expected according to other Neotropical rainforest organisms from

Table 3 Results of evaluation of demographic scenarios of *Schiffornis virescens* using coalescence simulations using BAYESSC

Model description	Global <i>p</i> value	Model description	Global <i>p</i> value
A-Panmixia, stability	0.0620	D2-Panmixia, two bottlenecks, <i>Nt/No</i> = 10–100 %	0.0542
B1-Panmixia, bottleneck LGM, <i>Nt/No</i> = 1–10 %	0.3270	E-Expansion, 10–100X	0.0640
B2-Panmixia, bottleneck LGM, <i>Nt/No</i> = 10–100 %	0.0482	F1-Stable islands, <i>m</i> :0–0.01	0.0242
C1-Panmixia, bottleneck pre LGM, <i>Nt/No</i> = 1–10 %	0.8440	F2-Stable islands, <i>m</i> :0–0.001	0.0780
C2-Panmixia, bottleneck pre LGM, <i>Nt/No</i> = 10–100 %	0.1530	G1-Stable islands, <i>m</i> :0–0.01, origin 6 MYr ago	0.0980
D1-Panmixia, two bottlenecks, <i>Nt/No</i> = 1–10 %	0.3430	G2-Stable islands, <i>m</i> :0–0.001, origin 6 MYr ago	0.0540

LGM last glacial maximum; *Nt* and *No* effective population size at the end and at the beginning of the bottleneck, respectively; *m* migration rate parameter

the AF or from outside the AF (i.e., Cabanne et al. 2011; d’Horta et al. 2011; Carnaval et al. 2009; Bates 2002; Aleixo 2004; Cheviron et al. 2005; Nyari 2007), *S. virescens* did not present a strong phylogeographic structure.

The differences in matrilineal population structures among birds could not be explained by ecological divergence between *S. virescens* and the other AF birds. Ecology predicts levels of intraspecific genetic divergence in birds from Amazonia and likely from other regions (Burney and Brumfield 2009). For example, canopy birds tend to be less genetically differentiated than understory birds. In addition, very specialized bird taxa are more diversified than generalist birds (Salisbury et al. 2012). Even though comparative ecological studies of AF birds are scarce (i.e., Anjos 2006; Ribon et al. 2003), there are not remarkable ecological differences between *S. virescens* and the AF birds that present strong phylogeographic structure (Cabanne et al. 2008, 2011; d’Horta et al. 2011; Maldonado-Coelho 2012; Pessoa et al. 2006; Lacerda et al. 2007). *S. virescens*, as well as most of the other studied birds, inhabits the understory and is a specialist of well-preserved forests. A pattern of low genetic structure, such as the one found in *S. virescens*, is expected for species with high gene flow and low sensitivity to forest fragmentation. An example of such a species is *Basileuterus leucoblepharus* (Anjos 2006), which is a bird co-distributed with *S. virescens* whose mtDNA and nuclear DNA are not geographically structured and that presented demographic stability during the late Pleistocene (Batalha-Filho et al. 2012).

The mtDNA genealogical pattern of low diversity and no genetic structure of *S. virescens* could have been generated by a selective sweep (positive selection) (Nielsen 2005). However, we utilized specific tests for positive selection, HEW and DHEW, and the results did not support the hypothesis of selection (Table 2). Therefore, the genetic constitution of *S. virescens* could have been modeled by factors other than positive selection, such as demographic processes.

S. virescens is a relatively young species, as shown by its time to the most recent common ancestor (TMRCA)

(0.43 Mya; Fig. 3a), and thus not enough time may have passed for the evolution of intraspecific structure. However, *S. virescens* is not younger than other AF species that do present a strong phylogeographic structure, such as *Sclerurus scansor* and *Dendrocolaptes platyrostris* (d’Horta et al. 2011; Cabanne et al. 2011), therefore species age alone cannot account for the lack of phylogeographic structure.

The difference between the splitting time between *S. virescens* and *S. turdina* (6.2 Mya) and the TMRCA of *S. virescens* (0.43 Mya) seems to be high when compared to other pairs of sister taxa of the region. For example, in other AF species such as *S. scansor* (d’Horta et al. 2011) and *Xiphorhynchus fuscus* (Cabanne et al. 2008), the divergence with their sister clades are ~1.1 and ~3 Mya ago, respectively, while the maximum intraspecific divergence are about 0.6 and 0.8 Mya, respectively. Also, in *D. platyrostris*, another passerine from the AF and the gallery forests of central South America savannas, the divergence with its sister taxa is about 1.6 Mya while the intraspecific divergence is 0.22 Mya (Cabanne et al. 2011). We expected a higher intraspecific differentiation in *S. virescens*, and thus an older TMRCA, because of the old splitting time with *S. turdina* and because of the high intraspecific diversity documented within the latter species. The matrilineal constitution of *S. virescens* suggests an evolutionary history that resulted in low intraspecific diversification. Perhaps, lower diversification rates or higher extinctions rates in *S. virescens* could be responsible for this pattern (Ricklefs 2007).

The phylogeographic pattern of *S. virescens* could have been originated through the combined effects of gene flow and historical demography. Our results indicated that gene flow rates among populations are moderate to high, enough to avoid allele fixation and strong divergence (Wang 2004). Also, different analyses collectively revealed a recent population expansion. Finally, *S. virescens* presented lower levels of mtDNA genetic diversity than other AF birds (“Appendix 2”), suggesting that the demographic expansion may have occurred after a population bottleneck that

reduced genetic diversity. This bottleneck might have contributed to the extinction of intraspecific mtDNA lineages, leading to the observed lack of a strong phylogeographic pattern and low genetic diversity in *S. virescens*.

Different effective population sizes, demography, or the lack of recognition of different species in *S. turdina* might be responsible for the differences in mtDNA genetic diversity and structure between *S. virescens* and *S. turdina*. *S. turdina* inhabits all the Amazonian basin forests, part of Central America forests, and part of northeastern AF. Thus, its geographic range is nearly five times larger than that of *S. virescens*, which may imply a larger population and therefore higher genetic diversity for *S. turdina*. In addition, *S. turdina* has several highly divergent phylogroups that, with further study, might be considered as good species (Nyari 2007).

The phylogeographic pattern of a species can be shaped by the history of the biome in which it is found (Hewitt 2000, 2004). Our results, specifically the lack of a strong phylogeographic structure, the low genetic diversity, a most recent common ancestor in the mid-Pleistocene, and the evidence of demographic expansion for the whole species, suggest that the N_e of *S. virescens* was reduced greatly during the late Pleistocene, likely to a population restricted to a single continuous forest (refuge) of uncertain location. Interestingly, the magnitude of the demographic expansion of *S. virescens* ($\sim 21 \times$ initial N_e ; Fig. 3c) is similar to that described for some taxa from the northern hemisphere that were highly affected by the cycles of forest shrinkage—expansion that occurred during global glaciations (e.g., *Catharus ustulatus*; Rugg et al. 2006).

Most populations of *S. virescens* inhabit a region whose rainforest coverage fluctuated during the Pleistocene, which could be the reason why they presented a shared demographic history. Specifically, the majority of the range of the bird is located in the southern portion of the AF, which was the most affected by natural fragmentation and expansion of savanna-like vegetation during the global glaciations (Carnaval et al. 2009; Behling 2002; Behling et al. 2002). This is different from those taxa that persisted in the same period in more than one population, and whose geographic ranges also span historically stable forests in

central and northern AF (e.g., d'Horta et al. 2011; Cabanne et al. 2008, 2011; Thomé et al. 2010).

Our results were not fully compatible with the predictions derived from the Carnaval–Moritz model (Fig. 1b), but they were still in accordance with some aspects of the hypotheses of the AF history. Most published studies on AF organisms suggest that the Pleistocene dynamics produced stable populations in the central region and unstable populations in the southern portion. We did not recover this pattern in *S. virescens*, but rather documented that its association to the southern AF and relatively short age was crucial for this resulting pattern. The Carnaval–Moritz model is not powerful enough to predict the distribution of Pleistocene refugia of organisms restricted to the south-east–south portion of the AF (Porto et al. 2012), and our results suggested that its power to predict phylogeographic patterns of those organisms is also low. Independent of the origin of the phylogeographic structure within *S. virescens*, our results indicated that some AF taxa have had all their extant populations similarly affected by the recent history of the biome, contrary to what has been revealed from most of the other phylogeographic studies of species from the region. Therefore, the response of organisms to common histories may be idiosyncratic, and predictions about the history of the biome should take into account ecological characteristics and distribution of each specific taxa.

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Appendix 1

See Table 4.

Table 4 *Schiffornis virescens* and *S. turdina* sampling localities and sample information used in the present study

Taxa	Locality	Sample size	Tissue ID ^{TISSUE}
<i>S. virescens</i>	1-Bonito, Bahia. 11°56'S, 41°15'W (popl)	1	LGEMA P2263 ^b
	2-Ecological station Acauã, Leme do Prado, Minas Gerais (MG). 17°4'S, 42°40'W	1	B2284 ^b
	3-Turmalina, MG. 17°16'S, 42°43'W	7	B1990 ^b , B1989 ^b , B2279 ^b , B2280 ^b , B2281 ^b , B2282 ^b , B2283 ^b
	4-Jequitinhonha (Mata Escura), MG. 16°20'S, 41°00'W	1	B1091 ^b
	5-José Gonçalves de Minas, MG. 15°00'S, 40°25'W	1	B2285 ^b
	6-Nova Lima, MG. 19°50'S, 43°49'W	1	B1570 ^b
	7-São Bartolomeu (Grande Mata), MG. 20°17'S, 43°35'W	1	B1022 ^b
	8-Santa Teresa, Espírito Santo (ES). 19°56'S, 40°34'W	1	B1711 ^b
	9-Morro do Diabo State Park, São Paulo (SP). 22°30'S, 52°18'W	10	All from LGEMA: P1965 ^b , P1867 ^b , P1957 ^b , P1960 ^b , P1959 ^b , P1973 ^b , P2158 ^b , P1494 ^b , P1989 ^b , P1388 ^b
	10-Barreiro Rico, SP. 22°38'S, 48°13'W	2	LGEMA P1703 ^M , LGEMA P1686 ^M
	11-Itatiaia Natl. Park, Rio de Janeiro. 22°25'S, 44°36'W.	5	All from LGEMA: P1322 ^b , P1346 ^b , P1339 ^b , P1332 ^b , P1333 ^b
	12-Piedade, SP.	1	LGEMA P681 ^M
	13-Morro Grande State Park, SP. 23°42'S, 46°59'W	10	All from LGEMA: P519 ^b , P493 ^b , P542 ^b , P463 ^b , P435 ^b , P448 ^b , P461 ^b , P544 ^b , P618 ^b , P518 ^b
	14-Juquitiba, SP, 23°53'S, 47°00'W	3	LGEMA P714 ^M , LGEMA P773 ^M , LGEMA P713 ^M
	15-Pinhalão, Paraná (PR). 23°46'S, 50°3'W	1	LGEMA P869 ^M
	16-Wenceslau Braz, PR. 22°5'S, 48°47'W	2	LGEMA P958 ^M , LGEMA P980 ^M
	17-PETAR, Caboclos station, SP, 24°28'S, 48°35'W	3	LGEMA P1211 ^b , LGEMA P1220 ^b , LGEMA P1221 ^b
	18-State Park Urugua-í, Misiones, Argentina, 25°51'S, 53°55'W	5	All from LGEMA: 10369 ^M , 10341 ^M , 10348 ^M , 10359 ^M , 10370 ^M
	19-Rancho Queimado, Santa Catarina. 27°40'S, 49°1'W	2	LGEMA P1753 ^b , LGEMA P1756 ^b
<i>S. turdina</i>	Novo Airão, Amazonas. 1°57'S 62°21'W		LGEMA 9705 ^M

Samples have been deposited at the Laboratório de Genética e Evolução Molecular de Aves (LGEMA), Instituto de Biociências, Univ. de São Paulo, Brazil, and at the Departamento de Biologia Geral, Instituto de Ciências Biológicas, Univ. Federal de Minas Gerais, Brazil (B). All samples are from Brazil, except when otherwise indicated

TISSUE tissue type, ^b blood, ^M muscle

Appendix 2

See Table 5.

Table 5 Nucleotide diversity of cytochrome b (mtDNA) from studies of Atlantic forest passerines

Species	Π —nucleotide diversity	Sample size	Length alignment (bp)	Reference
<i>Xiphorhynchus fuscus</i>	0.01574 ($SD = 0.0022$)	35	1,110	Cabanne et al. (2008); Aleixo (2002); Irestedt et al. (2004)
<i>Dendrocolaptes platyrostris</i>	0.004454 ($SD = 0.0012$)	34	1,006	Cabanne et al. (2011); Irestedt et al. (2004)
<i>Automolus leucophthalmus</i>	0.00242 ($SD = ?$)	22	900	D’Horta (2009)
<i>Sclerurus scansor</i>	0.01044 ($SD = 0.0020$)	47	1,046	d’Horta et al. (2011)
<i>Thamnophilus ambiguus</i>	0.005 ($SD = 0.002$)	22	837	Lacerda et al. (2007)
<i>Thamnophilus pelzelni</i>	0.003 ($SD = 0.002$)	21	837	Lacerda et al. (2007)
<i>Scytalopus speluncae</i>	0.0395 ($SD = 0.006$)	31	357	Mata et al. (2009)
<i>Scytalopus pachecoi</i>	0.0056 ($SD = 0.0023$)	14	344	Mata et al. (2009)
<i>Scytalopus petrophilus</i>	0.0039 ($SD = 0.0019$)	12	344	Mata et al. (2009), Whitney et al. (2010)
<i>Schiffornis virescens</i>	0.00187 ($SD = 0.00062$)	28	840	This study
<i>Tachyphonus coronatus</i>	0.00512 ($SD = 0.00042$)	50	990	Cabanne and Miyaki, unpublished
<i>Basileuterus leucoblepharus</i>	0.0058 ($SD = 0.0010$)	55	914	Batalha-Filho et al. (2012)

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Anexo XXVI

Connections between the Atlantic and the Amazonian forest avifaunas represent distinct historical events

Henrique Batalha-Filho · Jon Fjeldså ·
Pierre-Henri Fabre · Cristina Yumi Miyaki

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Abstract There is much evidence to support past contact between the Atlantic and the Amazon forests through the South American dry vegetation diagonal, but the spatio-temporal dynamics of this contact still need to be investigated to allow a better understanding of its biogeographic implications for birds. Here, we combined phylogenetic data with distributional data using a supermatrix approach in order to depict the historical connection dynamics between these biomes for New World suboscines. We examined the variation in divergence time and then compared the spatial distributions of taxon pairs representing old and recent divergences. Our results pointed to two distinct spatiotemporal pathways connecting the Atlantic and the Amazonian forests in the past: (1) old connections (middle to late Miocene) through the current southern Cerrado and Mato Grosso and the transition towards the Chaco and palm savannas of Bolivia and Paraguay; (2) young connections (Pliocene to Pleistocene) that possibly occurred through the Cerrado and Caatinga in northeastern Brazil. We suggest that the main events that played

important roles in these connections were geotectonic events during the late Tertiary associated with the uplift of the Andes (old connections) and Quaternary climate changes that promoted the expansion of gallery forest through the Cerrado and Caatinga in northeastern Brazil (young connections). Our results provide the first general temporal and spatial model of how the Atlantic and Amazonian forests were connected in the past, which was derived using bird data.

Keywords Neotropical region · New World suboscines · Supermatrix · Worldmap · Biogeography · Miocene · Quaternary

Zusammenfassung

Verbindungen zwischen atlantischen und amazonischen Waldvogelfaunen spiegeln distinkte historische Ereignisse wider

Zahlreiche Hinweise belegen ehemalige Kontakte zwischen atlantischen und amazonischen Wäldern über die diagonal durch Südamerika verlaufende Trockenvegetationszone hinweg, allerdings bedarf die raumzeitliche Dynamik dieser Kontakte noch näherer Untersuchung, um deren biogeografische Auswirkungen auf Vögel besser verstehen zu können. Hier fassten wir phylogenetische Daten mit Verbreitungsdaten in einer Supermatrix-Analyse zusammen, um so die Dynamik der historischen Verbindungen zwischen diesen Biomen für Neuwelt-Suboscine abzubilden. Wir untersuchten die Variation im Zeitpunkt der Artentrennung und verglichen dann die räumliche Verteilung von Taxonpaaren, die alte und rezente Trennungseignisse repräsentieren. Unsere Ergebnisse deuten auf zwei distinkte raumzeitliche Verbindungswege zwischen

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H. Batalha-Filho (✉) · C. Y. Miyaki
Laboratório de Genética e Evolução Molecular de Aves,
Departamento de Genética e Biologia Evolutiva, Instituto de
Biociências, Universidade de São Paulo, Rua do Matão 277,
Cidade Universitária, São Paulo, SP 05508-090, Brazil
e-mail: henrique.batalha@usp.br; rickbio@hotmail.com

J. Fjeldså · P.-H. Fabre
Center for Macroecology, Evolution and Climate at the Natural
History Museum of Denmark, University of Copenhagen,
Copenhagen, Denmark

atlantischen und amazonischen Wäldern in der Vergangenheit hin: (1) alte Verbindungen (Mittleres bis Oberes Miozän) durch den heutigen südlichen Cerrado und Mato Grosso sowie den Übergang zum Chaco und den Palmsavannen Boliviens und Perus; (2) junge Verbindungen (Pliozän bis Pleistozän), möglicherweise durch die Cerrados und die Caatinga im nordöstlichen Brasilien. Unserer Ansicht nach spielten die folgenden Hauptereignisse eine wichtige Rolle für diese Verbindungswege: geotektonische Ereignisse während des späten Tertiärs im Zusammenhang mit der Auffaltung der Anden (alte Verbindungen) sowie Klimaänderungen im Quartär, die die Ausbreitung von Galeriewäldern durch Cerrado und Caatinga im nordöstlichen Brasilien begünstigten (junge Verbindungen). Auf der Grundlage von Vogeldaten stellen unsere Ergebnisse die erste allgemeine Näherung für Zeiträume und die Art und Weise dar, wie atlantische und amazonische Wälder in der Vergangenheit in Verbindung standen.

Introduction

It is well known that many sister species and lineages are disjunctly distributed in the Brazilian Atlantic Forest (AF) and in the Amazon Basin (AM), but this biogeographic pattern has never been explored in depth for birds. The two biomes are separated by a broad diagonal of more xeric habitats comprising the Chaco, the Cerrado uplands, and the Caatinga (Ab'Saber 1977), and together they encompass one of the most diverse tropical forests of the world. There is much evidence to support past contact between the AF and the AM through this dry vegetation diagonal (Auler et al. 2004; Auler and Smart 2001; Behling et al. 2000; Costa 2003; Oliveira et al. 1999; Patton et al. 1997; Patton and Costa 1999; Vilela et al. 2009; Por 1992; Wang et al. 2004; Willis 1992). Palynological and geological data from these studies cover the late Pleistocene (the last 0.9 mya) and indicate that there were wetter periods that allowed the expansion of gallery forest into the Cerrado and Caatinga, providing connections between the AF and the AM (Auler et al. 2004; Auler and Smart 2001; Behling et al. 2000; Oliveira et al. 1999; Wang et al. 2004). Costa (2003) compared the phylogeographic patterns of small mammals in the AF and the AM and found lineages from either the AF or the AM in the Cerrado. Costa (2003) also observed that some taxa from northern AF are more closely related to AM taxa than to southern AF forms. Silva (1996) showed that several birds from the AM and the AF show distributions that extend along the Cerrado gallery forests. However, there are several species with disjunct distributions in these forested biomes (e.g., see Ridgely and Tudor 1994 for birds; see Vivo 1997 for mammals; see Martini

et al. 2007 for plants), and this reinforces the idea that the dry vegetation diagonal plays an important role in the connection of these biomes. Similarly, there is evidence from Africa supporting episodes of faunal connection across the “arid corridor” in eastern Africa between the main rainforest tracts and those near the Indian Ocean (the African Rift; Couvreur et al. 2008; Tolley et al. 2011). However, questions concerning the timing and nature of this connection are still to be conclusively answered. Such questions include: were the AF and AM biota connected just once in the past? Was the connection related solely to Quaternary climate changes? If not, when did this (these) connection(s) occur? Were there different pathways connecting AM and AF?

Possible explanations for the observed patterns could be extinction, dispersal, or vicariance (Crisci et al. 2003). A basic assumption of vicariance biogeography (as well as panbiogeography) is that generalized patterns can be detected by searching for concordance in area cladograms (or tracks) across multiple lineages. Although there are numerous known cases of different historical events generating similar patterns (e.g., Tuomisto 2007), there is still a widespread tendency to treat similar patterns as indications of common biogeographic events. Besides, distinct co-distributed taxa could present similar cladogenetic splits if speciation and/or diversification are driven by vicariance, as the distributions of the ancestor populations would have been affected in a similar way when the barriers to dispersal formed.

However, it is important to distinguish true congruence, where the taxa and areas share the same history (Cracraft 1985, 1988) from “pseudocongruence” (Cunningham and Collins 1994; Donoghue and Moore 2003), where cyclical processes (such as orbitally driven climatic/vegetational changes; Bennett 1990; Hooghiemstra et al. 1993) result in the repetition of historical events. This could be a repeated process of range expansion and retreat. We also need to consider whether entirely different biogeographic events, superimposed over millions of years, may in some cases result in patterns that we perceive as being congruent.

Recently, the integration of distributional and phylogenetic data has given robust insights into diversification processes and biogeographical patterns of tropical forests (Fjeldså et al. 2007; Fjeldså and Irestedt 2009; Fjeldså and Rahbek 2006; Roncal et al. 2011). This kind of approach connects ecological and historical biogeographies and gives insight into community assembly and species richness patterns by considering the biogeographical history of the species and clades that make up these patterns (Wiens and Donoghue 2004).

In the work described in this paper, we applied this approach to New World suboscine birds, a monophyletic group (Barker et al. 2004) with 1,200 extant species. This

group evolved within South America, but some subclades recently expanded to colonize Central and North America during the great American exchange (Smith and Klicka 2010; Weir et al. 2009). Several phylogenies for this group have been published (Derryberry et al. 2011; Irestedt et al. 2002, 2004, 2009; Moyle et al. 2009; Ohlson et al. 2008; Tello et al. 2009), which provide a large data source for biogeographic inferences.

In this paper, taxa with disjunct Amazonian/Atlantic forest distributions were examined to investigate whether such disjunctions are the result of a single (or at the very least a recurrent) range fragmentation event or entirely different events. This was done in two steps: first by examining the degree of genetic divergence between them, and second by examining their geographical distributions.

Thus, we aimed to combine phylogenetic data using a dated phylogeny with distributional data in order to try to depict the historical connection dynamics between AF and AM. We examined the variation in divergence time and then compared spatial distributions of taxon pairs representing old and recent divergences. This allowed us to address two main questions. (1) When and where did this (these) connection(s) happen? (2) Which historical events were responsible for the interchange of avifauna between the AF and the AM?

Materials and methods

Taxa selection and phylogenetic analyses

The first step was to identify sister groups (species or lineages) with disjunct distributions in the AF and the AM. This was done by performing a comprehensive search of the *Handbook of the Birds of the World* (del Hoyo et al. 2003, 2004), and utilizing information on species and population distributions as well as their putative evolutionary relationships with published phylogenetic information (Derryberry et al. 2011; Irestedt et al. 2002, 2004, 2009; Moyle et al. 2009; Ohlson et al. 2008; Tello et al. 2009). Thus, we selected only pairs of sister taxa with AF and AM distributions that had a well-supported phylogenetic grouping (including cases where an AF species is sister to a small group of species with an AM distribution). We also included, as recent splits, disjunctly distributed species whose populations in the AF and the AM are slightly differentiated morphologically, suggesting a recent (Plio-Pleistocene) split (see below). Unfortunately, no DNA sequence data on these populations are available, and we acknowledge that it would be desirable to use such data to estimate their divergence times. However, we decided to include these groups in our analyses with the assumption

that the current species-level taxonomy reflects relationships accurately. While there may be some exceptions where subspecies in the AM and the AF are genetically more divergent than their subtle morphological differences would suggest, but we find it unlikely that such divergences would be older than the 5.5 million years (Plio-Pleistocene period) that we regarded here as a recent split.

Other New World suboscine species were also included to help strengthen the support for some phylogenetic relationships (see Table S1 of the Electronic supplementary material, ESM). Based on Barker et al. (2004), the outgroups used were: *Pitta baudii*, *Smithornis rufolateralis*, *Corvus corone*, *Parula americana*, *Menura novaehollandiae*, *Acanthisitta chloris*, and *Pterocles namaqua* (Table S1 of the ESM).

The sequences of six genes for which data are available for many species of New World suboscines were downloaded from the GenBank/EMBI databases: one mitochondrial—cytb; three nuclear introns—myo2, G3PDH and FIB5; and two nuclear exons—RAG-1 and RAG-2 (Table S1 of the ESM). Thus, we selected 83 species (including outgroups) to reconstruct the phylogenetic relationships of New World suboscines from the AF and the AM (Table S1 of the ESM). Sequences were aligned using MUSCLE (Edgar 2004) in MEGA5 (Tamura et al. 2011). All alignments were inspected and corrected manually when necessary. The genes were concatenated using SeaView4 (Gouy et al. 2010), and this alignment contained 7,092 characters (the percentage of the data that are missing for each gene is shown in Table S2 of the ESM).

The best-fit substitution model for each partition was estimated using MrModeltest 2.2 (Nylander 2004) based on Akaike's information criterion (AIC). We used Bayesian and maximum likelihood inferences based on six partitions (Table S2 of the ESM) to reconstruct phylogenies using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) and RAxML (Stamatakis et al. 2008), respectively. Bayesian runs consisted of 17 million generations of two independent runs with four chains of Markov chain Monte Carlo (MCMC) each. The first one million generations were discarded as "burn-in," and the trees were sampled every 500 generations. The burn-in and convergence diagnostics were estimated using the PSRF (potential scale reduction factor), where values close to one indicate a good convergence between runs (Gelman and Rubin 1992). We also assessed the chain convergence by checking the posterior distribution plots of each run using Tracer 1.5 (<http://beast.bio.ed.ac.uk/Tracer>), verifying that the ESS values were >200. We carried out RAxML analysis in the CIPRES Science Gateway (Miller et al. 2010) using the same partitions of Bayesian inference under the GTRAC model; invariable sites and the gamma distribution were estimated

for each partition during the run. Node supports for the maximum likelihood analyses were estimated by performing 100 bootstrap replications.

Date estimates were obtained by implementing a Bayesian relaxed clock model in BEAST 1.6.1 (Drummond et al. 2006; Drummond and Rambaut 2007) based on the concatenated dataset. Two independent runs with 50 millions of generations were obtained using the following parameters: the Yule prior, a random starting tree, the GTR+I+ Γ model, parameters sampled every 1,000 steps, a burn-in of 10 %, and an assumption of an uncorrelated relaxed lognormal clock. We used the geological split between New Zealand and Antarctica as the calibration point, as this has been related to the basal separation of the *Acanthisitta* lineage from the other passerines (Barker et al. 2002, 2004; Ericson et al. 2002). Thus, we used a normally distributed tree prior with a mean of 78 mya and a standard deviation of 7, as used by Irestedt et al. (2009). To check the convergence of the parameters between runs, we used Tracer 1.5, verifying that the ESS values were >200. The consensus topology with the divergence times was visualized in FigTree 1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree>).

Distributional analysis

We used the bird distribution for South America that is digitalized in Worldmap (Williams 1998), which was generated from a comprehensive global distribution database developed at the Center for Macroecology, Evolution and Climate (January 2011 version). The resolution of the map used was $1^\circ \times 1^\circ$ quadrates (latitude $\times$ longitude), following the approach outlined in Brooks et al. (2001) and in Rahbek and Graves (2001). Maps represent a conservative source of extent-of-occurrence for the breeding ranges based on museum specimens, published sighting records, and spatial distribution of habitats of documented records (>1,000 references used), and have been validated by many experts.

To estimate the possible different pathways of contact between the AF and the AM, we selected species from the young and old divergences based on Bayesian dating. Ranges of young and old divergences assumed here were: young—comprising divergence during the Pliocene and Pleistocene (the last 5.5 million years); old—comprising the Miocene period (ranging from 5.6 to 23 million years ago). Thus, we generated species richness maps for young and old divergences.

In order to assess the degree of co-distribution between the richness maps of young and old splits, we applied a correlation test (ρ , ρ) between the maps, and this was visualized by overlaying a two-color scheme on the species distribution (Williams and Gaston 1998).

Results

Phylogenetic reconstruction and dating

Our supermatrix consisted of 7,092 characters and provided a robust dataset to estimate the phylogenetic relationships and to date New World subspecies divergences. The best-fit model estimated for each gene partition as well as the length of each gene are shown in Table S2 of the ESM. The phylogenetic reconstruction inferred by the Bayesian and maximum likelihood (Fig. S1 of the ESM) approaches were congruent and agreed with previous published phylogenies (Irestedt et al. 2002, 2004, 2009; Moyle et al. 2009; Ohlson et al. 2008; Tello et al. 2009). All splits between the AF and the AM identified based on the supermatrix topologies showed strong node support values.

The dated chronogram generated by BEAST revealed that the majority of the splits between pairs of sister groups investigated here occurred before the Plio-Pleistocene (Fig. S2 of the ESM). The only exceptions were the younger splits: *Mionectes rufiventris* versus *M. oleagineus* and *M. macconnelli* (4.85 mya); *Chiroxiphia caudata* versus *C. boliviana* (4.17 mya); and *Dendrocolaptes platyrostris* versus *D. picumnus* (4.2 mya). In addition, it was possible to identify very old splits, such as *Piprites pileata* versus *P. chloris* (25.76 mya) and *Psilorhamphus guttatus* versus *Liosceles thoracicus* (21.63 mya).

Species included in our Worldmap analyses that showed young divergences provided a good aid to understanding the dynamics of past connections between the AF and the AM, since no sequences are available for most of these populations in GenBank. Furthermore, phylogeographic studies of bird species distributed disjunctly in these biomes suggested divergence in the Pleistocene (Marks et al. 2002; Miller et al. 2008; Nyári 2007).

Spatiotemporal patterns

We selected a total of 33 and 29 taxa to build the richness maps for young and old splits, respectively (Table S3 of the ESM). These taxon pairs represent 32 young and 10 old splits between the AF and the AM (Fig. S2 and Table S3 of the ESM). The few species that were used in the dating analyses but not included in richness maps exhibited uncertain sister species relationships between the AF and the AM or low node support in the phylogenetic reconstruction.

The overall species richness maps for old and young splits showed a disparity in the AF taxa distribution (Fig. 1). Interestingly, nearly all AF species that originated from old splits occur in central and southern AF (Fig. 1a), whereas taxa that originated from recent splits occur

Fig. 1 Species richness variation maps of New World suboscines for disjunctions between the Atlantic Forest and the Amazon Basin. **a** Geographical distribution of 21 taxon pairs representing old divergences (Miocene: 5.6–23 mya) between these biomes.

b Geographical distribution of 37 taxon pairs representing young divergences (Plio-Pleistocene: the last 5.5 mya) between these biomes. Bars in both maps indicate the species richness for each pixel

predominantly in northern AF (Fig. 1b). Also, there is a contrast between the distributions of old and young taxa in the AM. Old taxa are mainly found in the western part of the basin and in the Guianas, and in a rather restricted area along the lower Amazon. In contrast, taxa representing younger splits are predominantly present in the lower AM at the north of Pará and in the eastern Amazonian states of Brazil (Fig. 1b). The correlation between maps was moderate ($\rho = 0.78$), but the two-color map shows a clear distinction in the distributions of young and old splits of AF taxa (Fig. 2). However, more green (young splits) is evident for the Caatinga and northern Cerrado, whereas there is a predominance of blue in southern Cerrado as well as in northern Chaco (Fig. 2).

A large number of old AF species, mostly from the cooler southern uplands, have corresponding sister species in the tropical Andes, mainly in Bolivia and Peru (not shown), and some species have disjunct populations in the Andes and the AF (see Ridgely and Tudor 1994). Therefore, these biomes may have been connected by two spatiotemporally distinct pathways, as observed here for the AF and the AM. Percequillo et al. (2011) also suggested a close relationship historically between the AF and the Andes for mammals.

Discussion

In this study, the use of a dated avian molecular phylogeny in combination with distributional data provided an evolutionary time perspective on the dynamics of historical connections between the AF and the AM. In concordance with previous studies, we found three putative pathways connecting the AF and the AM (Auler et al. 2004; Costa 2003; Oliveira et al. 1999; Por 1991; Wang et al. 2004). To our knowledge, this is the first study to present a general spatiotemporal model for the biogeographical connections between the two forest biomes based on bird data (Figs. 2, 3).

Our data show that the Quaternary climatic changes were important in the interchange of avifauna between these two biomes, and that geological events during the Miocene, such as Andean uplift (Hoorn et al. 2010) and associated changes in the axial groove to the east—the establishment of broad foreland basins and marine incursions (Cozzuol 1996; Haq et al. 1987; Paxton and Crampton 1995; Räsänen et al. 1995; Wesselingh et al. 2006; Wesselingh and Salo 2006), may also have played significant roles (see below). Cracraft (1985), in his definition of areas of avian endemism in South America, postulated that these areas are primarily historical

Fig. 2 Correlation map comparing geographic distributions for taxon pairs comprising old and young divergences, $\rho = 0.78$. The colors show the level of overlap: green shades represent young splits, blue shades represent old splits, and white represents completely overlapping distributions (color figure online)

entities rather than simply manifestations of a single, coherent ecology or consequences of climatic–vegetational changes during the Pleistocene. Fjeldså (1994) postulated that massive radiations of South American terrestrial biota occurred during the upper Tertiary, but Rull (2008) and Derryberry et al. (2011) documented a continuous rate of diversification during the Neogene.

In our study, species richness maps show clearly early and late AM/AF disjunctions. These events seem to represent the signatures of at least two distinct biogeographic connections (Figs. 1, 2): (1) the old connections (middle to late Miocene) between these two biomes appear to have existed along the southern margin of the modern Cerrado and in Mato Grosso, or on the transition towards the Chaco and palm savannas of Bolivia and Paraguay (Fig. 3); (2) the young connections (Pliocene to Pleistocene) possibly occurred through the Cerrado and Caatinga in northeastern Brazil (Fig. 3). Furthermore, there seem to be differences in the spatial occupations of young and old divergences within the AF, with the old ones restricted to the southern AF, upland forests at southern Bahia, Minas Gerais, Espírito Santo, Rio Janeiro, and northern São Paulo, and lowland forests in the south, while taxa representing young AF/AM divergences occur in lowland coastal forests from northern Rio de Janeiro to northeastern Brazil (Figs. 1, 2). This pattern also involves two climatic settings, as forests in southern Brazil are cooler than lowland forests in the

northern part of the AF. However, most of the species of the upland forests of southern Brazil have their nearest relatives in the Andes, as the bending of the Bolivian orocline (Chapare Buttress) made contact with the Brazilian Shield up until the Plio-Pleistocene, when the continuous crust thickening in the Bolivian Andes caused geological subsidence and the formation of hydrologically unstable savannah plains that now form a barrier between the Andean and South Brazilian forest faunas (Hanagarth 1993; Silva 1994).

The old pathway connecting the AF and the AM

Based on the species richness map comprising old divergences between the AF and the AM (Figs. 1a, 2), we hypothesize that an old pathway connected these biomes during much of the Miocene along the western edge of the Brazilian cratonic shield, from the northern modern Chaco in the state of Mato Grosso in Brazil, west to Bolivia and Paraguay, and from the state of Rondônia southeast to the state of Paraná (Brazil) (Fig. 3). During the Miocene, the axial groove of the western Amazon Basin expanded to become broad foreland basins characterized by marine and fresh water flux (7–23 mya; see Hoorn et al. 2010; Hoorn and Wesselingh 2010; Lundberg et al. 1998; Wesselingh et al. 2006; Wesselingh and Salo 2006), and there was also a shift in the position of the watershed between the Amazon and the Paraná Sea (Lundberg et al. 1998; Marshall et al. 1993; Marshall and Lundberg 1996; Roddaz et al. 2006). Despite controversies, these paleoseas could have been connected during the late Miocene through the Bolivian/Paraguayan Chaco (Hulka et al. 2006; Roddaz et al. 2006). In these scenarios, we can hypothesize that a pathway may have connected the AF and the AM along these paleoseas and the Chapare Buttress (Lundberg et al. 1998; Roddaz et al. 2006). This pathway could have originated via elevated channel belts (late Tertiary) through flooded savannas that may have provided dispersal pathways for terrestrial organisms (Wesselingh and Salo 2006). Besides, it is well known that the Andean uplift was crucial for the evolution of Amazonian landscapes (Hoorn et al. 2010; Hoorn and Wesselingh 2010), and played a key role in the diversification of organisms in adjacent regions. Also, molecular studies on mammals (Galewski et al. 2005) and lizards (Pellegrino et al. 2011) have postulated old connections (Miocene) between the AF and the AM.

All 19 lineages of New World suboscines that diverged in the mid-Oligocene are present in the AF (data not shown). This observation raises the possibility that many New World suboscine relict lineages originated within the AF, and that only during the Miocene did they colonize the AM. Also, Irestedt et al. (2002) suggested that tracheophone birds originated in southern AF.

Fig. 3 Representation of the hypothetical historical pathways connecting the Atlantic Forest and the Amazon Basin. *Double headed arrows* represent pathways. The *dark gray arrow* corresponds to old contacts, whereas the *black arrows* correspond to young contacts. The *broken black arrow* shows a less commonly observed young pathway, while the *continuous black arrow* represents a more commonly observed young pathway (see text for more details). These pathways

are supported by other studies that are not based on bird data: Andrade-Lima (1982), Auler et al. (2004), Costa (2003), Oliveira et al. (1999), Por (1992), and Wang et al. (2004). The dots in the Caatinga are localities in which there is evidence of the presence of gallery forest during the late Pleistocene (>0.9 mya), based on paleopalynological and speleothems (*triangle* Oliveira et al. 1999; *circle* Auler and Smart 2001 and Wang et al. 2004)

Furthermore, Por (1992) suggested a pathway connecting the AF and the AM through the Paraná River basin, and this is in accordance with our hypothesis. Costa (2003) also showed that this route could have been important for small mammals.

Young pathways connecting the AF and the AM

Our analyses suggest the presence of young pathways connecting the AF and the AM through current distributions in northern Cerrado and Caatinga (Fig. 3). Based on previous evidence (Auler et al. 2004; Costa 2003; Oliveira et al. 1999; Por 1992; Wang et al. 2004), we can also

suggest two connection pathways in northeastern Brazil: one through the coastal zones of Maranhão, Piauí, Ceará, and Rio Grande do Norte (Brazil), and another through Tocantins and Bahia (Brazil; Fig. 3).

The most northern young pathway is congruent with the presence of the “Brejos de Altitude” in this area. These “brejos” are relict rain forests enclaved in the Caatinga, and they represent current forest refuges (Andrade-Lima 1982). However, this pathway possibly played a minor role in the young AF/AM connections, as most of the birds with disjunct distributions between the AF and the AM included in our study do not occur in these areas in the state of Ceará. Albano and Girão (2008)

and Girão et al. (2007) only registered *Xiphorhynchus guttatus*, *Dendrocolaptes platyrostris*, *Rhynchocyclus olivaceus*, *Capsiempis flaveola*, *Attila spadiceus*, *Myrmotherula axillaris*, and *Xenops minutus* in these areas. In addition, Cavalcanti and Tabarelli (2004) showed that “brejos” and lowland Atlantic forest from northeastern Brazil are distinct phytogeographical units. Nevertheless, it is conceivable that these “brejos” intermittently constituted suitable habitats during the Plio-Pleistocene for most species with AF/AM disjunctions, which have since gone extinct in these areas.

Some Plio-Pleistocene connections may have also existed further south during contractions and retractions of gallery forests during the Quaternary climate episodes. Such pathways are supported by paleopalynological and geological studies (Auler et al. 2004; Auler and Smart 2001; Oliveira et al. 1999; Wang et al. 2004) indicating the presence of gallery forest in the Caatinga in Bahia (Brazil) during the late Pleistocene (>0.9 mya; Fig. 3).

There are few molecular studies on the AF/AM disjunction, but their results are in accordance with the data shown here. Costa (2003) pointed out that some small mammals that occur in the AF and the AM diverged during the Pleistocene. Also, Cabanne et al. (2008) and Weir and Price (2011) suggested that woodcreepers species endemic to the AF diverged from its Amazonian sister species or group during the Pliocene ($\cong 3$ mya), and that the connection between these biomes possibly occurred through northeastern Brazil. However, Martins et al. (2009) observed that AF/AM populations of a vampire bat diverged in the Pleistocene, following the expansion of dry habitats between these two biomes.

These results have allowed us to propose the first general temporal and spatial model of the historical connections between the AF and the AM. Future historical demographic and phylogeographic studies of organisms with disjunct distributions in these biomes would provide fine-scale tests of this model, and uncover further complexities regarding the nature of the biogeographic connections between the two biomes that are not readily apparent in our study.

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