

Rafael Dantas Lima

**Geographic variation, population structure, behavioral discrimination, and taxonomy of the most polytypic antbirds (Aves, Thamnophilidae)**

**Variação geográfica, estrutura populacional, discriminação comportamental e taxonomia das chocas mais politípicas (Aves, Thamnophilidae)**

São Paulo

2024

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Dissertation presented to the Institute of Biosciences of the University of São Paulo as a requirement for the acquisition of a Master's Degree in Biological Sciences (Zoology).

Supervisor: Prof. Luís Fábio Silveira

São Paulo

2024

## Ficha Catalográfica

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Lima, Rafael

Geographic variation, population structure, behavioral discrimination, and taxonomy of the most polytypic antbirds (Aves, Thamnophilidae)

269 páginas

Dissertação (Mestrado) - Instituto de Biociências da Universidade de São Paulo. Departamento de Zoologia.

1. Systematics 2. Species limits 3. Neotropics I. Universidade de São Paulo. Instituto de Biociências. Departamento de Zoologia.

Comissão Julgadora:

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Prof(a). Dr(a).

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Prof(a). Dr(a).

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Prof(a). Dr.(a).

Orientador(a)

*“Those forms which possess in some considerable degree the character of species, but which are so closely similar to some other forms, or are so closely linked to them by intermediate gradations, that naturalists do not like to rank them as distinct species, are in several respects the most important to us.”*

(C. R. Darwin, 1859)

*“It is no exaggeration to say that if no instances of uncompleted speciation were discovered we would have to conclude either that evolution did not occur or that the formation of new species is instantaneous. What is a difficulty to the cataloguing systematist is a blessing to the evolutionist.”*

(T. H. Dobzhansky, 1958)

*“We must not expect too much of the term species. In the first place, we must not expect a hard-and-fast definition, for since most evolution is a gradual process, borderline cases must occur. And in the second place, we must not expect a single or a simple basis for definition, since species arise in many different ways.”*

(J. Huxley, 1942)

*“For certain purposes names, and discontinuous categories, are exactly what we need. [...] We often feel more comfortable if we can use separate names for things when we talk about them.”*

(R. Dawkins, 2004)

## Acknowledgements

I thank my supervisor, Luís Fábio Silveira, for providing the means for this work to be carried out, and for granting me the freedom to pursue my interests.

I am grateful to Fernanda Bocalini and Sergio Bolívar-Leguizamón for their constant assistance in generating the genetic data used in this work. I also greatly appreciate my labmates Natália Luchetti, Ana B. Navarro, Ruan Vaz, Maria Clara Irumé, and Rafael S. Nascimento for their help with various tasks. Foremost, I greatly appreciate their friendship and constructive discussions.

I thank Diego Cueva, Darwin Morales-Martínez, Laura Sepulveda, Orlando Carrillo-Bohórquez, and Samantha Rutledge for kindly hosting me during my stay in Baton Rouge. I am also very grateful to Robb Brumfield, Diego Ocampo, Andre Moncrieff, Anna Hiller, Eamon Corbett, Roberta Canton, Jackson Roberts, Rafet Öztürk, Sheila Rodríguez-Machado, Heru Handika, and Quinn McCallum for their assistance with lab work, technical advice, and car rides, all of which together made my stay in Baton Rouge very pleasant. Special thanks to Darwin Morales-Martínez and Diego Ocampo for their extensive help with lab work and many other tasks. For helpful tips about New York, I thank Rafaela L. Vendramel.

Rafael S. Marcondes shared important unpublished genetic data. Paul Smith provided relevant information about contact zones of Variable Antshrike subspecies in Paraguay, and Gustavo Malacco about sample collection sites in Minas Gerais and Goiás. Vítor Q. Piacentini, Barbara M. Tomotani, and Fábio R. Amaral kindly wrote recommendation letters supporting funding applications, which were essential for completing this project. Taran Grant kindly provided access to his lab's high performance computing cluster. Almir Almeida and Airton Cruz digitized the illustrations used in this work. I also thank Luís F. Silveira, Ana B. Navarro, Kelsie A. Lopez, Igor Alvarenga, and Alexandre M. Fernandes for their help and company during fieldwork.

This study would have been impossible to complete without the efforts of numerous individuals who collected study skins, tissue samples, and sound recordings. For taking care of and facilitating access to samples, I thank Helder Araujo, Taís Costa (Coleção de Aves Heretiano Zenaide), Lincoln Carneiro (Museu Paraense Emílio Goeldi), Marcelo Vasconcelos (Museu de Ciências Naturais da Pontifícia Universidade Católica de Minas Gerais), Fernando Perini, Marina Lodi, Leonardo Marujo (Coleção Ornitológica da Universidade Federal de Minas Gerais), Marcos Raposo, Alexia Granado, Tomás Capdevile (Museu Nacional), Nicholas Mason, Robb Brumfield, Steve Cardiff, Donna Dittmann (Louisiana State University Museum of Natural Science), Joel Cracraft, Paul Sweet, Peter Capainolo, Thomas Trombone, Eduardo Schultz, Augie Kramer (American Museum of Natural History), Henrique Batalha-Filho (Universidade Federal da Bahia), Fabrício Santos, Ana Cristina Lara (Universidade Federal de Minas Gerais), Vítor Piacentini (Universidade Federal de Mato Grosso), Mark B. Robbins (Kansas University Natural History Museum), Ulf Johansson (Swedish Museum of Natural History), Vanessa Powell, Jay McGowan (Macaulay Library), Bob Planqué, Willem-Pier Vellinga (xeno-canto), Luiz Pedreira Gonzaga (Arquivo Sonoro Elias Coelho), Simone Dena (Fonoteca Neotropical Jacques Vielliard), Reinaldo Guedes, and Wagner Nogueira (WikiAves). I am also grateful to Gerald Mayr, Dagmar Baumann (Senckenberg Museum), Hans-Martin Berg (Naturhistorisches Museum Wien), Serina Brady (Carnegie Museum of Natural History), Andrew Kratter (Florida Museum of Natural History),

Miguel Lentino (Colección Ornitológica Phelps), and Dnilson Ferraz (Museu Paraense Emílio Goeldi) for sending me photos of relevant specimens.

This work was primarily supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grants no. 2021/10339-1 and 2022/13077-0) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES 88887.659085/2021-00) through graduate research fellowships to myself. Fieldwork, travels to visit museum collections, and DNA extraction and sequencing were largely funded by a FAPESP thematic project (17/23548-2) to L. F. Silveira, four travel grants from my graduate program (CAPES-PROEX funds provided by the IB-USP), and two grants from the American Museum of Natural History to myself.

Finally, I would like to thank my family for their extensive support in numerous ways during my studies.

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### **Coauthorship Statement**

The research presented in Chapters 2 and 3 was conducted collaboratively. Therefore, I use plural pronouns (we/our) throughout these sections. Any errors, however, are my own.



# CHAPTER 1

## General Introduction

Accurate taxonomy is essential to a wide range of biological subdisciplines. The names we place on perceived discontinuities in geographic phenotypic and genotypic variation provide working hypotheses for much biological research and significantly shape our perceptions of biological diversity (Isaac et al. 2004). Phenotypic and genotypic discontinuities at the population level are taxonomically categorized into species and subspecies taxa (ICZN 1999), both of which play important roles in biological research. For example, biodiversity is commonly measured in terms of species richness (Magurran 2004), and such measures form the basis of major models in ecology (Gotelli and Colwell 2001, Gray and Cavers 2014). Similarly, subspecies taxa and the partitioned geographic variation that they represent have been successfully used in the study and description of important biological patterns and processes, such as areas of endemism (Cracraft 1985), speciation (Mayr 1963, Wake 1997), and adaptive evolution (Patten et al. 2004, Manceau et al. 2011, Clark et al. 2022). In addition to their broad utility in biological research, biodiversity management and conservation efforts are also often highly contingent on species and subspecies labels (Mace 2004, Agapow et al. 2004, Haig et al. 2006, Zink and Klicka 2022).

However, most species and subspecies were described before the establishment of modern criteria for their delimitation (Patten and Unitt 2002, Remsen 2005, 2010), and despite their critical role and widespread use in biology, many unresolved taxa persist even among the best-known groups of organisms such as birds (Lees et al. 2022). This problem is especially acute in tropical regions, where progress in taxonomy lags far behind that in temperate regions, creating a latitudinal taxonomy gradient that hinders our understanding of biodiversity (Freeman and Pennell 2021). In addition, taxonomic uncertainty is much more pervasive in the subspecies than in the species category. Currently accepted avian subspecies taxa comprise a variety of phenomena ranging from good biological species that are not recognized as such due to shortfalls in taxonomic knowledge (Freeman and Pennell 2021), to separately evolving or incompletely separated lineages that do merit taxonomic recognition as species or subspecies, to entirely different phenomena that are not worthy of taxonomic recognition, such as morphs, names based on phenotypes that represent individual variation rather than lineage differences, or arbitrary divisions of gradual clines (Lanyon 1982, Patten and Unitt 2002). Taxonomic revision is therefore badly needed.

Antbirds (family *Thamnophilidae*) stand out among birds that have undergone extensive taxonomic revision in recent decades. Previous classifications recognized 176 species of antbirds (Peters 1951), and now 237 are recognized by most authoritative taxonomic references (Clements et al. 2022, Gill et al. 2023). Most of this increase was due not to the description of newly discovered species but to the realization that differences in vocalizations are major proxies of species-level divergence in the family (Isler et al. 1998, Remsen 2005). Although the importance of vocalizations in bird species delimitation has long been recognized (Mayr 1963, Payne 1986), difficulties in obtaining and analyzing sound recordings precluded comprehensive analyses of acoustic trait variation in these birds in the past, and so their classification was traditionally based on analyses of morphological variation, with many morphologically similar antbird taxa being lumped into broadly defined species taxa (Cory and Hellmayr 1924, Peters 1951). As obtaining, storing, and analyzing bird sound recordings became feasible, numerous morphologically similar antbird taxa formerly recognized as conspecifics have proven to differ markedly in vocalizations and thus be

best classified as separate biological species (Pierpont and Fitzpatrick 1983, Willis 1988, Bierregaard et al. 1997, Isler et al. 1997, 1999, 2001a, b, 2002, 2007a, b, 2008, 2009, 2012; Whitney et al. 2000, Isler and Isler 2003, Braun et al. 2005, Isler and Whitney 2011, 2017; Mayer et al. 2014, Isler and Maldonado-Coelho 2017). The converse has also happened, with antbird taxa that were originally described as species being later reclassified as subspecies based on new vocal evidence (e.g., Isler and Whitney 2018). In multiple different ways, therefore, research on vocalizations has significantly changed antbird taxonomy. Additionally, several new species and subspecies of antbirds continue to be described (Gonzaga et al. 2007, Donegan 2012, Whitney and Cohn-Haft 2013, Whitney et al. 2013b, a; Buzzetti et al. 2014, Moncrieff et al. 2018, Lima et al. 2024), indicating that the diversity in the family remains incompletely documented.

Taxonomic practice is constantly changing to accommodate advances in understanding speciation, and recent research has added a new variable to antbird taxonomy that may usher in a new era of substantial revision of species limits in this group and, perhaps, birds broadly. Present taxonomic practice places much emphasis on premating reproductive isolation (Remsen 2005, Gill 2014). Remsen's (2005) assertion that "clearly, premating isolating mechanisms provide the primary barrier to gene flow in birds" epitomizes the prevailing opinion in avian systematics (Price 2008, Winker 2009, Gill 2014) and vertebrate speciation research in general (Kirkpatrick and Ravigné 2002, Coyne and Orr 2004). However, our understanding of bird speciation has refined substantially in recent years, and taxonomic practice has been changing accordingly (Winker 2021). Because premating isolating mechanisms were thought to be an essential component of reproductive isolation in birds, evidence of extensive hybridization at contact zones—or, in other words, evidence of weak premating isolation—was seen as evidence of weak overall reproductive isolation and so a clear indicator of a single biological species until recently (Remsen 2010, 2016). While premating isolation unquestionably plays an important role in bird speciation (Price 2008, Uy et al. 2018), recent work has shown that assortative mating alone is a very porous and often ineffective form of reproductive isolation (Pulido-Santacruz et al. 2018, Irwin 2020). There is a growing realization that other forms of reproductive isolation, such as postzygotic genetic incompatibilities and selection against hybrids, may effectively act to maintain the genotypic integrity of hybridizing lineages even in the presence of extensive hybridization (reviewed in Winker 2021, Anderson et al. 2023). Consequently, evidence for even extensive hybridization at contact zones has no longer been seen as an automatic indication of weak reproductive isolation (Brelsford and Irwin 2009, Toews et al. 2011, Walsh et al. 2020). Regarding antbird taxonomy in particular, recent genetic research on antbird hybrid zones has suggested that postzygotic isolating mechanisms may be a key component of antbird speciation, perhaps even more important than premating isolation mediated by differences in vocalizations (Pulido-Santacruz et al. 2018, Cronemberger et al. 2020). The finding that vocally distinct antbirds—which have been classified as separate biological species on the basis of acoustic trait differences—do hybridize where they come into contact indicates that premating isolation mediated by acoustic trait differences is not as strong as previously thought (Pulido-Santacruz et al. 2018, Cronemberger et al. 2020). Importantly, although prezygotic isolation appears to be weak between those vocally distinct antbird pairs, they evolved substantial genetic incompatibilities and form hybrid zones that are significantly narrower than expected under neutral dynamics (Pulido-Santacruz et al. 2018, Cronemberger et al. 2020). Taken together, these findings suggest that postzygotic isolating mechanisms in the form of genetic incompatibilities and strong selection against hybrids, rather than premating isolating barriers, may be the major responsible for the maintenance of species boundaries in antbirds (Pulido-Santacruz et al. 2018, Cronemberger et al. 2020). Although

hybridization occurs (prezygotic isolation is weak), introgression is negligible (postzygotic isolation is strong). The evidence suggests, therefore, that these antbirds have been correctly classified as separate biological species, but for the wrong reason. While the generality of this putative preeminence of postzygotic isolating barriers needs to be further investigated, it is possible that the population-level taxonomy of antbirds will enter a new era of substantial revision.

Regardless of the relative importance of premating and postzygotic isolation in antbird speciation, it seems clear that vocal and genetic divergence, rather than morphological or plumage trait differences, are the major proxies for species-level divergence in antbirds, and with this realization comes the need to reevaluate species limits in antbird taxa whose classification remains based on morphological evidence alone. Several antbird taxa remain whose vocal and genetic variation have never been thoroughly characterized, and whose classification remains largely or entirely based on early analyses of variation in morphological or plumage traits. In this context, the Plain Antvireo (*Dysithamnus mentalis*) and the Variable Antshrike (*Thamnophilus caerulescens*) stand out as prime candidates for taxonomic revision. They have unusually great amounts of geographic plumage variation (Marcondes and Brumfield 2020), reflected in as many as eighteen and twelve described subspecies, respectively (Cory and Hellmayr 1924, Zimmer and Isler 2003). They have each variably been treated as comprising either a single or multiple species taxa (Todd 1916, Cory and Hellmayr 1924, Zimmer and Isler 2003), and it is presently unclear to what extent their differentiated populations exist as discrete clusters versus as continua, with subspecies boundaries within both species complexes being admittedly tentative (Zimmer and Isler 2003, Dickinson and Christidis 2014). Importantly, despite the importance of vocalizations in antbird taxonomy, vocal variation has never been characterized across the entire range of both complexes. In addition, recent genetic work documented sizable genome-wide divergence between some populations (Bolívar-Leguizamón et al. 2020, 2024), though incomplete geographic sampling has precluded inferences about species limits.

In my two research chapters, I combine morphological, vocal, and genomic data to characterize geographic variation and population genetic structure and resolve species and subspecies limits in the Plain Antvireo and Variable Antshrike. My primary goals were to investigate the pattern and nature of phenotypic and genotypic variation in these two species complexes and to interrogate whether current taxonomic hypotheses accurately describe this variation. In other words, I asked: Does phenotypic and genotypic variation in the Plain Antvireo and Variable Antshrike form discrete clusters or do they exist as gradual clines? Are these two antbirds as polytypic as currently accepted in the literature or do part of their subspecies taxa reflect misapplications of the subspecies concept or artifacts of incomplete sampling? And might any of their differentiated populations be best treated as separate biological species? To answer these questions, I first examined morphological and plumage trait variation in study skins to test whether character differences originally used to describe all taxa are indeed discrete. I then used recordings of vocalizations to measure acoustic trait divergence among populations and conducted field playback experiments simulating secondary contact between vocally differentiated populations to assess the significance of vocal differences as a potential premating reproductive barrier. Finally, I used genome-wide genetic markers to measure population genetic structure and admixture across the ranges of the two species complexes.

My results significantly improve our understanding of the geographic variation and population structure of the Plain Antvireo and Variable Antshrike, and provide novel working hypotheses for downstream science. In the Plain Antvireo, I found that phenotypic and genotypic

variation do form several discrete or nearly discrete clusters, but several of the currently recognized subspecies taxa reflect misapplications of the subspecies concept. My results suggest that reproductive isolation ranges from weak to moderate at most between most Plain Antvireo populations, and although some populations within the “gray zone” of speciation may warrant recognition as separate species upon further study of newly identified contact zones, current evidence largely supports their continued treatment as conspecific. In the Variable Antshrike, my results reveal two new taxa and establish that one population has probably evolved substantial reproductive isolation from all others in the complex. Most of the remaining Variable Antshrike populations exhibited very low levels of reproductive isolation and are therefore kept as members of a single species, although some populations exhibiting sizable genome-wide divergence may also prove to merit species status upon further study of newly identified contact zones.

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## General abstract

Species and subspecies taxa are foundational to much biological research, making accurate taxonomy crucial across various biological disciplines. Many existing taxa, however, predate modern standards, underscoring the critical need for taxonomic revision. The speciose avian family *Thamnophilidae* has seen considerable taxonomic progress, driven largely by new insights into traits that indicate lineage divergence and affect reproductive isolation. Despite these advancements, many thamnophilid taxa remain that have not yet been thoroughly revised in light of these insights. The Plain Antvireo (*Dysithamnus mentalis*) and Variable Antshrike (*Thamnophilus caerulescens*) stand out as prime candidates for taxonomic revision within the family. They have unusually great amounts of geographic plumage variation, reflected in as many as eighteen and twelve described subspecies, respectively. They have each variably been treated as comprising either a single or multiple species taxa, and it is presently unclear to what extent their differentiated populations exist as discrete clusters versus as continua. Critically, despite the recognized importance of premating isolation mediated by divergence in vocalizations and postmating isolation in thamnophilid speciation, comprehensive characterizations of vocal variation and genetic structure across their entire ranges are lacking. Here, I combined morphological, vocal, and genomic data to characterize geographic variation and population genetic structure in the Plain Antvireo and Variable Antshrike. For *D. mentalis*, I identified 12–14 phenotypically diagnosable populations forming six distinct nuclear genomic clusters. In contrast with its geographically structured phenotype and nuclear genome, the *D. mentalis* complex exhibits no geographic structuring of mitochondrial haplotypes. My analysis also uncovered several previously unrecognized hybrid zones and suggest that premating reproductive isolation is generally weak or nonexistent among most lineages, thus largely supporting their continued classification as a single biological species. For *T. caerulescens*, I found nine phenotypically distinct populations, including seven previously recognized taxa and two newly described ones. My results establish that one lineage (*T. c. cearensis*) has evolved substantial reproductive isolation from all others in the complex and is thus best treated as a separate biological species. The remaining populations of the Variable Antshrike showed low levels of reproductive isolation and are therefore kept as members of a single species, although some show genome-wide divergence that could justify future species status upon further investigation of newly identified contact zones. In general, my results significantly advance our understanding of the geographic variation and population structure of the *D. mentalis* and *T. caerulescens* complexes and provide novel working hypotheses for downstream science.

## Resumo geral

Táxons no nível de espécie e subespécie são fundamentais para muitas pesquisas na biologia, de modo que uma taxonomia precisa é crucial para várias disciplinas biológicas. Muitos táxons existentes, entretanto, foram descritos antes do estabelecimento dos critérios atualmente aceitos para o seu reconhecimento, ressaltando assim a necessidade da revisão taxonômica. A família Thamnophilidae tem passado por considerável progresso taxonômico, impulsionado em grande parte por um entendimento refinado sobre os caracteres que melhor indicam divergência de linhagens e isolamento reprodutivo. Apesar destes avanços, ainda existem muitos táxons de thamnofilídeos que não foram revistos à luz deste entendimento refinado sobre a diversificação da família Thamnophilidae. A choquinha-lisa (*Dysithamnus mentalis*) e a choca-da-mata (*Thamnophilus caerulescens*) destacam-se como excelentes candidatos à revisão taxonômica. Ambos apresentam uma quantidade impressionante de variação geográfica na plumagem, que se reflete em respectivamente dezoito e doze subespécies descritas. Ambos foram historicamente tratados como compreendendo uma ou várias espécies, e atualmente não se sabe até que ponto suas populações diferenciadas existem como agrupamentos nitidamente separados ou contínuos de variação que foram categorizados arbitrariamente. Apesar da reconhecida importância do isolamento pré-zigótico mediado por divergência em vocalizações e do isolamento pós-zigótico na especiação dos thamnofilídeos, faltam caracterizações abrangentes da variação vocal e da estrutura genética em toda a distribuição geográfica da choquinha-lisa e da choca-da-mata. Aqui, eu integrei dados morfológicos, vocais e genômicos para caracterizar a variação geográfica e a estrutura genética populacional da choquinha-lisa e da choca-da-mata. Em *D. mentalis*, eu identifiquei 12–14 populações fenotipicamente distintas que formam seis agrupamentos genômicos nucleares. Em contraste com seu fenótipo e genoma nuclear geograficamente estruturados, o complexo *D. mentalis* não apresenta estruturação geográfica de haplótipos mitocondriais. A minha análise também revelou várias zonas híbridas e sugere que o isolamento reprodutivo pré-zigótico é no geral fraco ou inexistente entre a maioria das linhagens, apoiando assim em grande medida a classificação do complexo como uma única espécie biológica. Em *T. caerulescens*, eu encontrei nove populações fenotipicamente distintas, incluindo sete táxons previamente reconhecidos e dois descritos aqui. Meus resultados estabelecem que uma linhagem do complexo, *T. c. cearensis*, é substancialmente isolada reprodutivamente de todas as outras e, portanto, é melhor tratada como uma espécie biológica separada. As demais populações do complexo mostraram baixos níveis de isolamento reprodutivo e são, portanto, mantidas como membros de uma única espécie. Algumas apresentam níveis substanciais de divergência genética que pode justificar o reconhecimento como espécies separadas após uma investigação mais aprofundada das zonas de contato aqui identificadas. No geral, meus resultados avançam significativamente a nossa compreensão da variação geográfica e estrutura populacional dos complexos *D. mentalis* e *T. caerulescens* e fornecem novas hipóteses para a ciência a jusante.