

UNIVERSIDADE DE SÃO PAULO
FFCLRP - DEPARTAMENTO DE BIOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENTOMOLOGIA

**Spatial history and genetic-morphological variation of populations of
Belostoma angustum Lauck, 1964 (Heteroptera: Belostomatidae)
throughout Pampas highlands in Rio Grande do Sul, Brazil**

**História espacial e variabilidade genético-morfológica de populações de
Belostoma angustum Lauck, 1964 (Heteroptera: Belostomatidae) nas
serranias pampianas do Rio Grande do Sul, Brasil**

FABIANO STEFANELLO

Dissertação apresentada à Faculdade de
Filosofia, Ciências e Letras de Ribeirão Preto
da USP, como parte das exigências para a
obtenção do título de Mestre em Ciências,
Área: Entomologia

RIBEIRÃO PRETO - SP

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Orientador: Eduardo Andrade Botelho de Almeida

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Stefanello, Fabiano

Spatial history and genetic-morphological variation of populations of *Belostoma angustum* Lauck, 1964 (Heteroptera: Belostomatidae) throughout Pampas highlands in Rio Grande do Sul, Brazil. Ribeirão Preto, 2017.

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43 f.

Dissertação de Mestrado, apresentada à Faculdade Filosofia, Ciências e Letras de Ribeirão Preto/USP. Área de concentração: Entomologia.

Orientador: Almeida, Eduardo Andrade Botelho de.

1. Population genetics. 2. Phenotypic variation. 3. Demographic history. 4. Water bug.

1. Genética de populações. 2. Variação fenotípica. 3. História demográfica. 4. Barata d' água

Agradecimentos

Ao Programa de Pós-Graduação em Entomologia e ao Departamento de Biologia da Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto por disponibilizarem toda a infraestrutura necessária para a realização deste trabalho.

Ao *Department of Ecology and Genetics, Uppsala University*, por me receber e oferecer toda a infra-estrutura necessária para a execução do estágio de pesquisa no exterior.

À FAPESP pela bolsa de mestrado (proc. #2015/07166-7) e bolsa de estágio de pesquisa no exterior (BEPE, proc. #2016/12720-6). A pesquisa aqui realizada foi beneficiada também pelo projeto Auxílio a Jovens Pesquisadores, concedido ao Dr. Eduardo A. B. Almeida (proc. FAPESP #2011/09477-9).

Ao CNPq pela bolsa durante os primeiros meses do mestrado (proc. #130128/2015-5).

Ao orientador e amigo, Dr. Eduardo A. B. Almeida por ter aceito a minha proposta de mestrado mesmo não sendo o grupo alvo de sua pesquisa. Agradeço por todas as discussões diretamente ligadas a este projeto e a respeito de ontologia e epistemologia.

Ao Dr. José Ricardo I. Ribeiro, meu orientador durante a minha iniciação científica, por todo o apoio dado a realização deste trabalho desde o princípio, e constantes discussões a respeito dos métodos empregados e resultados obtidos neste trabalho.

Aos docentes do Programa de Pós-Graduação em Entomologia pelos ensinamentos, conselhos e discussões ao longo das disciplinas de mestrado.

Ao Dr. Göran Arnqvist por ter me recebido durante o estágio em Uppsala, Suécia. Agradeço imensamente essa oportunidade para discussão a respeito do desenvolvimento deste trabalho, principalmente sobre o estudo morfológico.

Aos colegas do 'The Arnqvist Lab' em Uppsala, Johanna Liljestrand-Rönn, David Berger, Ahmed Sayadi, Rafael Augusto, Helen Bayram, Ivan Martinossi-Allibert, Karl Grieshop e Zorana Kurbalija Novicic pelas críticas e sugestões a este projeto e pelo companheirismo em todos os memoráveis dias frios e escuros do inverno Escandinavo.

Ao Dr. Marco A. T. Marinho e Dr. Rodolpho S. T. Menezes por todo o aprendizado acerca dos procedimentos moleculares e filogeográficos durante a execução deste trabalho.

Aos colegas pós-graduandos do Laboratório de Biologia Comparada e Abelhas (LBCA), Daercio A. A. Lucena, Diego S. Porto, Fábio G. L. Oliveira, Stephania S. Arango e os Drs. Fábio B. Quinteiro e Anne M. Costa pela amizade e longas discussões sobre diversos temas, pelos bons momentos vivenciados, além das críticas e sugestões para este trabalho.

Aos companheiros do Laboratório de Morfologia e Evolução de Diptera, Caleb C. Martins, Diego A. Fachin, Gabriela Pirani, Maria Isabel Balbi pela amizade e todos os bons momentos vivenciados durante esse período.

Aos colegas e amigos do Programa de Pós-Graduação em Entomologia integrantes da comissão organizadora do Curso de Verão em Entomologia com quem tive a satisfação de compartilhar responsabilidades.

Ao Dr. Rodrigo A. S. Pereira e à Dra. Renata Brandt Nunes pelas críticas e sugestões durante o acompanhamento do relatório parcial referente ao projeto de mestrado.

Ao Dr. Tiago G. dos Santos e à Dra. Márcia R. Spies por apoiarem e estimularem a minha vinda para Ribeirão Preto, além de todas as informações básicas a respeito da cidade e da Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto.

Ao amigo Vitor F. Oliveira por todo o auxílio durante a expedição de coleta pela região do Pampa e fixação em campo dos espécimes coletados.

Aos amigos gaúchos que mesmo longe seguimos com longas conversas proporcionando muitos momentos felizes.

À minha filha Sofia, um lindo presente ganho durante o mestrado, e a sua adorável mãe que entende a minha ausência frequente devido aos compromissos acadêmicos. Agradeço por estar ao meu lado, pelo seu amor e dedicação a nossa pequena família.

Aos meus queridos pais, Flávio J. Stefanello e Dineva M. P. Stefanello, e irmã, Ana Flávia Stefanello, por todo o carinho, constante apoio, dedicação e confiança depositados em mim. Muito obrigado!

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ABSTRACT

We investigated the population dynamics of the giant water bug, *Belostoma angustum*, across highland sin the Pampas of southern Brazil. We evaluated genetic and morphological variation, as well as the demographic history of 18 populations. The overall range includes two highlands and a lowland between them, overall exceeding 400 kilometers along the longitudinal gradient. Genetic variation was assessed from mitochondrial and nuclear markers. The morphological variation was estimated using linear measurements of males and females, and from male genitalic structures using geometric morphometrics approaches. We evaluated the effect of the highland topography, drainage basins, and past climate changes on the population structure. Our results from multiple analyses of molecular variance (AMOVA) show that *Belostoma angustum* structures as a large panmictic population across the Pampas highlands range. Every most frequent haplotype is shared by individuals from all three sampled areas in genetic markers from the mitochondrial as well as from the nuclear locus. Differentiation among haplotypes was very low, not greater than two mutation steps. The congruent phylogeographical pattern in both markers indicate absent sex-biased migration rates. Furthermore, there was no evidence for isolation-by-distance (IBD) based on the mitochondrial data. The pairwise Φ_{st} was low and not significant, indicating historical gene flow among populations of the giant water bug studied throughout Pampas highlands. Our findings about the demographic history of panmictic population throughout Pampas highlands suggest it experienced recent and rapid population expansion that started in the Late Pleistocene period, approximately 15,000 years old after Last Glacial Maximum. The recent marked demographic expansion could explain the high percentage of the exclusive haplotypes and the very low mutational steps among them. We did not find morphological variation among populations of *B. angustum* throughout Pampas highlands reflected, except for some body dimensions. The overall phenotypic uniformity among populations becomes more likely if gene flow is hypothesized to homogenize populations. However, in the body size, specially, there was variation among populations potentially explained by phenotypic plasticity, thereby generating phenotypic diversity without genetic differentiation. Our genetic findings suggest indirectly that individuals of *B. angustum* are strong fliers able to overcome the topographical barriers of the sampled area.

RESUMO

Neste trabalho, investigamos a dinâmica populacional de uma barata d' água, *Belostoma angustum*, ao longo do Pampa no sul do Brasil. Foram avaliadas a variação genética, morfológica e a história demográfica de 18 populações. A área total amostrada inclui duas serranias e uma planície entre elas, excedendo 400 quilômetros ao longo de um gradiente longitudinal. A variação genética foi avaliada a partir de marcadores mitocondriais e nucleares. A variação morfológica foi avaliada utilizando medidas lineares de machos e fêmeas, e de estruturas genitais masculinas usando abordagens de morfometria geométrica. Testamos o efeito da topografia das serranias, das bacias de drenagem e das mudanças climáticas passadas sobre a estrutura das populações. Os resultados de múltiplas análises de variância molecular (AMOVA) mostram que *Belostoma angustum* forma uma grande população panmítica ao longo das serranias do Pampa. Todos os haplótipos mais frequentes são compartilhados por indivíduos de todas as três grandes áreas amostradas em marcadores genéticos dos loci mitocondrial e nuclear. A diferenciação entre haplótipos foi muito baixa, não excedendo dois passos mutacionais. O padrão filogeográfico congruente em ambos os marcadores indica taxas de migração não enviesada para um dos sexos. Além disso, não encontramos evidência de isolamento por distância (IBD) com base nos dados mitocondriais. Os valores de Φ_{st} par a par foram baixos e não significativos, indicando fluxo gênico histórico entre as populações da barata d'água estudada ao longo das serranias Pampianas. Os resultados sobre a história demográfica da população panmítica ao longo do planalto do Pampa sugerem que essa população experimentou uma expansão populacional recente e rápida que teve início no fim do período Pleistoceno (há aproximadamente 15.000 anos), após a última máxima glacial. A expansão demográfica recente e acentuada poderia explicar a alta porcentagem de haplótipos exclusivos e o número reduzido de passos mutacionais entre eles. Não encontramos variação morfológica entre as populações de *B. angustum* amostradas ao longo das serranias do Pampa, exceto em algumas dimensões corporais. A uniformidade fenotípica entre as populações torna-se mais provável na medida em que o fluxo de genes atue homogeneizando as populações. Entretanto, no caso do tamanho do corpo, especialmente, há variação entre populações potencialmente explicada por plasticidade fenotípica, gerando assim diferenciação fenotípica sem diferenciação genética. Nossos resultados genéticos sugerem indiretamente que os indivíduos de *B. angustum* possuem capacidade de voo suficiente para transpor as barreiras topográficas na área amostrada.

1. INTRODUCTION¹

Variation among populations across a species range is common and often serves as initial motivation for phylogeographical studies (Zamudio et al., 2016). Intraspecific research provides valuable insights regarding the role of historical and contemporary processes in genetic and phenotypic variation among populations (Avice, 2000). Geographic variation in morphological and molecular characters provides opportunities to understand evolutionary processes (Holwell, 2008). Studies evaluating correlations between morphology and genetics at the population level often require projection of patterns over geographic space and can provide fundamental information for the understanding of population dynamics (Habel et al., 2015). In the present study, we investigated the population genetic and morphological structure, and demographic history of a giant water bug in a mosaic of microhabitats in southern South America.

General body size is quite variable among and species of the giant water bug of the genus *Belostoma* Latreille, 1807 (Hemiptera: Belostomatidae), ranging from 9.5 mm to 50.0 mm (Lauck, 1962). In this work, we investigated *Belostoma angustum* Lauck, 1964, a representative of the *bifoveolatum* group (Lauck, 1964), which comprises small and cryptic species occurring in southern and southeastern South America, as well as along the southern portion of Andes, throughout an approximate area of 1,900,000 km². Representatives of *B. angustum* have an average body size ranging from 19.0 mm to 24.0 mm. Genitalic variation is also found among specimens of *B. angustum*. The life cycle of *Belostoma* species is fully dependent of freshwater habitats, mainly ponds and streams. These giant water bugs are known for their flight abilities, although the capacity and distance of flight remain unknown for *Belostoma* species.

Among models of gene flow for aquatic organisms, two models initially described to fishes can be characterized as follows: (1) the 'Death Valley Model' (DVM) was developed for isolated populations, unconnected hydrologically. In these cases, populations will be highly differentiated genetically without regard for landscape structure, resulting in absent gene flow (Miller, 1948; Finn et al., 2007); (2) The 'Stream Hierarchy Model' (SHM) was developed particularly to study desert fishes with some continuous hydrologic connections along dendritic networks (Meffe and Vrijenhoek, 1988). This model predicts gene flow according to hydrologic connections. Recent studies (e.g. Hughes et al., 1999; Hughes et al.,

¹ This work is formatted as a manuscript in preparation for submission to the *Journal of Zoological Systematics and Evolutionary Research* and will be entitled "Different genetic and morphological markers indicate panmixia in giant water bug populations throughout Pampas highlands".

2003; Wishart and Hughes, 2001; Wishart and Hughes, 2003) have invoked SHM as an overarching conceptual framework describing the genetic diversity according to drainage structure in aquatic insects.

The study area is part of the Pampa domain in southern South America, and the habitats where collecting was done are characterized by grasslands and forest mosaics with abundant permanent and temporary ponds and streams. The highlands altitude ranges between 300 and 600m. The Sul-Rio-Grandense highland raised during the Pre-Cambrian period and the Campanha highland, a more recent formation, was raised during the Triassic period (Holz and De Ros, 2000). The paleovegetation record indicates that grassland existed in the area under a relatively dry and cold climate during glacial times (about 23,000–17,000 year ago), and warm and dry conditions during the post glacial times (Behling et al., 2005). This vegetation remained in some formations due to a climate characterized by low precipitation and a long annual dry period as long as three months in this region at past time (Behling, 1997, 2002). Late Pleistocene grassland remains on the Pampas in southern Brazil.

Previous phylogeographical studies in Pampa domain are scarce and focused on plant or vertebrate species (e.g. Cossíos et al., 2009; Fregonezi et al., 2012). Moreover, there are only two documented studies conducted with belostomatid water bugs that provide some clues about migration capacity (*sensu* Williams, 1957), gene flow, and genetic population structure. Finn et al., (2007) proposed a new model for the gene flow in *Abedus herberti*, referred to as 'Headwater Model' (HM) based on mitochondrial data. This model was based on the geographic isolation and low gene flow rates among populations, possibly generated by conspicuous behavior of *A. herberti*, characterized just for the displacement on the soil in headwaters of streams (Lytle, 1999; Lytle and Smith, 2004). A distinct scenario was presented for the species *Appasus major* and *A. japonicus*, which showed different levels of population structure based on mitochondrial data. The structure found agrees with geographic barriers: the mountains of the Japanese archipelago and strait between Japan and South Korea (Suzuki et al., 2014).

Considering the geographic complexity, historical climatic changes of the Pampas region and the life cycle of the specimens fully dependent on freshwater habitats, we can expect some degree of genetic population structuring of *Belostoma angustum* occurring in the Pampas highlands. In this case, the structure should be either according to the DVM or SHM. Alternatively, these giant water bugs are strong fliers, and topography and other abiotic conditions do not constitute strong enough barriers, it would be expected little or no genetic population structure. If males and females have sex-biased migration rates, different degrees

of genetic structure among populations for each genetic marker would be expected, because inheritance differences. Yet, if molecular and morphological markers show convergent patterns, rapid and recent diversification processes could be invoked, as could phenotypic plasticity or gene flow in *B. angustum* populations. In a scenario that molecular and morphological markers show divergent patterns, we could propose explanations based on convergent local adaptation, phenotypic plasticity, balancing selection or gene flow (Zamudio et al., 2016).

2. OBJECTIVES

The main goals of this study were the following:

1. To evaluate the genetic structure of *Belostoma angustum* populations throughout Pampas highlands using mitochondrial and nuclear markers;
2. To evaluate the morphological variation of *B. angustum* populations throughout Pampas highlands using body and genitalic measurements;
3. To assess the historical demography changes of *B. angustum* populations in Pampas highlands and the effect of the past climate conditions in the Pleistocene period on these population;
4. To test the effect of the highland topography, drainage basins, and linear distances on the population structure of *B. angustum*.

3. MATERIAL AND METHODS

3.1. Population sampling

We sampled 204 specimens (102 males and 102 females) of *Belostoma angustum* from 18 populations in three areas in south Brazil. The study areas include the Pampas highlands, composed by the Campanha highland (ranging from 190–370 m a.s.l.), Sul-Rio-Grandense highland (350–400 m a.s.l.) and an intermediate lowland located between them (70–130 m a.s.l.). The intermediate area comprise part of the Central and Campanha Depressions in Rio Grande do Sul state, Brazil. This region represents a section of the total of distribution of the species but includes topographical variation across a wide enough area to evaluate the hypotheses herein posed. Specimens were collected on the South-Rio-Grandense highland along a 110 km long north-south gradient, and 210 km long east-west gradient; on the Campanha-highland the ranges were 95 x 80 km long north-south and 80 km long east-west; and on the lowland area the ranges were 115 km long north-south and 40 km long east-west. The total area sampled for the research was approximately 110,000 km². Populations of *Belostoma angustum* were initially conceived operationally as the set of individuals of this water bug collected in the same pond or in closely located pond). The average approximate distance among populations was about 40 km. The map with sampled localities, geographic topography, and drainage basins is represented in Figure 1. Latitude and longitude data for each collection site were recorded using a handheld GPS unit. Voucher specimens were fixed in 100% ethanol and are deposited in Coleção Entomológica "Prof.J.M.F.Camargo" (RPSP), Departamento de Biologia, FFCLRP/USP (Ribeirão Preto, São Paulo, Brazil).

Phylogeographic analyses were conducted considering about half of the total number of specimens collected (52 males and 52 females). These samples were randomly selected by sorting three males and three females from each deme. For outgroup, in the phylogenetic analyses, we sampled two individuals of the *Belostoma oxyurum* collected in the same area.

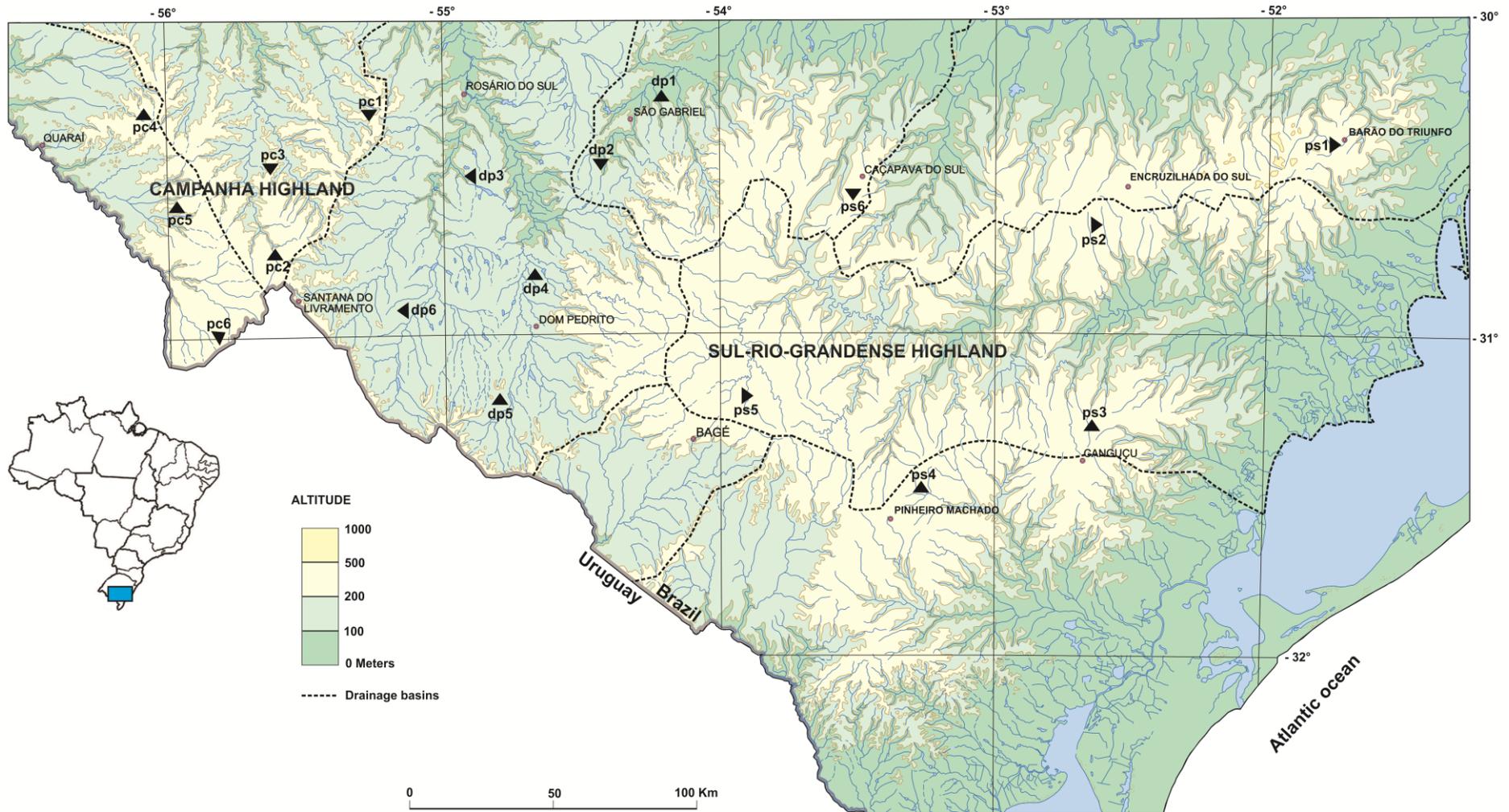


Figure1. Sampling localities of *Belostoma angustum* specimens (black triangles), topographical features of the Pampas highlands, and drainage basins. The three large sampled regions are designated by 'ps', 'dp' and 'pc'(Sul-Rio-Grandense highland, valley and Campanha highland, respectively), the six populations sampled each region are represented by black triangles.

3.2. Molecular procedures

3.2.1. Molecular data collection

Total genomic DNA was extracted from hind leg tissue using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Total DNA was used to amplify by polymerase chain reaction (PCR), two mitochondrial fragments: Cytochrome oxidase subunit I (COI) and 16S rRNA; and the ribosomal Internal Transcribed Spacer 1 (ITS1 rDNA). These molecular markers are often used in phylogeographical studies, and have successfully provided genealogical and historical information about evolutionary process and population dynamics. We used previously described primers for a COI loci (Germain et al., 2013) and for 16S rRNA (Takiya et al., 2006), and designed a newly designed set of primers for ITS1 (Figure S1). The primers sequences and PCR conditions are provided in Table 1. PCR products were purified using the GE Illustra GFX PCR DNA[®] kit (GE Healthcare, Buckinghamshire). Sequencing reaction of purified DNA was prepared with BigDye[®] Terminator v3.1 Cycle Sequencing kit (Thermo Fisher), and sequences generated by a first-generation automatic sequencer ABI 3730 XL DNA Analyser (Applied Biosystems) at *Centro de Recursos Biológicos e Biologia Genômica (CREBIO), Universidade Estadual Paulista 'Júlio de Mesquita Filho'* (Jaboticabal, São Paulo, Brazil).

3.2.2. Sequence alignment and phylogenetic analyses

Sequence edition and contig formation were done with GeneiousR7 (Kearse et al., 2012). Alignments were then conducted using default parameters of MUSCLE algorithm (Eggar, 2004). Aligned sequences were 650 base pair (bp) long for COI, 420 bp long for 16S rRNA fragment, and 1735 bp long for ITS1. Sequence data will be submitted to GenBank[®] database.

Each gene fragment was assigned a suitable substitution model by Akaike Information Criterion (AIC) (Posada and Buckley, 2004; Mazzerolle, 2006) in jModelTest v. 2.1.10 (Darriba et al., 2012). Gene trees were inferred using MrBayes 3.2 (Ronquist et al., 2012) and with TNT (Goloboff et al., 2008), followed by analyses of the concatenated data matrix also under Bayesian posterior probability and parsimony criteria. MrBayes analyses were run for 50 million generations with a 20% burn-in for COI + ITS1 genes combined using remotely CIPRES server (Miller et al., 2010). TNT searches used a traditional algorithm, assuming equal weights, with random seed = 0, 5,000 replications, and 10 trees saved per replication. Consensus trees were edited in FigTree v. 1.4.2 (Rambaut, 2014).

Table 1. Primers used in this study to amplify different mitochondrial regions and ITS1rDNA region of *Belostoma angustum* specimens.

Locus - direction	Sequence (5'→3')	Primer name	Reference
COI - for	TGTA AAAACGACGGCCAGTTTTCAACTAAYCATAAARGATATYGG	LCO1490	Germain et al., (2013)
COI -rev	CAGGAAACAGCTATGACTAAACYTCAGGATGACCAAAAAAYCA	HCO2198	Germain et al., (2013)
16S - for	CCGGTYTGA ACTCARATCA	LR-J-12887	Takiya et al., (2006)
16S - rev	CRMCTGTTTAWCAAAAACAT	LR-N-13398	Takiya et al., (2006)
ITS1 - for	ATTCCCAGTAAGCGCGAGTCATAA	HemiptITS1_1717	This work
ITS1 - rev	GCCGAGTGATCCVCCGYCAGGGT	HemiptITS1_2609	This work

The PCR was conducted in a 25 μ L mix with 11.0 μ L of H₂O Milli-Q®, 2.0 μ L of 25 mM MgCl₂, 5.0 μ L of 5 \times Golden Taq PCR buffer, 3.0 μ L of 1 mM dNTPs, 0.8 μ L of 16 mM each primer, 0.4 μ L of Golden Taq DNA Polymerase and 2.0 μ L of DNA template for **COI** and **16S rRNA**. PCR conditions were the following: initial denaturation at 94 °C–3 min; 35 cycles (denaturation at 94 °C–30 s, primer annealing at 55 °C–45 s and extension at 72 °C–1.5 min); final extension at 72 °C–7 min. For **ITS1**, PCRs were conducted in 25 μ L mix with 8.5 μ L of H₂O Milli-Q®, 2.0 μ L of 25 mM MgCl₂, 5.0 μ L of 5 \times Golden Taq PCR buffer, 3.0 μ L of 1 mM dNTPs, 0.8 μ L of 16 mM each primer, 2.5 μ L of 5 mg/mL BSA (Bovine Serum Albumin), 0.4 μ L of Golden Taq DNA Polymerase and 2.0 μ L of DNA template. PCR conditions for **ITS1** were the following: initial denaturation at 95 °C–3 min); 35 cycles (denaturation at 95 °C–30 s; primer annealing at 58 °C–45 s; extension at 72 °C–1.5 min); final extension at 72 °C–for 8 min.

3.2.3. *Phylogeographic analyses*

To estimate DNA polymorphisms in each population and in the whole set of populations, haplotype and nucleotide diversities were calculated using DNAsp v. 5.10.01 (Rozas et al., 2009). Haplotype networks were generated with the Median Joining (MJ) algorithm in Network v.5.0 (Bandelt et al., 1999). We implemented an analysis of molecular variance (AMOVA) in Arlequin v. 3.5.2.2 to calculate the percentage of genetic variation explained by grouped populations based on the identified highlands and drainage basins (Excoffier and Lischer 2010). For the AMOVA test, we used a Tamura-Nei distance (Φ_{st}) with 10,000 permutations. A Mantel test with pairwise genetic Slatkin's linearized distances generated in Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010) was implemented in IBDWS v. 3.23 (Jensen et al., 2005). We used 10,000 permutations to investigate distance isolation (linear geographic distance) patterns in the mitochondrial data. We also investigated whether there were recombination events in ITS1 rDNA dataset using DNAsp v. 5.10.01.

3.2.4. *Historical demography*

We used a mismatch distribution approach to reveal the demographic history of *B. angustum* from Pampas highlands with defined groups of populations by AMOVA. Additionally, three neutrality tests were implemented in DNAsp 5.10.01 (Rozas et al., 2009) to detect departures from the mutation-drift equilibrium that would be indicative of changes in historical demography and natural selection (*i.e.*, bottlenecks or expansions): Tajima's D (Tajima, 1989), Fu and Li's D (Fu and Li, 1993), and Fu's Fs (Fu, 1997) tests. Neutrality tests assume that the population has been in mutation-drift balance for a long period of evolutionary time (Nei and Kumar, 2000). When the population is not under a mutation-drift equilibrium, as a result of a sudden expansion in population size, these indexes tend to exhibit significantly negative values and an excess of polymorphisms at low frequency. By contrast, positive values reflect the elimination of rare alleles after genetic bottleneck or processes such as a population subdivision (Ramos-Onsins and Rozas, 2002). In case of recombination just Tajima's D and Fu and Li's D were implemented, because these class 1 tests are more powerful when recombination events are detected (Ramírez-Soriano et al., 2008).

Furthermore, we inferred population demographic changes over time using a Bayesian coalescent-based method with Extended Bayesian skyline plot (EBSP) (Heled and Drummond, 2005) in BEAST 2.4.5 (Bouckaert et al., 2014). The best fit nucleotide substitution models were the same used for Bayesian phylogenetic analysis. The EBSP approach incorporates uncertainty in the genealogical inference by using Markov chain Monte

Carlo (MCMC) integration under a coalescent model, providing information about effective population sizes (N_e) through time (Drummond et al., 2005). The analysis was run for 200 million generations with default mutation rates, and evaluated with Tracer v. 1.6 (Rambaut et al., 2014) to ensure convergence has been achieved and ESSs were larger than 200, after discarding the first 20% of the run.

3.3. Morphological measurements and procedures

Upon dissections of male and female genitalia, different measurements were made in each case. Three different components of male genital morphology were considered: two of which are intromittent portions, the sclerite of the phalloteca, known as “phallosoma” (PHA), and ventral part of phalloteca, known as “diverticulum” (DV), and one nonintromittent, the lateral sclerite of the right paramere (PA) (Figure 2). All measurements and imaging were made using a Leica M205C stereomicroscope with a coupled Leica DFC450 camera. Stacking was made using software module LAS Montage, and these male genitalic structures were analyzed with geometric morphometrics techniques (Bookstein, 1991; Rohlf and Marcus, 1993). Measurements of width and length of the female genital chamber and valves, and nine nongenitalic traits of male and female were documented (Figure 3). Measurements of these traits were made using a caliper rule (accuracy = 0.01 mm) or a ruler mounted on the stereomicroscope ocular.

One-third of specimens were re-measured to estimate the percentage of error obtained when first measuring morphological structures. The percentage measurement error was defined as the ratio between the estimated variance of the values within each group and the sum of the components of the estimated variance values between and within groups (Claude, 2008). These variance components (*i.e.*, the repeatability) were derived from the one-way ANOVA approach, considering the individual factor as a source of variation.

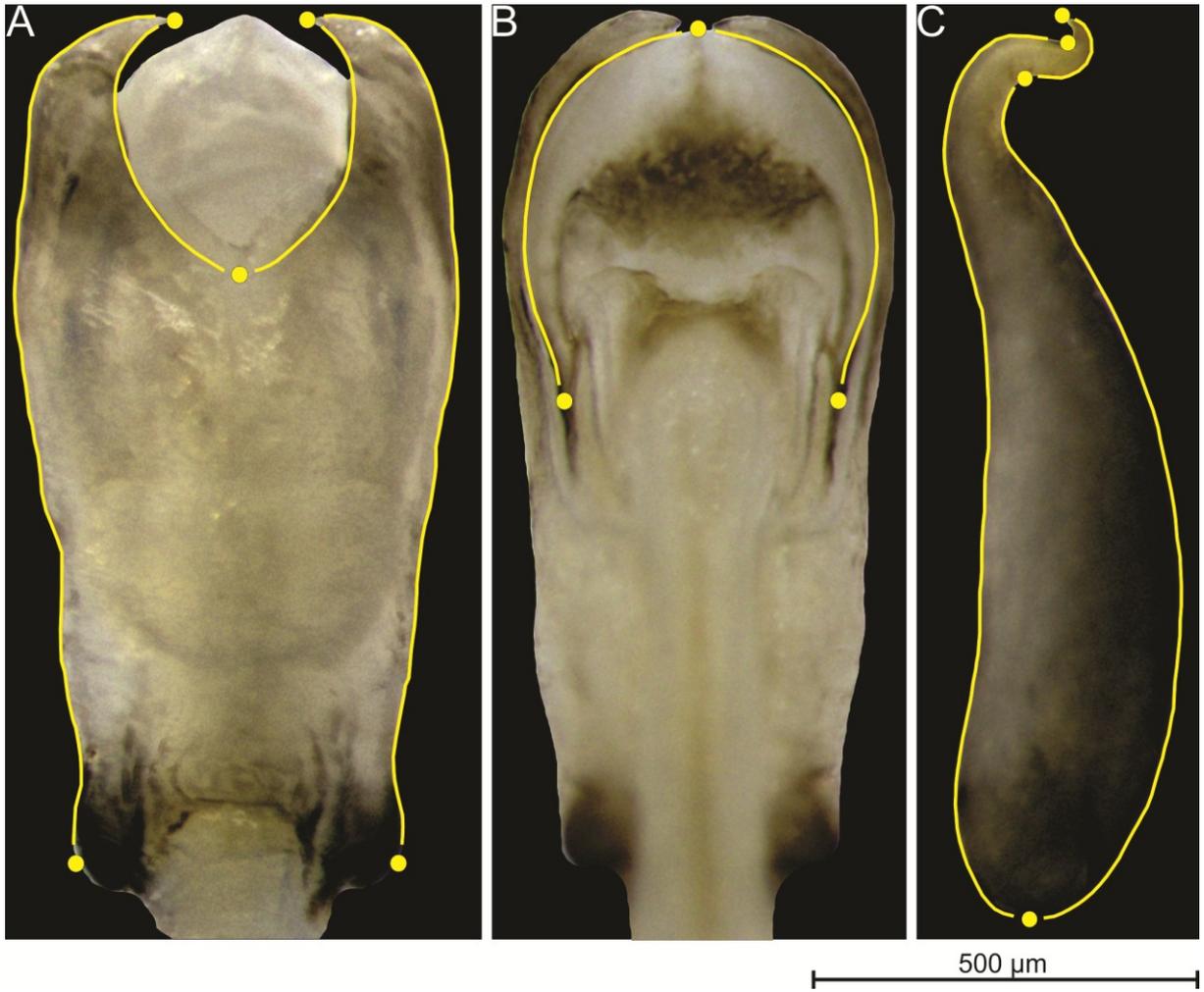


Figure 2. Male genitalia of *Belostoma angustum*: (A) phallosoma in dorsal view; (B) diverticulum of phallosoma and (C) right paramere. Yellow circles indicate the landmarks and the lines show the sampled curves (semilandmarks).

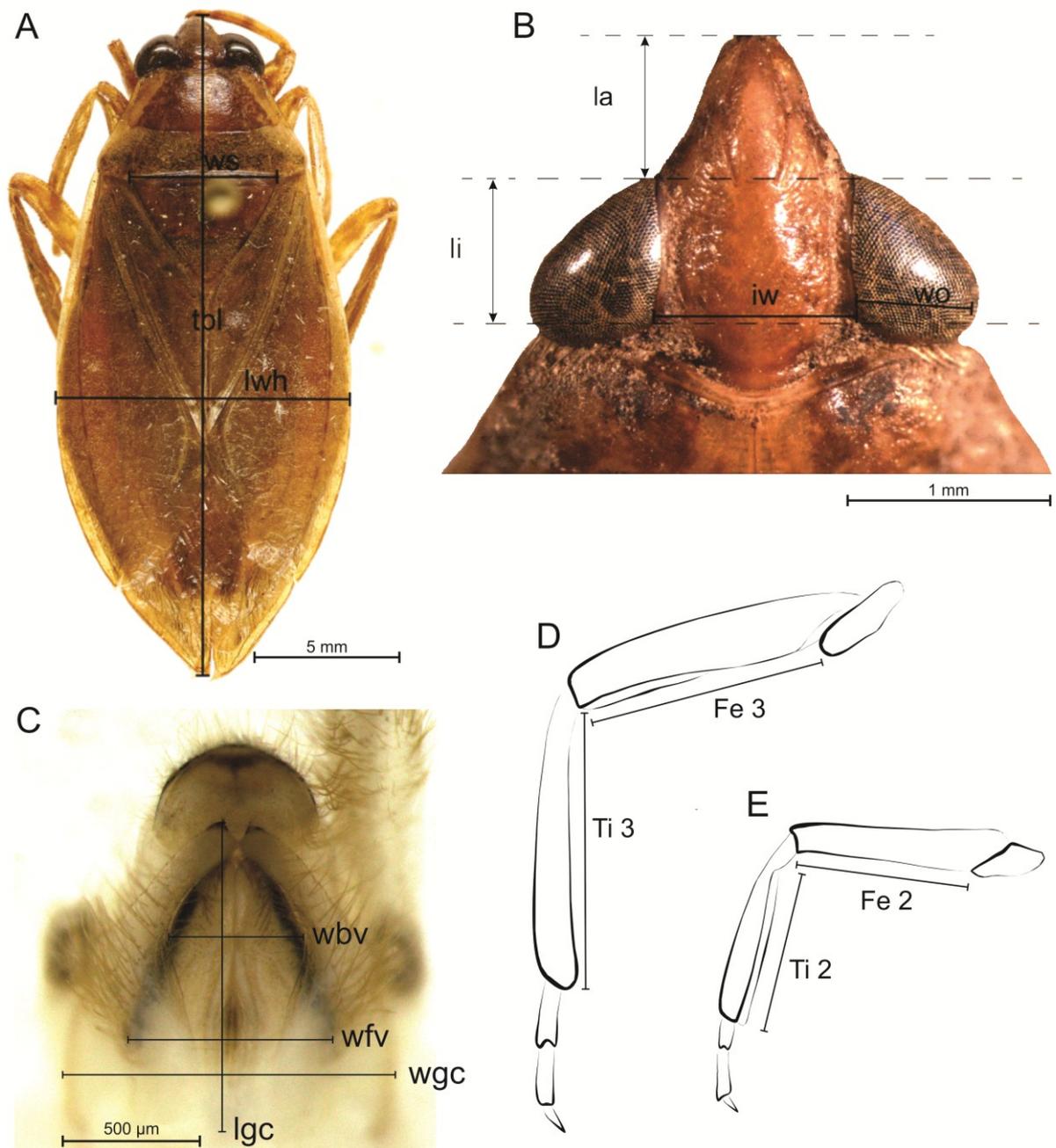


Figure 3. Linear measurements of *Belostoma angustum*. (A) body measurements: total body length (tbl) and largest width of hemelytron (lwh); (B) head measurements: interocular width (iw), width of ocular globe (wo), interocular length (li) and anteocular length (la); (C) female genital chamber measurements: genital chamber length (lgc), width chamber length (wgc), width of the first pair of valve (wfv); and width between valvifers (wbv); (D) hind leg measurements: hind femur (Fe3) and hind tibiae (Ti3); (E) mid leg measurements: mid femur (Fe2) and mid tibiae (Ti2).

3.3.1. Procrustes superimposition

Morphometric variation was quantified mostly based on the outlines because genitalic structures are curved and have few points that can be considered homologous to establish reliable morphometric landmarks. Bi-dimensional coordinates of landmarks and semilandmarks were obtained using the software TPSdig 2.16 (Rohlf, 2010). Superimposition of landmarks and semilandmarks was conducted in R 3.3.0 (R Development Core Team, 2016) using the package *geomorph* (Adams and Otárola-Castillo, 2013). In addition to optimally translating, scaling, and rotating the landmarks, the semilandmarks were slid along the outline curve until they matched the positions of the corresponding points along an outline, considering a reference specimen (Adams et al., 2004). The method of Minimum Bending energy criteria (BE) was used to slide landmarks (Perez et al., 2006). The number of principal shape components (PCs) to be analyzed was selected by measuring the correlation between the matrix of Procrustes shape distances in the full shape space and pairwise Euclidean distances in the reduced shape space, according to Cardini et al., (2007). Six PCs were explained 88.0% of the shape variation in PHA; five PCs explained 89.2 % shape variation in DV; six PCs explained 87.5 % of the shape variation in PA and three PCs explained 89.6 % of the shape variation in the second valva of the female genital chamber.

3.3.2. Residual allometry, fluctuating asymmetry and estimated error—geometric morphometric dataset

Residual allometry was assessed based on shape coordinates on centroid size, since effects of size on shape (*i.e.*, allometry) can affect integration and obfuscate modular structure (Klingenberg, 2016). We also evaluated the degree of random deviation from bilaterally symmetric traits, a phenomenon known as 'fluctuating asymmetry' (FA). This phenomenon is often assumed to be an important signal for environmental stress or reflection of small accidents during structural development (Rasmuson, 2002; Habel et al., 2015). Both PHA and DV are bilaterally symmetric regarding their respective central axes. We re-measured one-third of the specimens randomly, took new photos, plotted and superposed the land-semilandmarks to estimate the error. Fluctuating asymmetries were computed as the individuals vs. side interactions (or individuals vs. reflection interactions), whereas measurement error was computed from the variation among replicated land-and semilandmarks. We also considered here that there is an equal amount of variation around each semilandmark (*i.e.*, this variation is isotropic) (Klingenberg et al., 2002). Only symmetric components in PHA and DV were considered when they were present.

3.3.3. *Multicollinearity and geographic variation*

First, we tested multicollinearity among variables (linear measurements: body, leg and genital chamber of females) using VIF correlation (Variance Inflation Factor) (Marquardt, 1970) with R package *usdm* (Naimi, 2014). The variables with $r > 0.8$ were not included in the posterior analysis. Total body length (TBL) was highly correlated with other measurements; in this case, we excluded TBL from this dataset and evaluated the remaining measurements separately. For the leg dataset, we used two PCs axis, explaining 94.5 % of total variation in this structure because the correlation was very strong among parts of the legs. The measures of female chamber were not highly correlated variables ($r < 0.8$).

We assessed morphological variation in three levels: (1) among populations, (2) among drainage basins and (3) among highlands (groups of populations in last cases) by multivariate analysis of variance (MANOVA). We examined the pattern of shape variation on genitalic traits by canonical variate analysis (CVA), along with thin-plate spline (TPS) visualizations of shape variation and jackknifed assignment tests. This analysis assesses how well populations can be distinguished by shape. For the variable traits, we implemented Trend Surface Analysis (TSA) models (Borcard et al., 2011) using orthogonal polynomials to investigate both linear and curvilinear gradients of variation. The best model of morphological variation was selected by Akaike Information Criterion (AIC) (Posada and Buckley, 2004; Mazzerolle, 2006). These analyses were implemented with *vegan* (Oksanen et al., 2016) and *packfor* (Dray et al., 2016) packages in R 3.3.0 (R Development Core Team, 2016). Additionally, we evaluated the altitudinal effect on morphological traits. Considering the low altitudes of the areas sampled (between 70 and 400 meters) however, no altitudinal effect is expected.

3.3.4. *Spatial autocorrelation*

Analyses involving the study of relations with physical space can be affected by spatial autocorrelation, although no statistical test has detected this effect in a significant way (Legendre 1993; Diniz-Filho et al., 2008). Autocorrelation is a problem in spatial analysis of landscapes because it violates the data independence hypothesis and restricts the breadth of comparability of replicates (Dale and Fortin, 2002). We assessed the spatial correlation using orthogonal polynomials of geographic coordinates with distance classes, previously established (Sturges, 1926; Scott, 2009) to all morphological traits, using a Mantel correlogram analysis (Legendre and Legendre, 2012) in the *vegan* R package.

4. RESULTS

4.1. Genetic patterns of variation

4.1.1. Genetic polymorphism and gene trees

For the mitochondrial dataset, 650 bp of the protein-coding COI gene were obtained from 98 individuals and 420 bp of the 16S rRNA from 54 individuals. For the COI, we found 34 polymorphic sites, including 15 parsimony informative sites. The best fit substitution model for COI was HKY+I+ Γ model. For the 16S rRNA, only three polymorphic sites and one parsimony informative site were recognized. The selected substitution model for 16S rRNA was HKY (Table 2). The mitochondrial region 16S rRNA was not included in the subsequent phylogeographical analyses due to the very low information about genetic variation among populations of *B. angustum* throughout Pampas highlands. The nuclear dataset was constituted by 1735bp of the ITS1 rDNA generated for 102 individuals, including 34 polymorphic sites and 14 parsimony informative sites. The best fit substitution model for ITS1 was GTR+I+ Γ model (Table 2). Two events of recombination were detected between sites (1045-1454) and (1454-1493) in the ITS1 rDNA dataset.

A total of 42 haplotypes for the COI, which 35 are exclusive, and 33 haplotypes for the ITS1, which 23 are exclusive, were recognized among all individuals collected from 18 populations throughout the highlands. The haplotype and nucleotide diversities estimated were 0.901 and 0.00340 for COI and 0.878 and 0.00117 to ITS1 for whole dataset (Table 3). The haplotype and nucleotide diversities for each population did not show significant variation.

Gene trees inferred with Bayesian and parsimony criteria are shown in Figures S2, S3, respectively. Based on genealogical relationships from mitochondrial and nuclear dataset, we observed absent genetic population structure. There were not reciprocal monophyly among highlands or drainage basins populations.

We obtained similar phylogeographical patterns with different mtDNA (COI) and ITS1 rDNA markers, even though, there was a difference in nucleotide diversities between these markers. However, the remarkable feature was found in 16S rRNA amplified fragment for the *B. angustum* populations. This fragment is not informative, once we found only three polymorphic sites. We used the 16S rRNA because it has different substitution rates compared to COI and ITS1. In a previous phylogeographical study, the use of 16S rRNA in giant water bug populations was successfully evaluated (Suzuki et al., 2014). However, for our dataset the 16S rRNA was not a suitable marker.

Table 2. Substitution models selected for the parametric probabilistic analysis of the gene loci amplified for *Belostoma angustum* specimens.

Loci	Selected model	BP	Sample	S (%)	PIS (%)	%A	%C	%G	%T
COI	HKY+I+ Γ	650	98	34 (5)	15 (2)	30	19	16	35
16S	HKY	420	54	3 (1)	1 (0)	36	14	14	36
ITS1	GTR+I+ Γ	1735	102	34 (2)	14 (1)	21	29	31	19
COI +ITS1		2385	94	67 (3)	29 (1)	24	26	27	23

S = Segregation sites; PIS = Parsimony Informative sites.

Table 3. Nucleotide polymorphism and neutrality tests in panmictic population of *Belostoma angustum* based on mitochondrial and nuclear dataset. NHap = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity.

Parameter	Mitochondrial data (COI)	Nuclear data (ITS1)
Sample size	98	102
NHap	42	33
Hd	0.901	0.878
π	0.00340	0.00117
Tajima's D	-2.08825*	-2.12349*
Fu's Fs	-51.781**	***
Fu and Li's D	-3.86106**	-4.21937**

*P < 0.05; **P < 0.001; ***Test not implemented because recombination.

4.1.2. Phylogeographic structure, haplotype networks and gene flow

Overall, results from multiple analyses of molecular variance (AMOVA) for COI and ITS1 dataset indicated that *Belostoma angustum* forms a large panmictic population across the area studied. The expected effect of the highlands or drainage basins on genetic population structure of these giant water bug populations was null. The AMOVA analyses of our datasets indicated about 0% of the variation explained by these sources of variation (Table 4).

Table 4. Summary of the analysis of molecular variance (AMOVA) for the mitochondrial (COI) and nuclear (ITS1) datasets of *Belostoma angustum* populations. Populations were grouped based on the Pampas highlands and drainage basins.

Loci	Source of variation	G.L.	SS	% Variance	Fixation indices
COI	Among highlands (HG)	2	1.961	-0.28	$\Phi_{CT} = -0.00275^{ns}$
	Among populations within HG	15	16.238	-0.78	$\Phi_{SC} = -0.00781^{ns}$
	Within populations	81	91.581	101.06	$\Phi_{ST} = -0.01058^{ns}$
	Among drainage basins (DB)	6	6.897	0.78	$\Phi_{CT} = 0.00780^{ns}$
	Among populations within DB	11	11.303	-1.66	$\Phi_{SC} = -0.01676^{ns}$
	Within populations	81	91.581	100.88	$\Phi_{ST} = -0.00882^{ns}$
ITS1	Among highlands (HG)	2	1.702	-1.23	$\Phi_{CT} = -0.01232^{ns}$
	Among populations within HG	15	19.294	3.64	$\Phi_{SC} = 0.03089^{ns}$
	Within populations	84	84.941	97.59	$\Phi_{ST} = 0.03413^{ns}$
	Among drainage basins (DB)	6	6.288	-1.66	$\Phi_{CT} = -0.01954^{ns}$
	Among populations within DB	11	14.708	3.20	$\Phi_{SC} = 0.02392^{ns}$
	Within populations	84	84.941	98.46	$\Phi_{ST} = 0.03543^{ns}$

^{ns} = non significant.

The haplotype networks did not indicate genetic structure in populations of *B. angustum* from highlands based on mitochondrial nor nuclear markers. All most frequent haplotypes are shared by individuals from all three sampled areas. The differentiation among haplotypes was low—only two mutation steps. The haplotype network from COI is available in Figure 4. The nuclear sequence data showed lower genetic diversity than the mitochondrial data, and reinforced the lack of phylogeographical structure pattern (Figure 5).

There was no evidence of isolation-by-distance (IBD) in *B. angustum* populations based on mitochondrial data. The correlation of the Mantel test between genetic Slatkin's linearized distance and Euclidean geographic distance was not significant ($r = 0.0443$; $P = 0.6587$) (Figure 6). The pairwise Φ_{st} was low and not significant, indicating historical gene flow among populations of this giant water bug throughout the study area. However, the historical gene flow was asymmetric, with lower rates in populations *ps3* and *ps4*. These populations were more distantly isolated (Figure 1), and their pairwise Φ_{st} among populations were the highest ($\Phi_{st} > 0.15$) encountered (Figure 6).

4.1.3. Historical demographic changes

The negative and significant values of neutrality tests (Tajima's D , Fu and Li's D , Fu's F_s) from mitochondrial locus, and negative and significant values (Tajima's D , Fu and Li's D) from the nuclear locus, suggested an overall past panmictic population expansion (Table 3). The unimodal mismatch distributions were found for both markers (COI and ITS1) and can be interpreted as characterizing expanding populations (Harpending, 1994). In the Extended Bayesian skyline plot of the combined COI + ITS1 datasets, we observed an event of demographic growth after a prolonged phase of substantial demographic stability, which is estimated to have started about 50,000 years ago (50 kyr). In the Last Glacial Maximum (LGM), around 23 kyr, the panmictic population decreased its growth reaching almost a stabilizing. After the LGM, around 15kyr, a marked population growth can be noticed, which agrees with the increase in the number of coalescent events in time observed between 15–10 kyr (Figure 7).

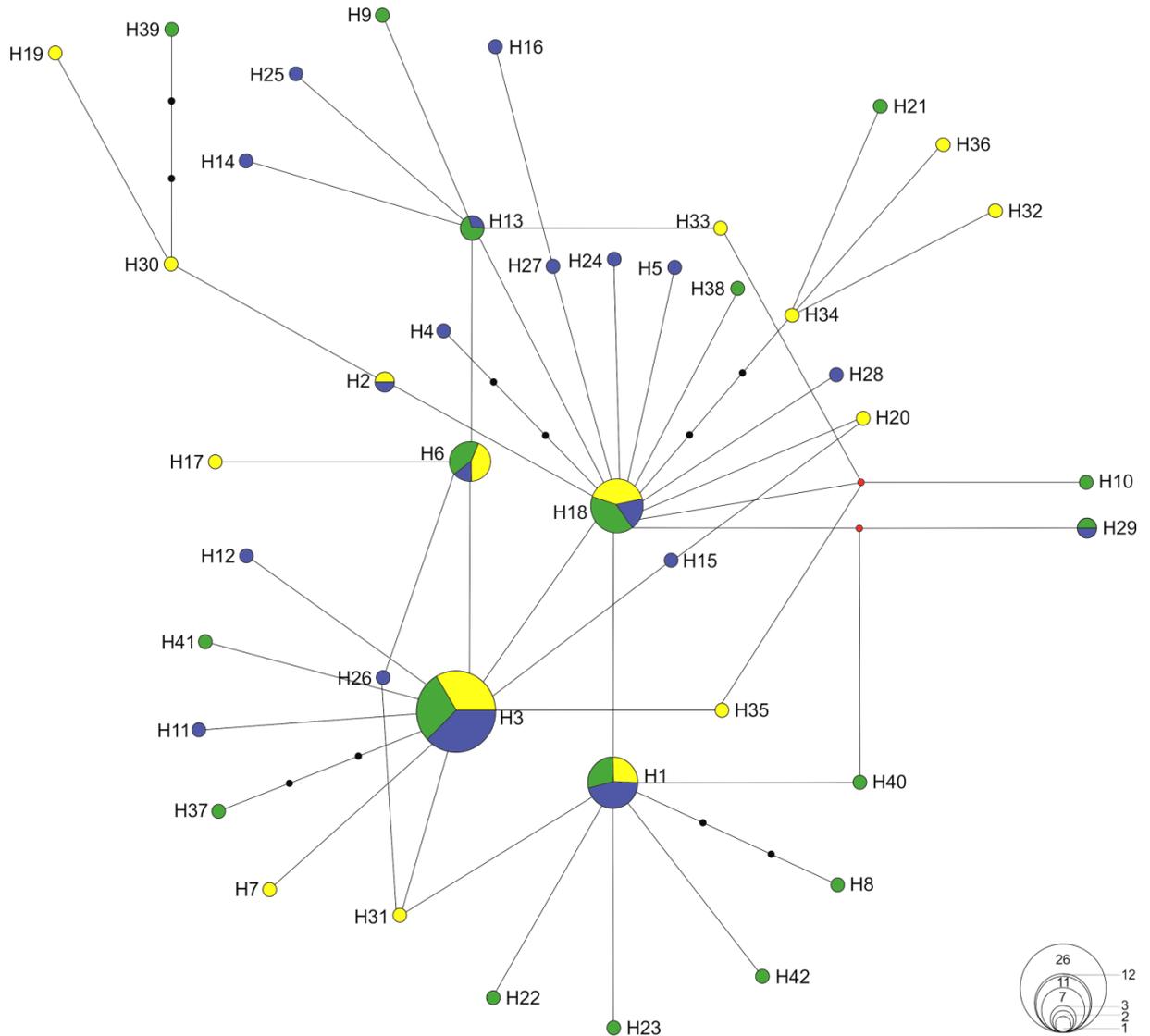


Figure 4. Median joining haplotype network of *Belostoma angustum* populations estimated from the mitochondrial marker COI. Haplotype circle size is proportional to the number of sampled individuals. Lines and black dots represent one mutational step. The colors indicate the three sampled areas: Blue = Sul-Rio-Grandense highland; Yellow = valley between highlands; Green = Campanha highland.

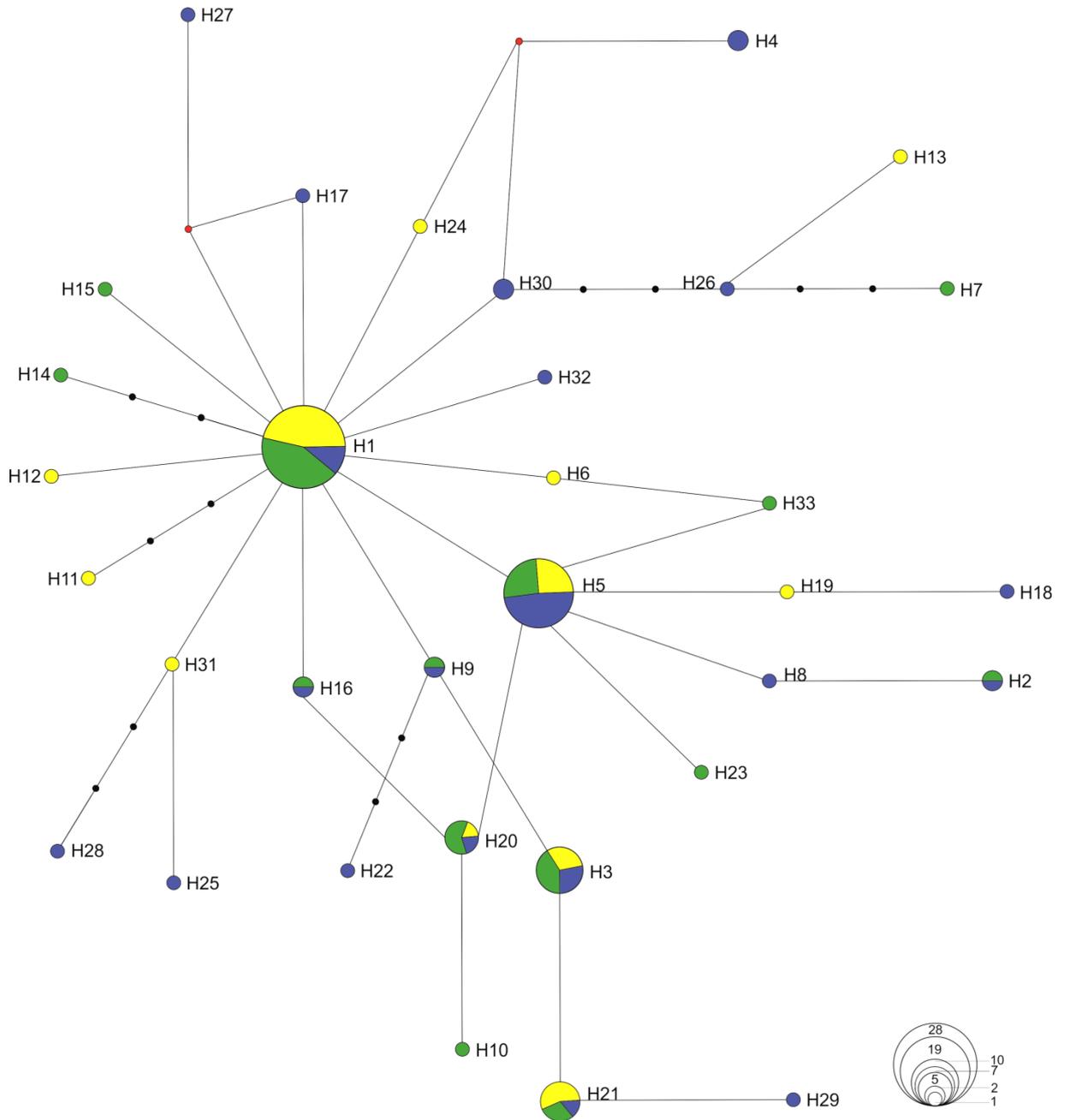


Figure 5. Median joining haplotype network of *Belostoma angustum* population estimated from the nuclear marker ITS1 rDNA. Haplotype circle size is proportional to the number of sampled individuals. Lines and black dots represent one mutational step. The colors indicate the three sampled areas: Blue = Sul-Rio-Grandense highland; Yellow = valley between highlands; Green = Campanha highland.

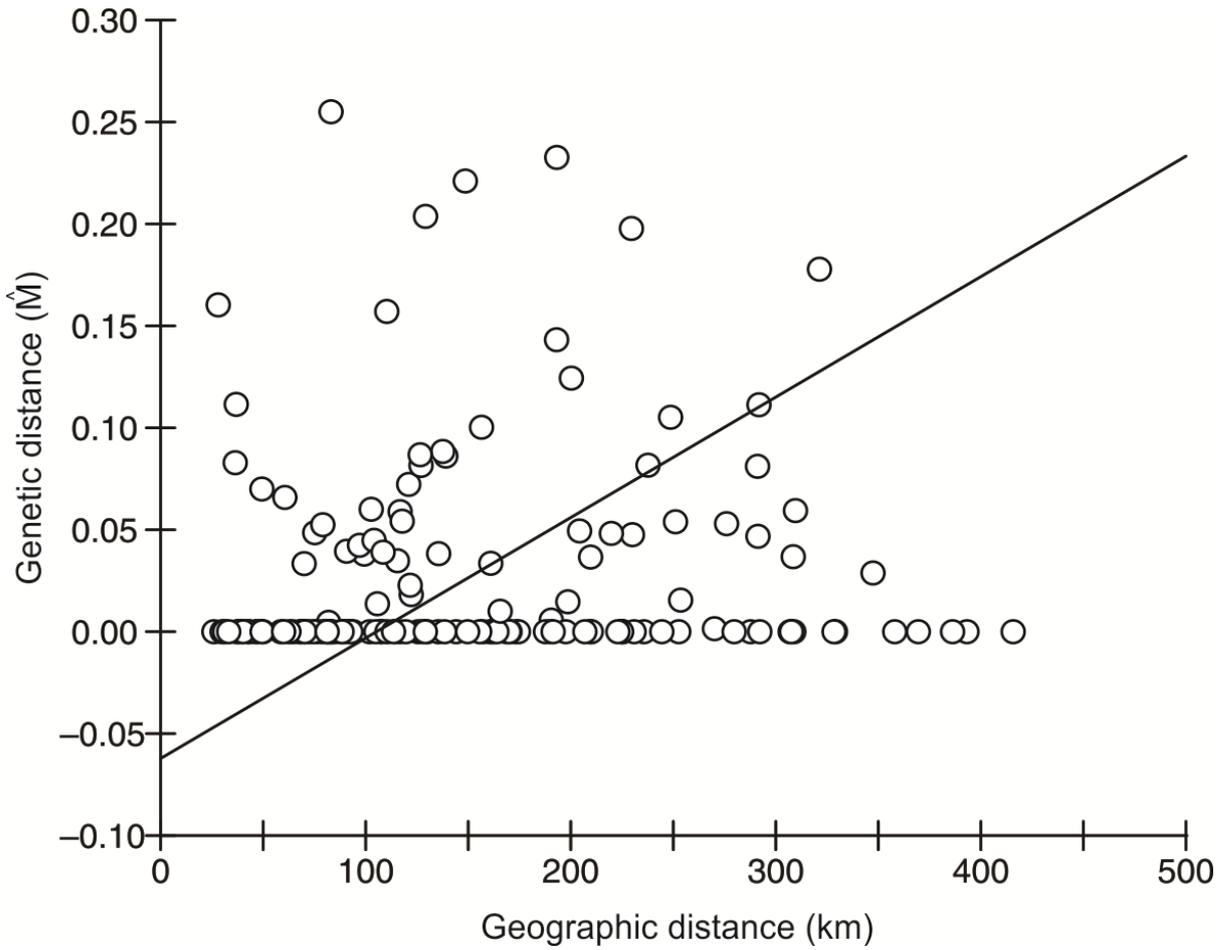


Figure 6. Scatter plot showing the relationship between genetic Slatkin linearized distances (\hat{M}) and geographical distances (km) based on COI data set in *Belostoma angustum* populations. $\hat{M} = \Phi_{st}/(1 - \Phi_{st})$.

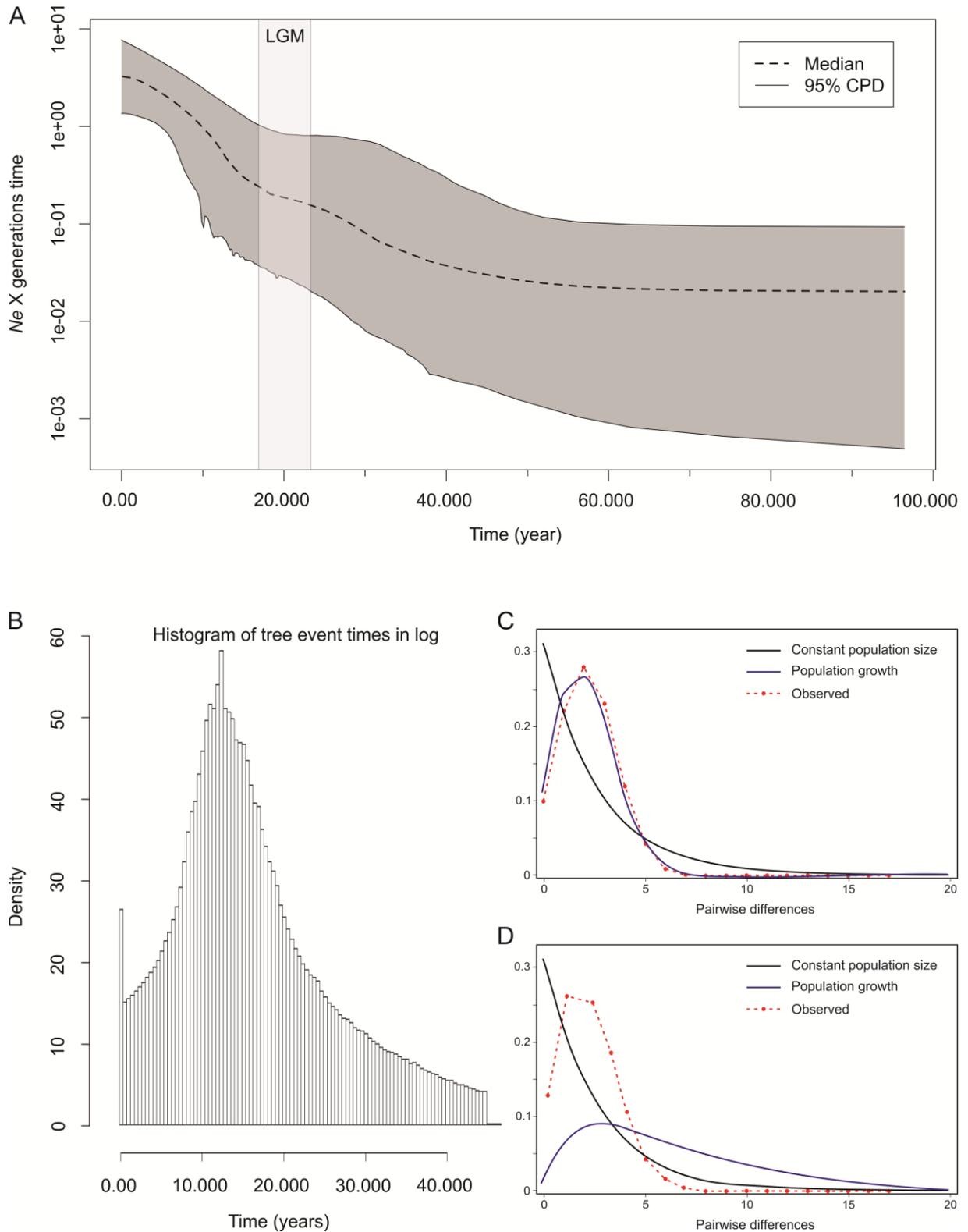


Figure 7. Demographic history of *Belostoma angustum*. (A) Extended Bayesian skyline plot based on COI + ITS1 markers. X-axis is the timescale before present, and Y-axis is the estimated effective population size. Solid curves indicate median effective population size; the shaded range indicates 95% highest posterior density intervals. LGM represents Last Glacial Maximum. (B) Histogram of density of the coalescence distribution (Y-axis) through time (X-axis). (C) Observed mismatch distributions and their fit to expected model of demographic history based on mitochondrial marker (COI) and (D) based on nuclear marker (ITS1).

4.2. Morphological patterns of variation

4.2.1. Body measurements

The estimated error associated with the inferred size measurements indicated that there was no influence of the measured sessions upon each measurement. Also, the estimated variation between measured session is smaller than the variation within each session based on the one-way ANOVA ($F_{\text{between groups}} = 0.137$, $P = 0.771$; $F_{\text{within group}} = 22.571$, $P < 0.01$). The estimated error was therefore negligible to assume the existence of variation in taking measurements of the same individual. The total percentage error was estimated to be only 2.11%.

There was significant variation in total body length (TBL) among the populations of *B. angustum* sampled ($F = 2.1136$, d.f. = 17, 185, $P = 0.0057$). Moreover, there was significant sexual dimorphism in these individuals sampled ($F = 8.1243$, d.f. = 1, 185, $P = 0.0017$), females being larger than males. The average of TBL of males and females in each population is shown in Figure 8. The largest width of hemelytron (LWH) and interocular width (IW) were other examples of body traits with interpopulation variation: LWH ($F = 3.0350$, d.f. = 17, 185, $P < 0.01$); IW ($F = 2.3060$, d.f. = 17, 185, $P < 0.01$).

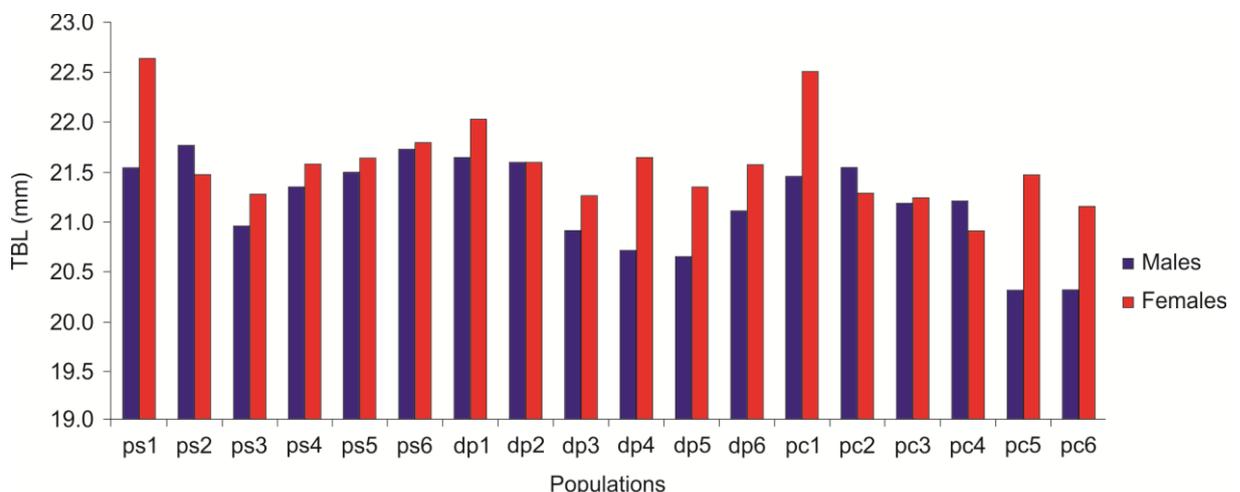


Figure 8. Average of total body length (TBL) in millimeters (mm) for each population of *Belostoma angustum* separated by sex. PS1–PS6, DP1–DP6, PC1–PC6 represent the 18 populations sampled for the analyses — PS = South-Rio-Grandense highland, PC = Campanha highland; DP = valley located between highlands.

The percentage of explanation among and within each population, drainage basins and highlands related to each body trait is given in Table 5. Patterns of TBL, LWH and IW indicated longitudinal variation. The only significant term of the orthogonal polynomial of the TSA was Y, indicating individuals from Sul-Rio-Grandense highland (ps) to be larger than others on the population average. As for leg length, we did not find interpopulation variation or among groups of populations (highlands or drainage basins); only sexual dimorphism was

detected ($F = 4.1870$, $d.f. = 1, 185$, $P = 0.0174$). There was no spatial autocorrelation in any of the distance classes evaluated in these body traits.

Table 5. Percentage of total variance explained by differences among and within populations, and others sources of variation in an ANOVA framework for each of the measured and variable morphological traits of *Belostoma angustum* specimens.

Source of variation	Morphological character		Among (% variance)	Within (% variance)
Populations	TBL		16.4	83.6
	Body measurements	LWH	21.9	78.1
		IW	17.5	82.5
	Diverticulum	PC1	15.3	84.7
		PC2	17.6	82.4
Highlands	TBL		3.2	96.8
	Body measurements	LWH	5.8	94.2
		IW	3.6	96.4
	Diverticulum	PC2	7.6	92.4
	Drainage basins	TBL		11.6
Body measurements		LWH	15.9	84.1
		IW	10.2	88.8
Diverticulum		PC2	17.6	82.4

TBL = total body length; LWH = largest width of hemelytron; IW = interocular width.

4.2.2. Genital structures of males and females

The main variation across PCA axis (PC1 and PC2) of the male genitalic intromittent and non intromittent structures in *B. angustum* specimens can be visualized in the Figure 9. We did not detect fluctuating asymmetry in male symmetric structures (PHA and DV) or significant error in the shapes of genital structures. The residual allometry of centroid size on shape was slightly significant in DV, but this factor did not change the sequential results. In the shape of PHA or PA, we did not detect residual allometry. There was no spatial autocorrelation in any of the distance classes evaluated in genitalic traits.

The shape of diverticulum of the phallosoma was the only genitalic trait with interpopulation significant differences. More specifically, the variation occurs in PC1 and PC2 among populations, and in PC2 among drainage basins and highlands. However, the percentage of explanation was very low, about 15% (Table 5). Based on the CVA analysis of the diverticulum, we did not discriminate the shapes across population, just 10% of correct classification (Figure 10). Shapes of phallosoma and paramere showed no variation among populations. The centroid size of the male genitalic structure also showed no variation among populations or other sources. The same pattern was observed in dimensions of the genital chamber of females. None of the measurements showed significant variation among populations, neither for drainage basins or highlands.

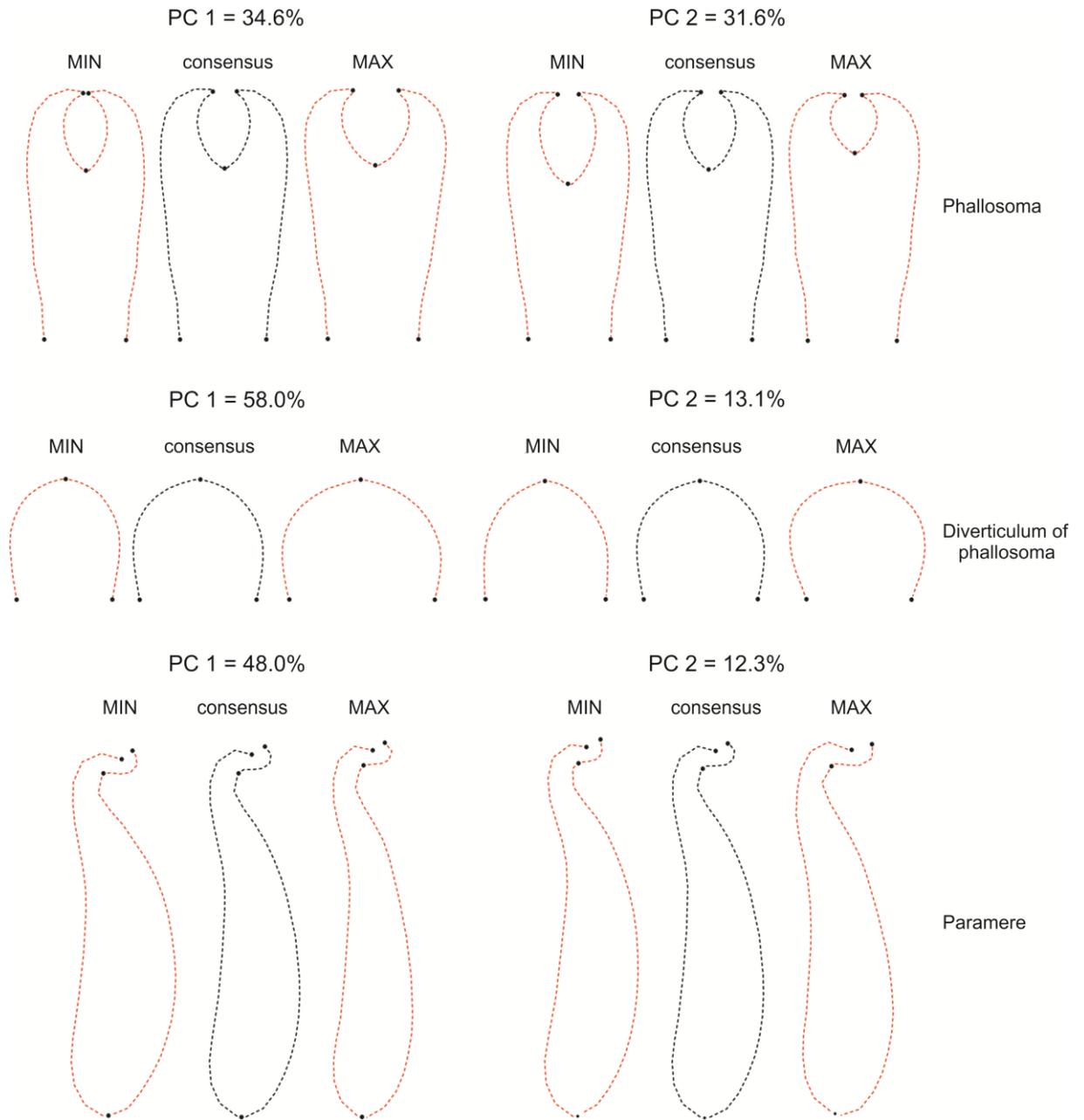


Figure 9. Extreme shapes across PC1 and PC2 and the explanation percentage for each axis in the males genitalic intromittent and nonintromittent structures of *Belostoma angustum*. Black dots indicate landmarks and dashed lines indicate curves (*i.e.*, sliding semilandmarks).

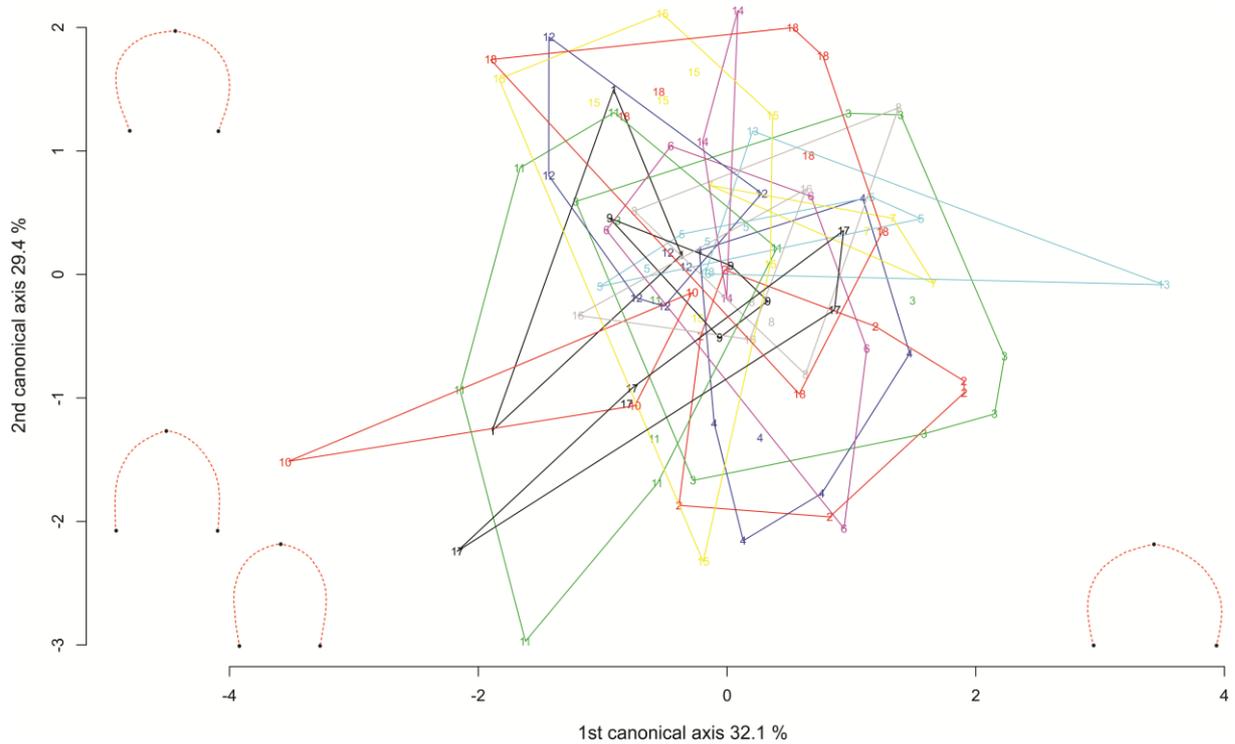


Figure 10. Plot of the CVA analysis of the shape of diverticulum of *Belostoma angustum* and the extreme shapes deformations across axes 1 and 2. Lines connect individuals from the same population. The diverticulum corresponds to an intromittent sclerite in the male genitalia of this giant water bug located ventrally on the phallosome.

4.3. Morphological variation linked with genetic or altitudinal variation

Based on Mantel's test with covariation distance matrix, all evaluated morphological structures showed different patterns of correspondence when compared with previous genetic variation. The altitudinal effect was not significant in none of the evaluated morphological traits. Yet, the genetic and morphological pattern of variation were very similar (not structured), and the correspondence between them was null.

5. DISCUSSION

5.1. Genetic structure and gene flow

Combining evidence from two different genetic markers, the mtDNA (COI) and ITS1 rDNA, we evidenced that there is no genetic population structure in *Belostoma angustum* throughout the Pampas highlands. This water bug is genetically organized as a panmictic population across the evaluated range. An absence of isolation-by-distance indicate that gene flow was not restricted by distance. Gene flow was likely to occur between neighboring populations as well as distant populations, in a range of 40 km to 400 km. The absence of any pattern of isolation by distance in a species, which it was supposed to be found, suggests that the species is far from an equilibrium, and may have recently invaded the area it occupies (Slatkin, 1993). Furthermore, the low Φ_{st} values found in this experiment suggest high historical migration rates among the sampled populations of *B. angustum*. Nevertheless, the historical rates of gene flow among these populations were asymmetric.

Populations *ps3* and *ps4* (Figure 1) had the lowest rates, showing that they are more isolated than others. We suggest a potential historical effect of the drainage basin over the *ps4* population and linear distance over the *ps3* population. Genetic drift would result in substantial local differentiation if $Nm < 1$, but not if $Nm > 1$ (Slatkin, 1987). Regarding the Nm based on Slatkin's linearized Φ_{st} values to the populations *ps3* and *ps4* about 1, it is probable that genetic drift was acting on these populations. However, the low Φ_{st} values do not imply current gene flow and not necessarily give reasonable estimates of Nm (Whitlock and McCauley, 1999). Although genetic analyses cannot replace direct studies on individual migration rates and demographic connectivity, they can contribute to a broader perspective upon the dynamics among and within populations (Peel et al., 2013). Our surprising findings about panmictic pattern in *B. angustum* across the highlands indicate that these giant water bugs are strong fliers, as suggested by Cullen (1969) for general Belostomatidae specimens.

Panmixia is a common pattern in species that representatives of populations have high migration ability, which is favored by flight ability in continental species (e.g., Küpper et al., 2012; Peel et al., 2013). Although the evaluated water bugs can be considered strong fliers, the occurrence range of *B. angustum* extends further than the sampling sites of this study and occurs in other physiognomies of open vegetation habitats. Additional sampling would be required to assess the limits of panmixia in this studied species.

Despite representatives of *B. angustum* being aquatic insects, previously proposed models for aquatic organisms previous described (*i.e.*, DVM, SHM or HM) proved to be unsuitable to explain the phylogeographic pattern of this species. Conversely to these models,

our results indicate that the linear distance, the highland topography, and drainage basins do not appear to represent barriers capable to prevent historical gene flow and, consequently, to structure the studied *B. angustum* populations. The high flight capacities of *B. angustum* specimens allow them to overcome these geographical barriers (linear distance, highlands and drainage basins). Moreover, the capacity of overcoming environmental barriers of these specimens was not sex-biased, once the genetic population structure was based on uniparentally and biparentally inherited markers and show a very similar phylogeographical pattern. There is no direct evidence about different sexes showing different flight abilities in *B. angustum* or even in *Belostoma*.

5.2. Historical demographic changes

Demographic dynamics of populations can be investigated by statistical tests of neutrality of mutations (Fu, 1997). Our findings about neutrality tests with high negative and significant Tajima's D , Fu's F_s and Fu and Li's D values indicate that *B. angustum* panmictic population throughout Pampas highlands experienced recent population expansion (Table 3). According to the Extended Bayesian skyline plot (EBSP), *Belostoma angustum* effective population size (N_e) started an expansion at about 50,000 years ago. During the Last Glacial Maximum (LGM), at about 23,000–17,000 years ago, N_e seems to have stabilized. After the LGM, in the late Pleistocene (15,000–12,000 years ago) we detected a marked population growth in *B. angustum* from Pampas highlands, corroborating the by neutrality tests and estimated mismatch distributions. The historical panmictic population trend seems to indeed fit the climate changes in Pleistocene periods in the Pampas.

The reduced growth during LGM can be explained by the cold and dry conditions at this period in the sampled area. The transition from the late glacial to the Holocene, according to radiocarbon date, occurred 10,460 years ago, and reflects a change from cold and dry to warm and dry conditions (Behling, 2005). In this period, the Pampas region in Rio Grande do Sul state was naturally covered by Campos vegetation (Behling, 1997, 2002, 2005). A similar conclusion for the Pampas from Argentina was found by Iriondo and Garcia (1993), which suggested that during the late Pleistocene and into the early Holocene (18,000–10,000), the Pampas were arid and cool. The past climatic conditions certainly affected *B. angustum* populations because the number of freshwater habitats available and resources of food potentially decreased. These conditions were not enough to lead this species to a local extinction, nor were they able to result in relatively deep splits of populations. We believe that the high capacity of migration by flight of *B. angustum* specimens could provide them the

possibility to find new and farther remaining habitats, keeping high rates of gene flow. The grasslands vegetation could be another factor which would facilitate the long flight because of the lower environmental physical resistance. Therefore, if gene flow remains strong compared to drift, a pattern reflecting panmixia would persist, the homogenizing effects of gene flow would spread genetic variants throughout the region without regard to the extent of geographic separation (Hutchison and Templeton, 1999).

During the early Holocene (18,000–10,000), the Campos vegetation still dominant, the subtropical gallery forests were not apparent and, but the increased presence of Cyperaceae reflects the development of wetlands (Behling, 2005). The marked recent panmictic population expansion of *B. angustum*, according to EBSP analysis, started about 15,000 years old, after LGM, but before Holocene period when the conditions were more wet, as proposed by Behling (2005). However, the highest density of coalescent events observed was about 12,000, when the climatic conditions were already warmer and wetter than LGM. These conditions resemble current ones and may have favored the demographic expansion due to the increasing potential habitats and food resources. The recent marked demographic expansion could explain the high percentage of the exclusive haplotypes and the very low mutational steps among them, in the mitochondrial and nuclear haplotype networks.

5.3. Morphological variation

We found variation in the dimensions of *B. angustum* body size throughout populations in the Pampa highlands. Based on the average total body length (TBL), largest width of hemelytron (LWH) and interocular width (IW) in each population, we identified that specimens are larger in the Sul-Rio-Grandense highland than other areas. Although there is variation in these body traits among populations and other sources (such as drainage basins and highlands), the variation found was higher within populations, reaching 80% or more, indicating strong individual variation. Previous studies on body size variation in the giant water bugs indicated phenotypic plasticity driven by quantity and quality of food (Cullen, 1969; Pereira and Melo, 1998) or temperature (Pelegrin, 2006). In fact, analyzing the current climate variables of Pampas region, we detected higher daytime temperature in South-Rio-Grandense highland than in the other areas. So, the higher daytime temperature in this region could affect directly the food abundance and consequently the increase of the body lengths of individuals in this populations. Considering the phenotypic plasticity in these organisms, a different result may be possible, depending on the food resources and climate conditions. The sexual dimorphism

in body size is common in insects, the male is usually smaller than the female, which reflects adaptation to their different reproductive roles (Fairbairn 1997).

Leg length variation was only detected within populations, as well as sexual dimorphism, with no sign of variation among populations. Sexual dimorphism in legs is a characteristic well known to all *Belostoma* species, which could reflect a generalized status in this genus. In general terms, the middle and hind legs are relatively longer in males compared to females (Iglesias, 2012). Males of most back-brooding genera (belostomatid males carrying eggs on back) aerate eggs through brood pumping, an active pushup behavior that increases the water flow over the surface of eggs. In addition to pushups, males aerate eggs by brood-stroking, or rhythmically brushing their hind legs over their charges, again to increase water flow over the brooded eggs (Smith, 1976). This behavior was observed in *B. angustum* during the collections of the specimens. In fact, the legs variation is not structured by populations, but caused by individual factors, such as body dimensions and indirectly by the back-brooder behavior (results not shown).

Our findings suggest the existence of variation among populations in the shape of diverticulum of the phallosoma (DV), although it is not clear because the explanation by source of variations is very low (Table 5). In the CVA analysis it was not possible to classify correctly the shapes across populations. When considering population averages, DV did not show geographic variation. Size of the male genitalia did not exhibit spatial autocorrelation nor a clear pattern of differentiation between population groups. There are several mechanisms potentially involved in the size and shape variation of the male genitalic traits. Three main hypotheses have been proposed: the lock-and-key hypothesis, the pleiotropy hypothesis, and the sexual selection hypothesis (reviewed by Eberhard, 1985; Arnqvist, 1997).

5.4. Linking historical, genetic and morphological variation in the present and in the future

The genetic data pointed to a lack of population structure in *Belostoma angustum* throughout the Pampas highlands, which can be interpreted as panmictic population across the evaluated range maintained by migration and therefore gene flow. Phenotypic uniformity among populations will be more likely if gene flow homogenizes populations (Lenormand, 2002). When environmental conditions are temporally and spatially consistent, phenotypic variation may become fixed at an optimum, without phenotypic variation across a species range (Kawecki and Ebert, 2004; Zamudio, 2016). Phylogeographic structure will not accumulate

over time if individuals consistently migrate and exchange alleles with other populations (Zamudio et al., 2016). Moreover, the climatic changes over the past, in last geological period, have profound effects on the demography of many extant species, as well as, in the patterns and levels of genetic diversity of them (Hewitt, 1996; Avise, 2000).

Most of the morphological variation of *B. angustum* throughout Pampas highlands reflected the underlying absent of population genetic structure. In the body size, specially, there is variation among populations potentially explained by phenotypic plasticity under observed spatially different environmental regime, thereby generating phenotypic diversity without genetic differentiation.

Our genetics findings indicate indirectly that representatives of *Belostoma angustum* species are strong fliers. However, expanding the phylogeographic studies for the other species of genus *Belostoma* might reveal distinct patterns of population structure. Perez Goodwyn (2001) found different levels of regression of flight muscles in *B. elegans* (Mayr, 1871) (TBL = 19.0 mm and 22.0 mm) and *B. oxyurum* (Dufour, 1863) (TBL = 15.0 mm to 18.0 mm), concluding that the first species has a higher flight capacity than second due to the presence of larger and non-degenerate muscles. Therefore, the distinct migration ability by flight capacity could reflect the idiosyncratic phylogeographic pattern in *Belostoma* species or for each group of species (according Lauck, 1964). Furthermore, *Belostoma* species experienced different past climate regimes based on current known occurrence. For example, species occurring in temperate region and tropical region of the South America or species occurring in South America and North America. The historical climate regimes probably affected the historical demography of these species and resulted in distinct phylogeographical patterns.

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SUPPLEMENTARY INFORMATION

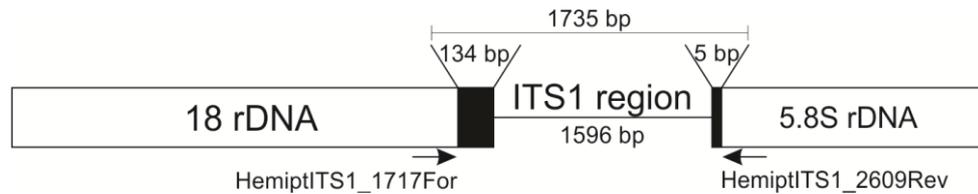


Figure S1. Schematic representation of rDNA specific region (18S – 5.8S) indicating the primers designed to amplify Internal transcribed spacers ITS1 in *Belostoma angustum* specimens.

Description of newly set of ITS1 primers

After sequence edition, we obtained 1735 base pairs (bp) from this region including 134 bp from conservative region of the 18S rDNA and five bp from conservative region of the 5.8S rDNA. This set of primers was designed based on eight previous hemipteran sequenced regions (partial sequence of 18S rDNA + ITS1 + 5.8S + ITS2 + partial sequence of 28S rDNA) available in GenBank. This target region was successfully amplified and sequenced using the newly ITS1 primers. The ITS1 region is quite variable across hemipteran insects based on previous GenBank sequences (e.g. 186 bp in Mesoveliidae [KJ461234] and 1140 bp in Reduviidae [KM278219]). In *Belostoma angustum* (Belostomatidae) we determinate the length of 1596 bp for this region.

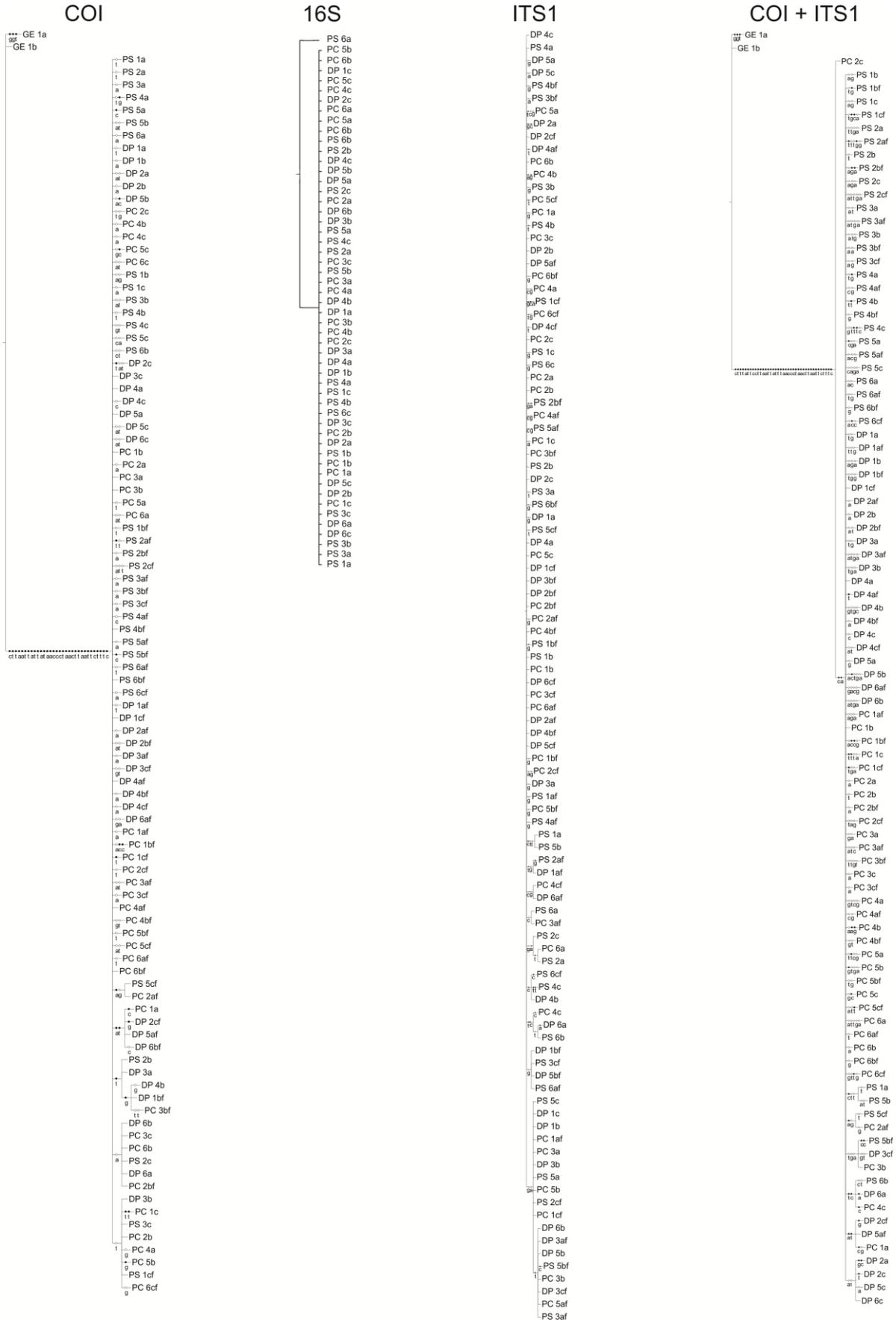


Figure S2. Gene trees based on parsimony criterion from *Belostoma angustum* genetic dataset. PS = South-Rio-Grandense highland, PC = Campanha highland; DP = valley located between highlands.

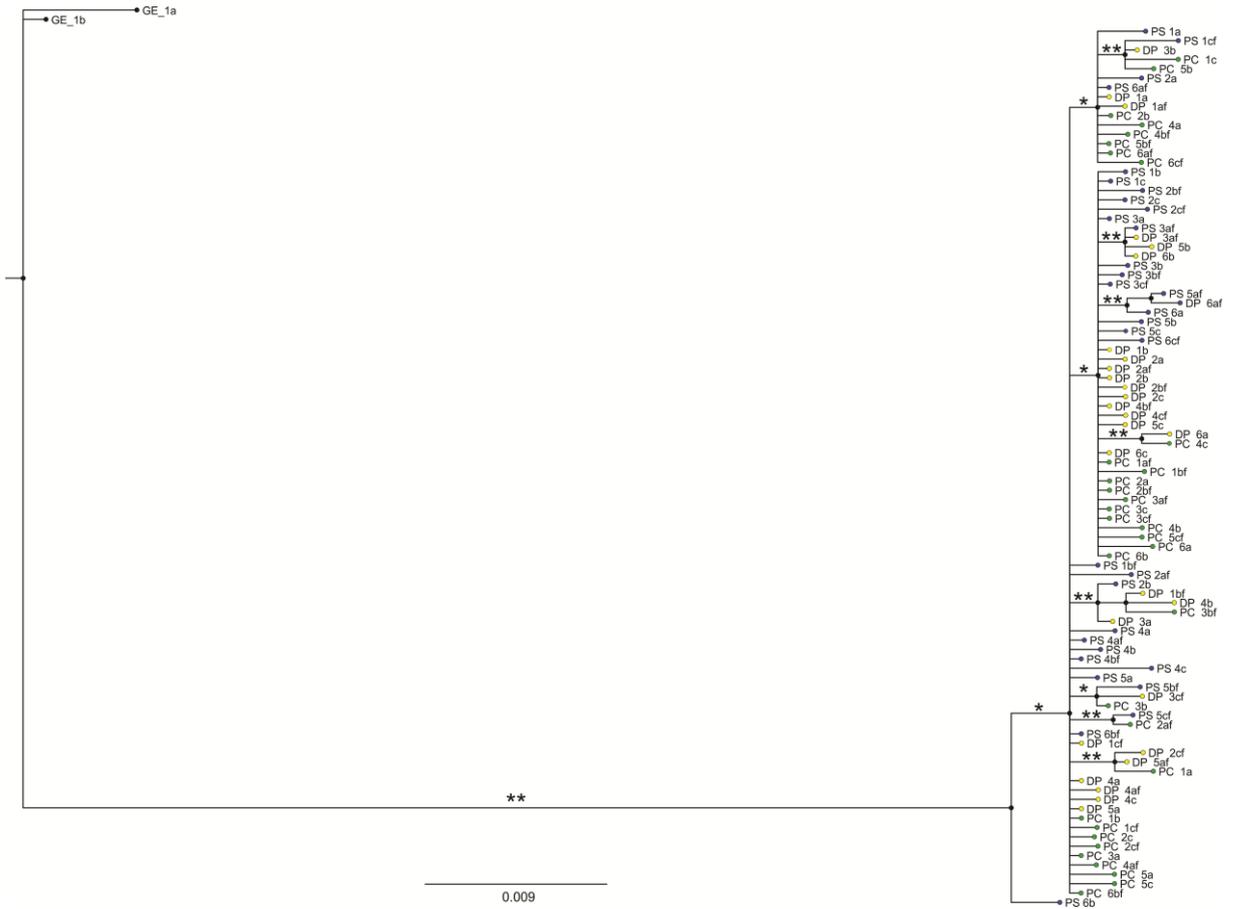


Figure S3. Combined COI + ITS1 with Bayesian inference. PS = South-Rio-Grandense highland, PC = Campanha highland; DP = lowland located between highlands. The * indicate the posterior probabilities: * > 0.8; ** > 0.99.