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**Desvinculando as etapas da especiação: fatores geográficos levam a
mudanças no sinal sexual de uma rãzinha-de-liteira por meio da variação
do tamanho corporal**

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Manaus, Amazonas

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Sinopse:

Estudamos a variabilidade multicaráter (genética, acústica e morfológica) e sua relação com o gradiente ambiental ao longo de um transecto latitudinal. Foi avaliado como a distância ambiental afeta o fenótipo e genótipo e suas implicações para diversificação populacional da espécie *Allobates sumtuosus* (Aromobatidae).

Palavras-chave: Amazônia, Anura, distancia ambiental, evolução, fenótipo, genótipo.

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RESUMO

As distâncias geográfica e ambiental influenciam a divergência de caracteres entre populações biológicas, especialmente em escala macroespacial, dificultando a interpretação da contribuição individual dessas variáveis preditoras no processo de diferenciação entre populações. Os anuros são excelentes modelos para estudos evolutivos de vários caracteres, devido à sua baixa vagilidade e frequente territorialidade, fazendo com que certas mudanças ambientais resultem em barreiras que isolam populações. Nesse sentido, propomos testar a correlação de distâncias ambientais e geográficas com caracteres fenotípicos e genotípicos populacionais na ausência de barreiras vicariantes evidentes, usando como modelo de estudo um sapo de liteira na Amazônia. Testamos a hipótese geral de que as diferenciações geográficas e ambientais afetam a variação nos caracteres morfométricos, acústicos e genéticos de *Allobates sumtuosus* ao longo de um gradiente latitudinal em uma escala espacial fina. Todos os conjuntos de dados, com exceção dos que envolvem distância genética, apresentaram correlação. Controlando o efeito dos diferentes conjuntos de dados, observamos que a distância geográfica apresentou o maior número de correlações, com uma força explicativa sempre superior a 60%. A distância geográfica associada à distância ambiental provavelmente molda os caracteres acústicos das populações de *A. sumtuosus* por meio de pressões no tamanho corporal. Isso ocorre porque o gradiente ambiental climático ao longo do gradiente latitudinal resulta em alterações na relação área-volume dos indivíduos, maximizando a taxa de sobrevivência e resultando em tamanhos corporais maiores em locais mais próximos do Equador. Conseqüentemente, os sinais acústicos divergem devido a variações nos órgãos envolvidos na vocalização. Apesar de mostrar a presença de uma estrutura genética intrapopulacional para esta espécie, essa estrutura não foi associada a variações fenotípicas. Em resumo, este estudo divide os estágios de especiação em um sapo da Amazônia, demonstrando que fatores ambientais podem levar a alterações no sinal sexual de uma rãzinha-de-liteira devido à variação no tamanho do corpo.

ABSTRACT

Geographical and environmental distances influence the divergence of characters between biological populations, especially on a macro spatial scale, making it difficult to interpret the individual contribution of these predictor variables in the process of differentiation between populations. Anurans are excellent models for multi-character evolutionary studies, due to their low vagility and frequent territoriality, causing certain environmental changes to result in barriers that isolate populations. Accordingly, we propose to test the correlation of environmental and geographical distances with phenotypic and genotypic population characters in the absence of evident vicariant barriers using as a study model an Amazonian litter-frog. We tested the general hypothesis that geographic and environmental differentiations affect the variation in the morphometric, acoustic and genetic characters of *Allobates sumtuosus* along a latitudinal gradient at a fine spatial scale. All data sets with the exception of those involving genetic distance showed correlation. Controlling the effect of the different data sets, we observed that the geographical distance showed a greater number of correlations, with an explanatory force always greater than 60%. The geographical distance associated with the environmental distance likely shapes the acoustic characters of populations of *A. sumtuosus* by means of pressures on body size. This is because the climatic environmental gradient along the latitudinal gradient results in changes to the area-volume relationship of individuals, maximizing survival rate and resulting in larger body sizes at locations closer to the Equator. Consequently, the acoustic signals diverge due to variations in the organs involved in vocalization. Despite showing the presence of an intrapopulation genetic structure for this species, this structure was not associated with phenotypic variations. In summary, this study breaks down the stages of speciation in an Amazonian frog, demonstrating that environmental factors can lead to changes in the sexual signal of a litter-frog due to the variation in body size.

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INTRODUÇÃO GERAL

A diversificação intraespecífica é altamente influenciada por fatores da paisagem como a heterogeneidade ambiental e barreiras vicariantes ao fluxo gênico (Leão-Pires et al. 2018; Maia-Carvalho et al. 2018). O papel da paisagem como indutora de diferenças populacionais, podendo levar a especiação, e assim afetando a composição das assembleias locais, tem sido amplamente testado e corroborado em regiões tropicais (Morales-Jimenez, et al. 2015; Dal Vechio et al. 2018; Naka and Brumfield 2018; Ribas et al. 2018). Tanto a capacidade adaptativa dos organismos quanto a deriva podem resultar em diferenciações fenotípica e genotípica entre populações, as quais podem experimentar restrição ou até mesmo interrupção de fluxo gênico entre si, levando à formação de novas espécies (Tryjanowski et al. 2006; Cortázar-Chinarro et al. 2017; van Rensburg et al. 2018). Além disso, o surgimento de novidades evolutivas não obedece a uma ordem fixa ao longo do processo de separação de linhagens, de modo que a seqüência de aparecimento de novidades evolutivas é essencialmente idiossincrática (De Queiroz 2007). Contudo, estudos multicaráter abordando diferenciação entre populações distribuídas ao longo de paisagens contínuas (i.e. na ausência de barreiras vicariantes ou mudanças ambientais abruptas) ainda são escassos.

Tanto a distância geográfica quanto a ambiental influenciam a divergência de caracteres entre populações biológicas. Normalmente, estes fatores variam em conjunto, especialmente quando analisadas em uma macro escala espacial, tornando difícil a interpretação da contribuição individual destas variáveis preditoras no processo de diferenciação das populações (Kaefer et al. 2013; Maia et al. 2017). Desta forma, entender o efeito desses fatores sobre múltiplas classes de caracteres em menores escalas espaciais, auxilia na compreensão do papel da paisagem na evolução de traços genéticos e fenotípicos das populações (Mullen et al. 2009; Spurgin et al. 2014; Valenzuela-Sánchez et al. 2015).

Anfíbios anuros são excelentes modelos para estudos evolutivos multicaráter, já que a sua baixa vagilidade, frequente territorialidade e baixa capacidade dispersiva (Zimmerman and Bierregaard 1986; Ernst and Rödel 2008; Menin et al. 2007; Keller et al. 2009) tendem a gerar marcantes assinaturas de variações espaciais e ambientais em suas características fenotípicas e genotípicas. Diversos estudos em fina escala espacial demonstram que anfíbios tendem a possuir populações altamente estruturadas geneticamente, com fluxo gênico diminuindo conforme o aumento da distância geográfica entre estas populações (Newman and Squire 2001; Lampert et al. 2003; Marchesini et al. 2017; Kobayashi et al. 2018). Isto é intensificado pelo

fato de anuros possuírem sítios reprodutivos espacialmente restritos, fazendo com que alterações ambientais resultem em barreiras intransponíveis para indivíduos, isolando populações (Kobayashi et al. 2018). Entretanto, poucos estudos têm abordado a distância geográfica e seu efeito sobre as mudanças fenotípicas e genotípicas de anuros e outros estudos em relação a distância ambiental, porém abordada de forma categórica (Kaefer and Lima 2012; Maia et al. 2017; Ortiz et al. 2018).

Além disso, anuros são apropriados para estudos integrativos pelo fato de se reconhecerem e selecionarem reprodutivamente por meio de sinais vocais (Beebee 2005; Zeisset and Beebee 2008). As características dos cantos desses organismos estão intimamente ligadas ao seu tamanho corporal (Hoskin et al. 2009; Gingras et al. 2013), já que traços espectrais são afetados pelo tamanho dos órgãos envolvidos na vocalização (e. g. massa das cordas vocais, musculatura da laringe) que por sua vez estão correlacionados com o tamanho do corpo (Martin 1972; Jaramillo et al. 1997). Sabe-se que a relação entre tamanho corporal e frequência do canto é negativa: indivíduos ou espécies de tamanhos corporais maiores tendem a cantar em faixas de frequência mais baixas (Gingras et al. 2013, Tonini et al. 2020). Diferenças nos sinais acústicos podem afetar os limites de reconhecimento no espaço acústico (Amézquita et al. 2011). Desse modo, a falta de reconhecimento ou seleção do sinal sexual do macho por parte da fêmea pode levar a processos de especiação pela formação de barreira reprodutiva pré-zigótica (Boul et al. 2006).

Fatores ambientais de ordem estrutural (e.g. vegetação e solo), e climática (e.g. temperatura e precipitação) atuam diretamente sobre a ocorrência e abundância de anuros amazônicos, potencialmente facilitando ou restringindo o fluxo gênico interpopulacional (Maia et al. 2017; Ortiz et al. 2018; Ferreira et al. 2018). A heterogeneidade ambiental ao longo de gradientes latitudinais pode afetar o tamanho corporal de anuros, onde variações na temperatura e precipitação são as principais reguladoras deste caráter, bem como da duração da estação reprodutiva e disponibilidade de presas (Ficetola and Maiorano 2016). Ainda, populações de anuros que vivem em locais com maior sazonalidade de temperatura apresentam maior tamanho corporal médio (Valenzuela-Sánchez et al. 2015).

OBJETIVOS

Propomos testar a correlação entre distâncias ambiental e geográfica na ausência de barreiras vicariantes evidentes e caracteres populacionais fenotípicos e genotípicos usando como modelo de estudo um anuro de liteira amazônico. Nosso principal objetivo é testar a hipótese geral que as diferenciações geográfica e ambiental afetam a variação nos caracteres morfométricos, acústicos e genéticos de *A. sumtuosus* ao longo de um gradiente latitudinal em fina escala espacial. Nossas previsões são: (1) as distâncias geográfica e ambiental serão positivamente relacionadas com a diferenciação em tamanho corporal, o qual, por sua vez, deverá ser negativamente relacionado com a frequência do sinal sexual acústico; (2) as distâncias fenotípicas (i.e. tamanho corporal e frequência do sinal sexual) serão positivamente relacionadas à distância genética entre as populações amostradas, indicando uma variação conjunta de genótipo e fenótipo.

Capítulo 1

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Unlinking the speciation steps: geographical factors drive changes in sexual signals of an Amazonian nurse-frog through body size variation

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Abstract

Geographical and environmental distances influence the divergence of characters between biological populations, especially on a macro spatial scale, making it difficult to interpret the individual contribution of these predictor variables in the process of differentiation between populations. Anurans are excellent models for multi-character evolutionary studies, due to their low vagility and frequent territoriality, causing certain environmental changes to result in barriers that isolate populations. Accordingly, we propose to test the correlation of environmental and geographical distances in the absence of evident vicariant barriers with phenotypic and genotypic population characters using as a study model an Amazonian litter-frog. We tested the general hypothesis that geographic and environmental differentiations affect the variation in the morphometric, acoustic and genetic characters of *Allobates sumtuosus* along a latitudinal gradient at a fine spatial scale. All data sets with the exception of those involving genetic distance showed correlation. Controlling the effect of the different data sets, we observed that the geographical distance showed a greater number of correlations, with an explanatory force always greater than 60%. Therefore, we suggest that there is a combined effect of geographical and environmental distances on phenotypic characteristics in *A. sumtuosus*. These distances shape the acoustic characters of this species through pressures on body size. This is because the climatic environmental gradient occupied by the species promotes changes in the area-volume relationship of individuals, resulting in larger body sizes towards the Equator. Additionally, a regression analysis showed that larger body sizes resulted in lower-spectral frequency acoustic sexual signals. Although we observed a pronounced intrapopulation genetic structure, it was not associated with phenotypic variations. In summary, this study breaks down the stages of speciation in an Amazonian frog, demonstrating that environmental factors lead to changes in the sexual signal of a litter frog due to the variation in body size.

Introduction

Intraspecific diversification is highly influenced by landscape factors such as environmental heterogeneity and vicariant barriers to gene flow ([Leão-Pires et al. 2018](#); [Maia-Carvalho et al. 2018](#)). The role of the landscape as an inducer of population differences, which can lead to speciation, and thus affect local assembly species composition, has been widely tested and corroborated in tropical regions ([Morales-Jimenez et al. 2015](#); [Dal Vechio et al. 2018](#); [Naka and Brumfield 2018](#); [Ribas et al. 2018](#)). Both the adaptive capacity of organisms and genetic drift can result in phenotypic and genotypic differentiations between populations, which may experience restriction or even interruption of gene flow between them, leading to the formation of new species ([Tryjanowski et al. 2006](#); [Cortázar-Chinarro et al. 2017](#); [van Rensburg et al. 2018](#)). In addition, the stages of differentiation of classes of genotypic and phenotypic characters do not obey a fixed order throughout the process of lineage separation, so that the sequence of appearance of evolutionary novelties is essentially idiosyncratic ([De Queiroz 2007](#)). In this context, multi-character studies addressing differentiation between populations spread across continuous landscapes (i.e. in the absence of biogeographical barriers or abrupt environmental changes) are still scarce.

Both geographic distance and environmental variation influence the divergence of characters between biological populations. Usually, these factors co-vary, especially when analyzed at a macro-spatial scale, making it difficult to interpret the individual contribution of predictor variables in the population differentiation process ([Kaefer et al. 2013](#); [Maia et al. 2017](#)). Thus, understanding the effect of such factors on multiple character classes at smaller spatial scales helps clarify the role of the landscape in the evolution of genetic and phenotypic traits of populations ([Mullen et al. 2009](#); [Spurgin et al. 2014](#); [Valenzuela-Sánchez et al. 2015](#)).

Anuran amphibians are excellent models for multi-character evolutionary studies, since their low vagility and frequent territoriality, associated with a short generation time ([Zimmerman and Bierregaard 1986](#); [Ernst and Rödel 2008](#); [Menin et al. 2007](#); [Keller et al. 2009](#)), tend to generate remarkable signatures of spatial and environmental variations in their phenotypic and genotypic characteristics. Several studies at the fine spatial scale demonstrate that amphibian populations tend to be highly structured genetically, with gene flow decreasing as the geographical distance between these populations increases ([Newman and Squire 2001](#); [Lampert et al. 2003](#); [Marchesini et al. 2017](#); [Kobayashi et al. 2018](#)). This is intensified by the fact that frogs have spatially restricted reproductive sites, causing environmental changes to result in insurmountable barriers for individuals, further isolating populations ([Kobayashi et al. 2018](#)). However, few studies have tested how phenotypic and genotypic changes in anurans occur in relation to geographical and environmental distances, and often the landscape is simply treated categorically ([Kaefer and Lima 2012](#); [Maia et al. 2017](#); [Ortiz et al. 2018](#)).

In addition, anurans are suitable for integrative studies because they recognize and selectively reproduce through vocal signals ([Beebee 2005](#); [Zeisset and Beebee 2008](#)). The characteristics of the call of these organisms are allometrically linked to their body size ([Hoskin et al. 2009](#); [Gingras et al. 2013](#)), since spectral features are affected by the size of the organs involved in producing vocalizations (*e.g.* mass of vocal cords, laryngial musculature) which are, in turn, correlated with body size ([Martin 1972](#); [Jaramillo et al. 1997](#)). It is known that the relationship between body size and call frequency is negative: individuals or species with larger body sizes tend to call at lower frequency ranges ([Gingras et al. 2013](#)). Differences in the acoustic signals can affect the recognition limits in the acoustic space ([Amézquita et al. 2011](#)). Thus, the lack of recognition or selection of the male's sexual signal by the female can lead to speciation processes via the formation of a pre-zygotic reproductive barrier ([Boul et al. 2006](#)).

Environmental factors of structural (*e.g.* vegetation and soil), and climatic natures (*e.g.* temperature and precipitation) act directly on the occurrence and abundance of Amazonian anurans, potentially facilitating or restricting the interpopulation gene flow ([Maia et al. 2017](#); [Ortiz et al. 2018](#); [Ferreira et al. 2018](#)). Environmental heterogeneity along latitudinal gradients can affect the body length of frogs, where variations in temperature and precipitation are the main regulators of this character, since they control important aspects of life-history, such as reproductive season duration and prey availability ([Ficetola and Maiorano 2016](#)). Also, populations of frogs that occupy environments with greater temperature seasonality have a larger average body size ([Valenzuela-Sánchez et al. 2015](#)).

Accordingly, we propose to test the correlation of environmental and geographical distances in the absence of evident vicariant barriers with phenotypic and genotypic population characters using as a study model an Amazonian litter-frog. Our main objective was to test the general hypothesis that geographic (drift) and environmental (selection) differentiations affect the variation in *A. sumtuosus* morphometric, acoustic and genetic characters along a latitudinal gradient at a fine spatial scale. Our predictions were: (1) Geographic and environmental distances will be positively related with differentiation in body size, which should be negatively related to the frequency of an acoustic sexual signal; (2) Increase in phenotypic distances (*i.e.*, body size and frequency of sexual signal) will imply an increase in genetic distance between sampled populations.

Material and Methods

Study area

This study was carried out along a latitudinal gradient composed of sampling points in the northern and southern hemispheres of the Amazonian biome ranging 446 km, in the Brazilian states of Amazonas and Roraima (Figure 1, Table 1). The vegetation matrix that covers the

sampling area, as well as a large part of the latitudinal segment, is low-lying humid tropical forest, characterized by a closed canopy and a poorly-lit understory with abundant palm trees ([Ribeiro et al. 1999](#)). According to the climate classification proposed by [Peel et al. 2007](#), extent of the sampling area are the Af domain (tropical rainforest) in the southern hemisphere and the Am domain (tropical monsoon) in the northern hemisphere sector. The rainy season in the southern hemisphere extends from December to March, while in the northern hemisphere rains peak in mid-June and July. Field data collection took place from January to July 2019, in the morning (05:30–11:00 h), peak time of vocal activity of the study species ([Simões et al. 2013](#)).

Model species

The species *Allobates sumtuosus* ([Morales 2002](#)) (Anura, Dendrobatoidea, Aromobatidae) is terrestrial and diurnal and, as is common for members of the genus, reproduces mainly at the peak of the rainy season ([Simões et al. 2013](#)). In the reproductive period, males vocalize in the vicinity of isolated puddles, mainly in riparian environments ([Jorge et al. 2016](#)). The species was chosen because of its functional characteristics, including small size, reproduction with egg deposition in leaf litter, and high territoriality of males, which have small home ranges as well as occurring in a larger area that involves latitudinal environmental gradients ([Simões et al. 2013](#)). Such characteristics are known to reduce individual dispersion and promote molecular and/or phenotypic differentiation in the face of physical or environmental barriers ([Moraes et al. 2016](#)), and so being appropriate for studies involving historical and ecological biogeography.

Recently, the taxonomic status of the taxon *Allobates sumtuosus* has been reevaluated, and characteristics such as advertisement call, color-in-life, larval morphology and reproductive aspects have been explored in unprecedented detail ([Simões and Lima 2012](#); [Simões et al. 2013](#)). The attribution of several new populations under this name resulted in an expansion of

its geographical distribution to an extensive portion of the Amazonian biome ([Simões et al. 2013](#)). The type locality for *A. sumtuosus* is the Trombetas River Biological Reserve, Pará, Brazil, and it occurs in other locations in the northern region of Brazil, in the states of Pará, Amazonas and Roraima ([Simões et al. 2013](#)), via Guyana ([Alonso et al. 2016](#)) to Suriname ([Fouquet et al. 2015](#)), while records of the species in the western Amazon (Peru) need to be reexamined as they constitute potentially the result of erroneous taxonomic designation ([Simões et al. 2013](#)).

Morphological data

The individuals collected were sacrificed by applying a topical anesthetic (5% benzocaine) to the ventral surface, labeled, fixed in 10% formaldehyde, and then preserved in 70% ethanol. With the aid of a Leica stereomicroscope (model S8APO) coupled to a Leica DFC295 camera, the following measurements were made with a precision of 0.001 mm (except for SVL, which was measured with digital calipers at a precision of 0.01 mm) following [Simões et al. \(2013\)](#): snout-to-vent length (SVL), length of the head from the tip of the snout to the posterior edge of the maxillary joint (HW), width of the head at the level of the maxillary joint (HL), length from the tip of the snout to the anterior corner of the eye (SL), eye-nostril distance from the anterior corner of the eye to the center of the nostril (EN), internarial distance (IN), eye length from the anterior to the posterior corner (EL), interorbital distance (IO), diameter maximal tympanum (TYM), length of the forearm from the proximal edge of the palmar tubercle to the outer edge of the flexed elbow (FAL), length of the arm from the front of the corner of the arm insertion to the outer edge of the flexed elbow (UAL), lengths from the proximal edge of the palmar tubercle to fingertips I, II, III and IV (respectively HAND I, HAND II, HAND III, HAND IV), width of the disc on finger III (WFD), width of the third phalanx of finger III (WPF), maximum diameter of the palmar tubercle (DTP), maximum diameter of the tenar tubercle (DTT), leg length from the posterior end of the coccyx to the outer edge of the flexed knee (LL), tibia

length from the outer edge of the knee to the flexed heel (TL), foot length from the proximal edge of the external metatarsal tubercle to the tip of the toe IV (FL) and width of the disc on toe IV (WTD). Specimens were deposited in the Amphibian and Reptile Collection of the Brazilian National Institute of Amazonian Research (See Supplementary Material).

Acoustic data

Vocalizations of collected individuals were recorded on average for three minutes, using a Sony PCM – D50 digital recorder coupled to a Sennheiser ME66 directional microphone. Recordings were made approximately one meter away from the subject, and air temperature was measured after the recording ended. Calls were analyzed using Raven Pro software, version 1.5 ([Charif et al. 2010](#)), where 10 notes were sampled, evenly distributed across the recording time ([Simões et al. 2013](#)). To do this, we divided the total number of notes in each record by 10 and defined the resulting fraction as the sampling interval. For each record, we measured the note duration (ND), peak frequency (NPF), lowest frequency (NLF) and highest frequency (NHF) of the notes. Spectral analyzes were performed with a frequency resolution of 82 Hz and 2048 points, using the Blackman window format ([Kaefer and Lima 2012](#)). Frequencies emitted by the notes were measured 20 dB below the peak frequency, as this represents the lowest point at which the energy transmitted by vocalization is distinguishable from background noise during recordings ([Erdtmann and Amézquita 2008](#)). Air temperature was measured at the time of each recording and showed no relationship with acoustic properties of the calls ($p > 0.05$).

Genetic data

Muscle tissue samples were taken from the thighs of collected individuals and preserved in absolute alcohol, before fixing the animal. Extraction and isolation of genomic DNA followed the salt extraction protocol with a final volume of 50 μ L ([Aljanabi and Martinez 1997](#)). The extraction product was quantified in NanoDrop by the Nucleic Acid program where the

concentration of DNA in the solution ($\mu\text{g/ml}$) and concentration of impurities (proteins and carbohydrates remaining in the cell digestion process) were verified. The 16S region of the extracted DNA went through a mitochondrial DNA amplification process using the polymerase chain reaction (PCR), with primers 16sar (5'-CGCCTGTTTATCAAAAACAT-3') and 16sbr (5'-CCGGTCTGAACTCAGATCACGT-3') ([Palumbi 1996](#)). This gene was selected for its extensive previous use studies of amphibian taxonomy and phylogeography, showing satisfactory inter- and intraspecific resolution ([Vences et al. 2005](#); [Fouquet et al. 2017](#)). The PCR reaction material contained 4.4 μL of distilled and deionized water; 2.0 μL dNTPs; 2.3 μL MgCl₂; 1.5 μL of Tris-HCl buffer; 0.3 μL of BSA; 1.5 μL of each primer; 0.5 μL of Taq DNA polymerase and 1 μL of DNA. The thermocycling process was performed according to the following steps: initial DNA strand denaturation at 92°C for 30s; 35 denaturation cycles at 92°C for 10s, annealing primers to the DNA strand at 50°C for 35s, extension of the new free nucleotide strands and fragments at 72°C for 90s and final extension at 72°C for 10 min. PCR products were purified by reaction with EXO-SAP, following the protocol suggested by the manufacturer (Thermo Fisher). For sequencing reactions we use the Big Dye kit (Applied Biosystems), following the manufacturer's instructions. Sequenced products were precipitated in EDTA/ethanol and analyzed in an ABI3500 automatic capillary sequencer (Applied Biosystems). All procedures were performed at the Animal Evolution and Genetics Laboratory (LEGAL), at the Federal University of Amazonas (UFAM).

The new sequences obtained (n=39) were edited and inspected in Geneious Prime 2.3 program ([Kearse et al 2012](#)) and we complemented the molecular database with the download of 38 sequences of the species with precise location information deposited in the online GenBank repository, totaling 77 sequences. To align these sequences, we used MAFFT 7 program on an online platform ([Katoh et al. 2019](#)) with default parameters, except for the use of the E-INS-i strategy, due to the presence of multiple conserved domains and long gaps

([Kato and Standley 2013](#)). The new sequences generated were deposited on GenBank (see Supplementary Material for accession numbers).

Phylogenetic and population analyzes

We reconstructed phylogenetic trees implementing both Bayesian Inference and Maximum Likelihood. The GTR + G nucleotide substitution model was selected as the most suitable for molecular data according to the PartitionFinder 2.1.1 program ([Lanfear et al. 2017](#)) under Akaike's corrected information criterion (AICc; [Hurvich and Tsai 1991](#)). Sequences of *Allobates* species most closely related to *A. sumtuosus* (*A. bacurau* and *A. paleovarzensis*) were selected as external groups for analysis and obtained via GenBank. For the Bayesian analysis conducted in the MrBayes 3.2.6 program ([Ronquist et al. 2012](#)), we conducted four independent runs of 5 million generations, starting with random trees and four Markov chains, sampled every 1,000 generations, discarding 25% of generations and trees as burn-in. Parameter convergence (Estimated Sample Size, ESS > 200) was assessed using the Tracer 1.7 program ([Rambaut et al. 2018](#)). Maximum Likelihood analysis was conducted with the RaxML 8.2.10 program ([Stamatakis 2014](#)), researching the most likely tree 100 times and with support assessed through 1,000 non-parametric bootstrap repetitions.

The most likely number of genetic clusters formed by the sampled mtDNA sequences was inferred through a Bayesian analysis of population structure using the BAPS version 6 program ([Corander et al. 2008](#)). Based on the nucleotide frequencies, this model seeks to generate groups of individuals, so that those assigned to the same group are as genetically similar to each other as possible. We executed the mixture model with a range of 2–9 k and four replicates. The result of this model was used to run the admixture model ([Corander and Marttinen 2006](#)) using 100 iterations, 200 reference individuals and 10 iterations per individual. Values of $p < 0.05$ were considered significant evidence of admixture. To visualize the spatial structure among the

sampled haplotypes, we built a haplotype network with the HaploView 4.2 program ([Barrett et al. 2005](#)), using the top likelihood tree topology. Finally, we estimated the Φ_{ST} index of pairwise differentiation between the populations sampled using *adegenet 2.0.0* package of the R program ([Jombart 2008](#)).

Environmental variables

We used three environmental variables to understand how the latitudinal environmental gradient affects the *A. sumtuosus* phenotype and genotype: (1) average annual thermal amplitude, which directly affects the amphibian immune system and can alter the susceptibility of these organisms to diseases ([Raffel et al. 2006](#)); (2) Average Annual Precipitation, representing the relative humidity of the environment, which affects amphibians by maximizing or decreasing the rates of evaporation and loss of body water through their semipermeable skin ([Mitchell and Bergman 2016](#)); (3) Percentage of Sand, since soils of greater granulometry tend to retain less water and have more frequent saturation, which may affect the desiccation rate of *A. sumtuosus* eggs, which are deposited in leaf litter in locations with predominantly sandy soil ([Juo and Franzluebbers 2003](#)). In addition, *A. sumtuosus* has an occurrence relationship with bodies of water that are in turn correlated with higher percentages of sand as it approaches the water body ([Menin et al. 2011](#)).

We obtained the variables of mean annual thermal amplitude (BIO7) and mean annual precipitation (BIO12) for the period 1950-2000 from the online WorldClim database ([Hijmans et al. 2005](#)). The percentage of sand in the soil at a depth of 15 cm was obtained from the SoilGrids database ([Hengl et al. 2014](#)). All raster files used were interpolated for 30 arc-sec (~1km²), cut on the R platform ([R Team 2019](#)) using the "*raster*", "*sp*" and "*rgeos*" packages and the "*extend*" and "*crop*" functions, with the data extracted using the "*extract*" function ([Hijmans et al. 2015](#)).

Distance matrices

To construct a latitudinal distance matrix, we calculated the difference in latitude among all sampling points. For the geographic distance matrix, we calculate the linear distance in kilometers between sampling points with the aid of the Google Earth Pro program ([Google 2019](#)). To construct the genotypic distance matrix, we calculated uncorrected pairwise p-genetic distances, removing gaps through pairwise deletion with MEGA 6.6 ([Tamura et al. 2013](#)). For the other data classes (morphological, acoustic and environmental) distance matrices were generated using Principal Component Analysis (PCA) ([Jolliffe 2002](#)), which reduces the dimensionality of the data to points (scores) in a two-dimensional graph in which the main component axis 1 holds most of the important variations and similarities between the samples, and the consecutive main component axes tend to accumulate variations related to other factors ([Gauch Jr 1982](#)). From the PCA scores 1 and 2, we generated each Euclidean distance matrix pair by pair using the *vegan* package with the *dist* and *as.matrix* functions on the R platform ([R Team 2019](#)).

Statistical analyses

To access variation of environmental heterogeneity, geographic distance and latitudinal variation in relation to *A. sumtuosus* phenotypic and genotypic characters, we used the simple Mantel correlation test, using the Euclidean distance matrices of our groups of variables to test relationships that do not necessarily assume cause and effect (*e.g.* genotypic *vs.* phenotypic distances) ([Mantel 1967](#)). We also used partial Mantel tests to access correlation between two variables, while also controlling the effect of a third one (*e.g.* correlation between geographic and acoustic distances, excluding the effect of morphological distance), to disentangle the relative and joint force of geographic isolation and environmental heterogeneity impact on phenotypic and genotypic characters. We conducted both types of Mantel tests on the R platform using the *vegan* package with the functions *mantel* and *mantel.partial* ([R Team 2019](#)).

To access the relationship between the SLV and the peak frequency of the call, and the relationship between the frequency of the call and temperature, we performed simple regression analyses. In addition, we tested the direct effect of the latitudinal gradient on spectral characters of the acoustic signal of *A. sumtuosus* also through a linear regression test between latitude (predictor variable) and peak frequency of the call (response variable, which is correlated to all other measured spectral traits). These analyses were also conducted on the R platform using the *vegan* package.

Results

Phylogenetic and phylogeographic relationships

Both phylogenetic inference approaches generated congruent gene trees for the phylogenetic relationships of the analyzed sequences (Fig. 2). These analyzes recovered both the monophyly of *Allobates sumtuosus* (PP - posterior probability = 1.0, BS - bootstrap support = 100), and the presence of two reciprocally monophyletic subclasses contained in this name. Such subclades are segregated by a genetic distance of 3% and correspond to populations in the far West of the species distribution (states of Roraima and Amazonas) (PP = 0.9, BS = 80) versus the population at the type locality in the region of Rio Trombetas (TRM, State of Pará) (PP = 1.0, BS = 100). The different genotypic distances found in the species are equivalent to the intraspecific distances observed for other species of the genus *Allobates* ([Kaefer et al. 2013](#); [Maia et al. 2017](#)). Considering the West clade, despite their interrelationships receiving low support due to the high similarity between the sequences (maximum of 2% of genetic distance), a trend can be seen in the segregation of the sequences in our Northern sampling (SJB, NVC, VLE and VLJ, Roraima state) versus Center (VLN, Amazonas state) and South (BAL, PDF, STA, FUF, RDU AND UFA, Amazonas state), with the central population (VLN) being more genetically differentiated than the those in the second subgroup (Figure 2 and 3).

BAPS analysis resulted in the most likely number of genetic clusters (k) = 4, with no evidence of admixture, corresponding to the populations of: (1) Rio Trombetas (TRM, Pará state); (2) North; (3) Center; and (4) South (Figure 2). The obtained haplotypes network, corresponding to 13 different haplotypes, mirrored this result, with more subtle segregation between the North (2) and South (4) populations of the sample, and greater differentiation of those from the central population of our sample (3 - VLN) and from the Rio Trombetas (1 - TRM) (Figure 3). Results of the Φ_{ST} analysis demonstrate a high level of general genetic structuring among the populations (Table 2).

Correlation between environmental, geographical, phenotypic and genetic distances

Mantel tests showed correlation between all data sets, except those involving genetic distance matrices (Figure 4). Because the correlation between geographic distance and latitudinal distance was greater than 90%, and that geographical distance showed a greater number of correlations with the other matrices, we chose to exclude the latitudinal distance matrix from the other correlations to decrease the probability of type 1 error when making multiple statistical tests (Fig. 4i). When we controlled the effect of the data sets on the correlations in the partial Mantel test, we observed that the geographic distance matrix showed a higher number of correlations with the remaining matrices, with an explanatory force always above 60% (r 0.6; $p < 0.01$), with the exception of the correlation with the acoustic matrix excluding the effect of the morphology, which showed a determination coefficient of 29% ($r = 0.29$; $p < 0.05$) (Table 3). The relationship between SLV and peak calling frequency was 30% ($r^2 = 0.3$; $p < 0.01$) (Fig. 5a), while calling frequency and temperature showed no relationship ($r^2 = 0.05$; $p > 0.05$) (Fig. 5b). The effect of the latitudinal gradient about spectral characters of the acoustic signal showed force of 39% in the variation in peak frequency ($r^2 = 0.394$; $p < 0.05$) (Fig. 5c).

Discussion

We interpret the results of the correlational and regression test as a combined effect of geographic, latitudinal and environmental distance, both exerting pressure on the morphological and acoustic characteristics of the *A. sumtuosus* populations. Despite the genetic structure found in the present study, we did not find evidence that this structure was linked to phenotypic variations, a fact observed by the absent or small correlations between geographic and genetic distance. For geographic distance, although our study was performed at a spatial mesoscale, the effect is similar to that found in large scale studies that addressed the effect of latitude on species morphology, showing that there is a tendency for anurans to body-length increase in size when approaching the Equator ([Liu et al. 2018](#)). Environmental variations are also known as major drivers of phenotypic changes, since these characteristics provide the link between the evolutionary process and the environment, via the influences from physical-climatic factors ([Tryjanowski et al. 2006](#); [Ng et al. 2013](#)).

Our sampling extent comprised a climatic gradient in which precipitation levels tend to be lower at latitudes closer to the Equator (VLJ, VLE and NVC locations) and the average annual temperatures tend to be higher when compared to the locations sampled further south ([Liberato and Brito 2010](#)). It is known that these variables directly affect the body size of frogs, via loss- and gain-based thermal equilibria, modifying the surface-volume ratio of local populations. Larger body sizes have smaller area-volume ratios and this is advantageous in warmer and relatively drier habitats or those with an open vegetation matrix, as it counteracts the negative effect of water loss through skin evaporation, which in such case, will occur more slowly ([Bevier et al. 2008](#); [Mitchell and Bergman 2016](#)). The development of larger bodies can be explained by factors such as the extended longevity of individuals which, associated with a slower but steady growth rate, will lead to an increase in body volume ([Liao et al. 2012](#); [Liu et al. 2018](#)), this itself being selectively controlled by the climatic characteristics of the occupied

environment. Characteristics of each environment along the latitudinal gradient tend to select for different body length, which may, over time, lead to the expression of larger body length in *A. sumtuosus* populations closer to the Equator.

An increase in the body size of frogs is known to be able to promote changes in the acoustic characters, especially a general lowering on the frequency of the notes involved in calls (see [Gingras et al. 2013](#) for an example at the interspecific level). This allometric relationship was observed in *A. sumtuosus* study populations, as we found populations from the more southerly sample locations housed individuals of smaller body size, which tended to have acoustic sexual calls with a higher peak frequencies compared to northern locations. The most striking differences were noted when we compared the RDU locality population, in the south of the sampling area, which had a mean peak calling frequency of 6744 ± 336 Hz (6380 - 7080 Hz; $n = 8$), with population at locality NVC, to the north, whose peak frequency reached 6094 ± 246 Hz (5762 - 6546 Hz; $n = 7$). In a study involving the acoustic recognition space in dendrobatoids, including the genus *Allobates*, [Amézquita et al. \(2011\)](#) showed that the maximum limiting acoustic dis-similarity in this genus ('acoustic space') is around 1000 Hz at the intraspecific level. The existence in our study of such variations for *A. sumtuosus* populations indicate that the most acoustically divergent populations may be close to the limit of acoustic recognition at the intraspecific level. When we refined the results at an individual level, it became apparent that this intraspecific recognition limit was exceeded, since some individuals in the south of the sampled area have a peak frequency of 7181 Hz while some individuals in the north reach 5762 Hz, generating a 1419 Hz divergence between sexual signal frequencies. Thus, based on the results of [Amézquita et al. \(2011\)](#), we hypothesize that populations at the southern and northern ends of the sampled area would not recognize each other acoustically in case of secondary contact, but that need to be further tested.

The acoustic adaptation hypothesis proposes that in areas with high forest density, the spectral properties of the song tend to have lower values due to the fact that lower frequencies tend to be less attenuated and degraded in this type of environment allowing a more effective propagation of the sexual signal ([Morton 1975](#); [Erdtmann and Lima 2013](#)). However, when the effect of the environment on morphological and acoustic variation is also addressed, it can be seen that morphology is more affected by environmental variation than acoustic variation ([Bevier et al. 2008](#)), which supports our interpretation that environmental variation (in the case of the present study, latitudinal) acts as a mediator for morphological changes and that these, in turn, modulate the acoustic signal of *Allobates sumtuosus*.

As acoustic recognition is essential for the reproductive success of anurans ([Boul et al. 2006](#)), it is possible that populations at the northern and southern ends of *A. sumtuosus* in the sampled area may be isolated reproductively at present or over evolutionary time, thus achieving the pre-zygotic isolation criterion according to the classic (biological) species delimitation model ([De Queiroz 2007](#)). Alternatively, within the genus *Allobates*, there are interspecific overlaps in spectral acoustic characters, suggesting that sexual recognition and choice do not depend exclusively on acoustic characters, but on additional aspects such as visual and tactile signs ([de Luna et al. 2010](#); [Montanarin et al. 2011](#)), which still require study throughout the *A. sumtuosus* distribution.

Several studies have shown that the evolution of phenotypic and genotypic variation of single evolutionary entities with wide geographic distribution are often decoupled ([Amézquita et al. 2009](#); [Montanarin et al. 2011](#); [Duarte et al. 2015](#)). Sometimes, the evolutionary process of diversification is more quickly noticed in genotypic variation while at others it is in phenotypic, as the latter is more associated with selective environmental pressures ([Rojas et al. 2019](#)). Although we cannot rule out that the decoupling reported here between genotypic and

phenotypic variation is the result of bias arising from the choice of a non-coding mitochondrial molecular fragment, this has already been widely reported as a good predictor of *Allobates* intraspecific and interspecific diversification ([Simões et al. 2014](#); [Maia et al. 2017](#)). Thus, we suggest that, since there is an absence of a genetic signal in the process of *A. sumtuosus* morphometric and acoustic speciation that these characters are shaped primarily by the geographical and environmental distance between the sampled populations.

Conclusion

The geographical distance associated with the environmental distance likely shapes acoustic variation of populations of *A. sumtuosus* by means of pressures on body size. This is because the climatic environmental gradient along the latitudinal gradient results in changes to the area-volume relationship of individuals, maximizing survival rate and resulting in larger body sizes at locations closer to the Equator. Consequently, acoustic signals diverge due to variations in the organs involved in vocalization. Despite showing the presence of an intrapopulation genetic structure for this species, this structure was not associated with phenotypic variations. In summary, this study breaks down the stages of speciation in an Amazonian frog, demonstrating that environmental factors can lead to changes in the sexual signal of a litter-frog due to the variation in body size.

Conclusões

A distância geográfica associada à distância ambiental provavelmente molda os caracteres acústicos das populações de *A. sumtuosus* por meio de pressões no tamanho corporal. Isso ocorre porque o gradiente ambiental climático ao longo do gradiente latitudinal resulta em alterações na relação área-volume dos indivíduos, maximizando a taxa de sobrevivência e resultando em tamanhos corporais maiores em locais mais próximos do Equador. Consequentemente, os sinais acústicos divergem devido a variações nos órgãos envolvidos na vocalização. Apesar de mostrar a presença de uma estrutura genética intrapopulacional para esta espécie, essa estrutura não foi associada a variações fenotípicas. Em resumo, este estudo divide os estágios de especiação em um sapo da Amazônia, demonstrando que fatores ambientais podem levar a alterações no sinal sexual de uma rãzinha-de-liteira devido à variação no tamanho do corpo.

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Tables

Table 1. Locations sampled along the BR-174 highway, their respective acronyms used throughout the text, and number of samples obtained for the different sources of evolutionary data. Brazilian states: (AM) Amazonas; (PA) Pará; (RR) Roraima. Sample size molecular data: (NG) newly generated sequences, directly associated with morphological and acoustic data; (GB) sequences downloaded from GenBank.

Locality	Acronym	Coordinates	Morphology	Acoustic	mtDNA	
					NG	GB
Rio Trombetas (PA)	TRM	01°22'12"S, 56°51'08"W	–	–	–	4
Universidade Federal do Amazonas (AM)	UFA	03°06'0.85"S, 59°58'18.98"W	8	8	5	10
Reserva Florestal Adolpho Ducke (AM)	RDU	02°55'50.34"S, 59°58'28.68"W	8	8	5	3
Fazenda Experimental da UFAM (AM)	FUF	02°38'51.36"S, 60°03'13.43"W	7	7	6	18
Sítio Tamaga (AM)	STA	02°13'23"S, 60°03'55"W	–	–	–	1
Presidente Figueiredo (AM)	PRF	02°02'31.95"S, 60°01'46.98"W	7	7	5	–
Vila de Balbina (AM)	BAL	01°54'03.47"S, 59°24'52.12"W	7	7	4	–
Ramal Vila Nova - km 1062 (AM)	VLN	01°29'36.64"S, 60°14'25.12"W	7	7	4	–
Vila de Jundiá (RR)	VLJ	00°12'20.76"S, 60°41'46.54"W	7	7	6	–
Vila do Equador (RR)	VLE	00°07'24.93"N, 60°33'43.69"W	7	7	3	–
Nova Colina (RR)	NVC	00°34'55.23"N, 60°27'53.01"W	7	7	1	–
São João da Baliza (RR)	SJB	00°57'10"N, 59°55'43"W	–	–	–	2
Total			65	65	77	

Table 2. Pairwise Φ_{ST} fixation indexes (upper right matrix) and average (%) genetic distances (lower left matrix). Values calculated between the sampling localities for each *Allobates sumtuosus* population. NaN = Not sufficient data. See Table 1 for population acronyms.

Locality	UFA	RDU	FUF	PRF	BAL	VLN	VLJ	VLE	NVC
UFA	-	NaN	0.271	0.167	0.711	1.000	0.878	1.000	1.000
RDU	0.0	-	0.158	0.043	0.631	1.000	0.83	1.000	1.000
FUF	0.3	0.3	-	0.118	0.46	0.715	0.569	0.547	0.484
PRF	0.2	0.2	0.5	-	0.496	0.788	0.606	0.619	0.539
BAL	0.4	0.4	0.8	0.6	-	0.838	0.625	0.666	0.623
VLN	1.1	1	1.3	1.3	1	-	0.929	1.000	1.000
VLJ	0.5	0.5	0.9	0.8	0.5	1.1	-	0.156	0.319
VLE	0.3	0.3	0.6	0.8	0.7	0.6	0.0	-	NaN
NVC	0.4	0.6	0.4	0.9	1.1	0.4	0.1	0.2	-

Table 3. Simple Mantel (variable 1 x variable 2) and partial tests (variable 1 x variable 2. Covariate variable) correlating the geographical, environmental, phenotypic and genetic distances of *Allobates sumtuosus* from the sampled locations. Figures in bold indicate statistically significant relationships ($P \leq 0.05$). Model variables: (GeoD) geographical distance; (MorD) morphological distance; (GenD) genetic distance; (EnvD) environmental distance.

Model	R	P
GeoD x MorD	0.7628	0.001*
GeoD x AcoD	0.6496	0.009*
GeoD x GenD	0.1315	0.229
GeoD x EnvD	0.6368	0.011*
EnvD x MorD	0.5309	0.014*
EnvD x AcoD	0.3162	0.051*
EnvD x GenD	0.1303	0.291
MorD x AcoD	0.667	0.002*
GenD x AcoD	0.2714	0.072
GenD x MorD	-0.0324	0.538
MorD x AcoD . GeoD	0.3488	0.039*
MorD x AcoD . EnvD	0.637	0.002*
GeoD x GenD . EnvD	0.0634	0.356
GeoD x AcoD . MorD	0.2922	0.053*
GeoD x MorD . EnvD	0.65	0.001*
GenD x MorD . GeoD	-0.2071	0.825
GenD x MorD . EnvD	-0.121	0.704
GenD x AcoD . GeoD	0.2468	0.106
GenD x AcoD . MorD	0.3935	0.037*
GenD x AcoD . EnvD	0.2447	0.078
EnvD x AcoD . MorD	-0.04797	0.561

EnvD x AcoD . GeoD	-0.1649	0.809
EnvD x MorD . GeoD	0.0205	0.459
EnvD x GenD . GeoD	0.061	0.379

Figures

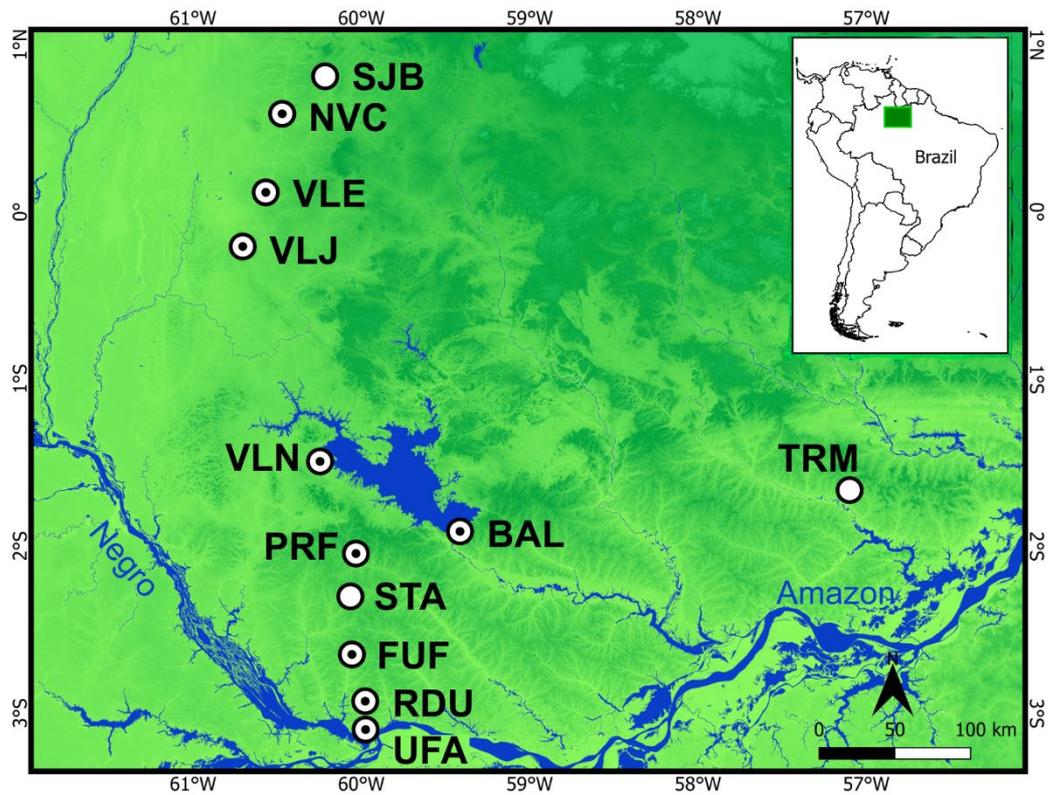


Figura 1. Geographical distribution of *Allobates sumtuosus* study sampling sites in northern South America. Black-dotted symbols indicate localities with associated morphology, acoustic and genetic data, and hollow symbols indicate localities represented only with genetic data. See Table 1 for population acronyms.

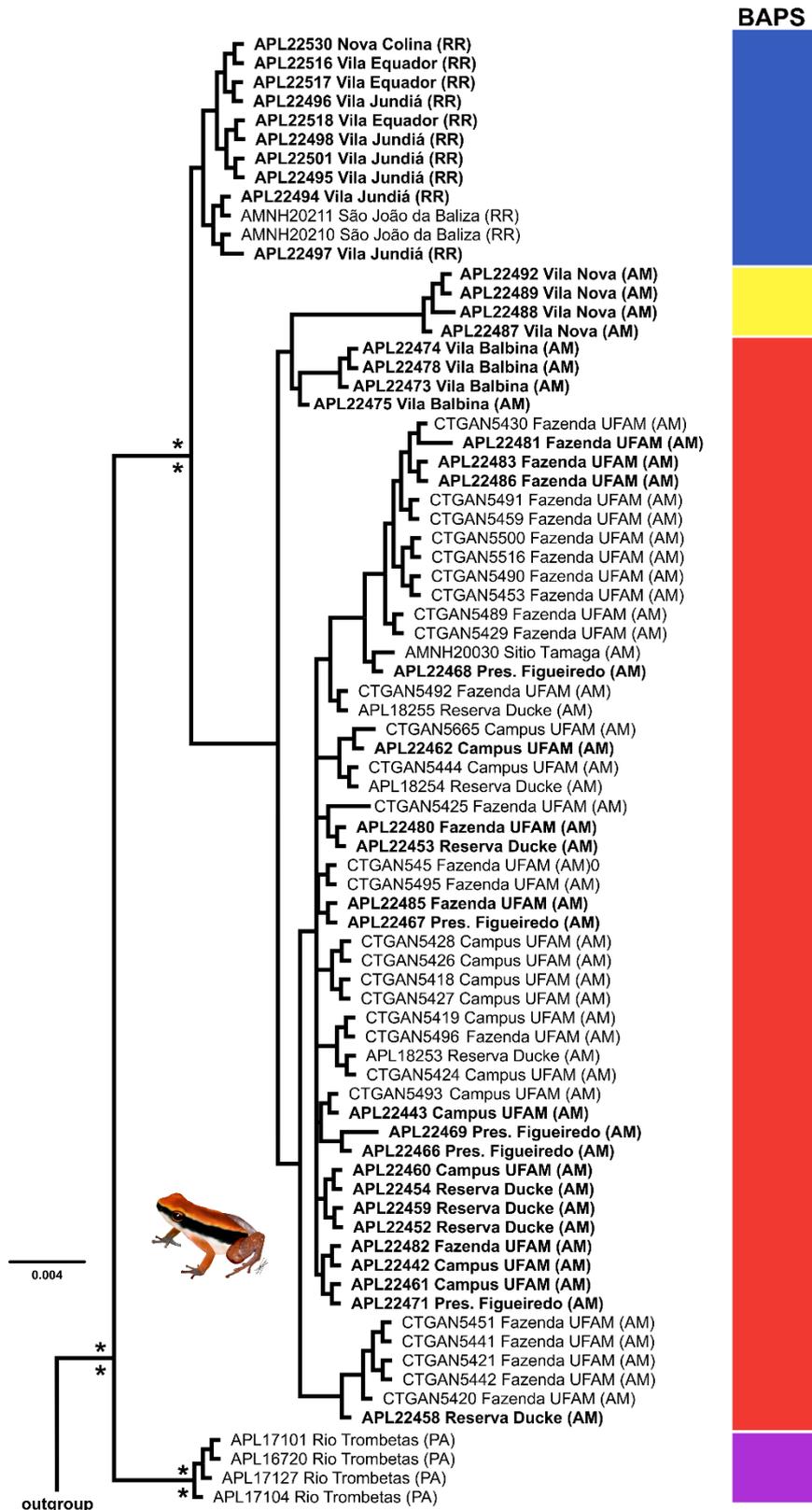


Figure 2. Phylogenetic tree of *Allobates sumtuosus* populations (on left) based on mtDNA (16S) variation, showing the two best supported major clades; and the result of population structure analysis (BAPS) (on right), showing the clusters recovered ($k = 4$). Newly sequenced specimens

are highlighted in bold. Asterisks represent support values of Bayesian inference between 0.9 and 1.0 (above branches) and Maximum likelihood between 80 and 100 (below branches). Branch scale is indicated in number of substitution per site.

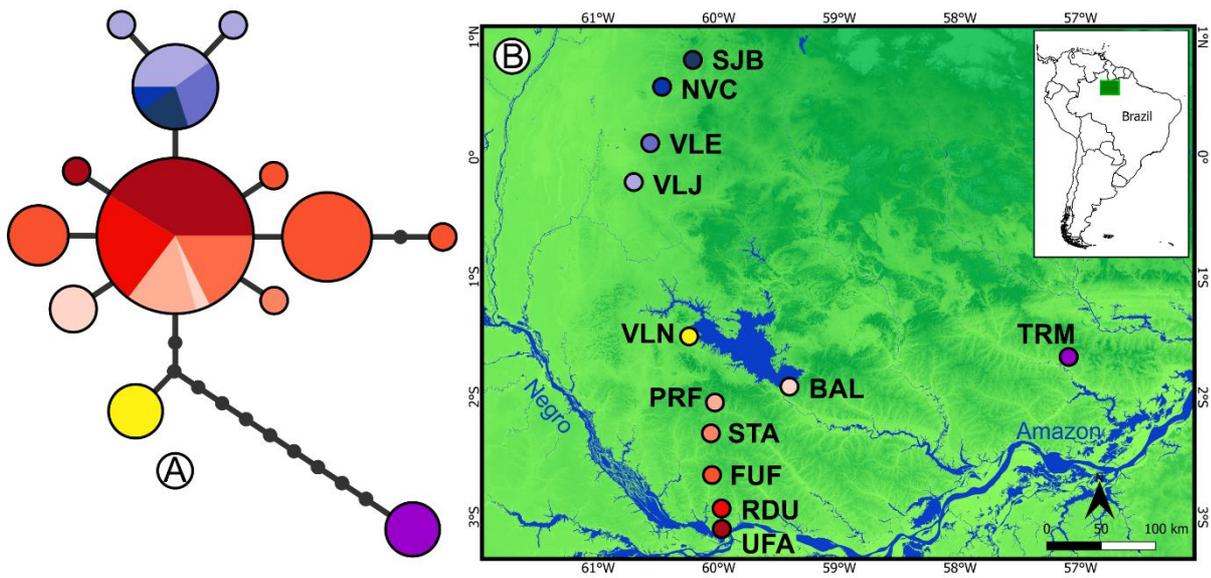


Figure 3. Haplotype network (A) generated with 77 rRNA 16S mtDNA sequences (xxx base pairs) for *Allobates sumtuosus* and their geographic origin (B). The size of the circles indicates the relative frequency of the haplotype and the color indicates the origin of the individuals corresponding to the geographic location in the sampled space (B). See Table 1 for population acronyms.

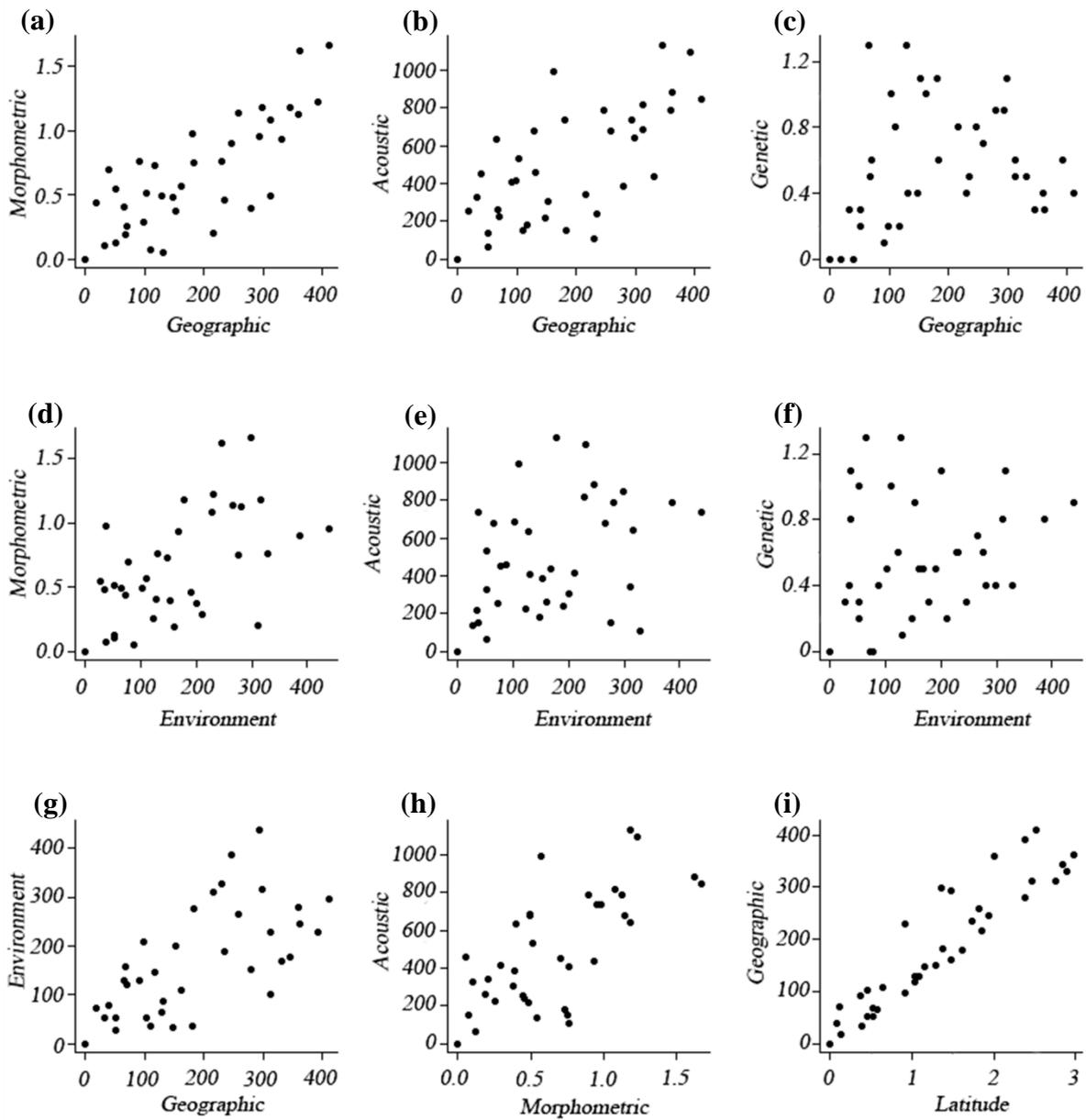


Figure 4. Correlation between p-distance matrices of each character class used in the Mantel tests. See the results of the correlations in Table 3.

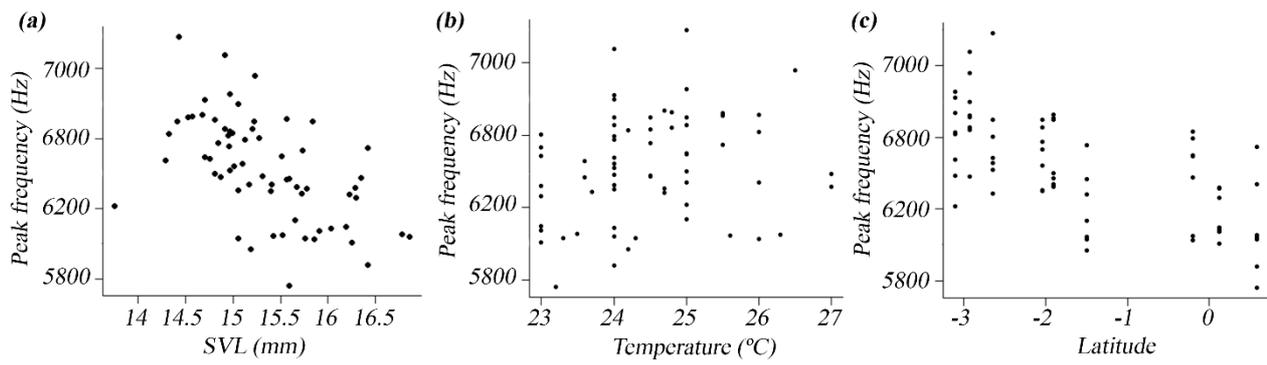


Figure 5. Effect of body length (SVL), temperature and latitude on the peak frequency of the advertisement call of *Allobates sumtuosus*.

Supplementary Material

SM1. Distribution of *Allobates sumtuosus* among 9 sampled localities sampled in Brazilian Amazonia. Collection numbers of vouchers (INPA-H) and GenBank accession numbers are provided concerning representative sequences.

Voucher (INPA-H)	APL	GenBank	Locality (Acronym)	Coordinates
INPA-H041200	22442	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041239	22443	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041256	22452	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041209	22453	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041258	22454	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041198	22455	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041202	22456	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041253	22457	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041222	22458	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041214	22459	XXX	Reserva Florestal Adolpho Ducke (RDU)	02°55'50.34"S, 59°58'28.68"W
INPA-H041216	22460	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041229	22461	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041223	22462	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041219	22463	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041224	22464	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041242	22465	XXX	Universidade Federal do Amazonas (UFA)	03°06'0.85"S, 59°58'18.98"W
INPA-H041248	22466	XXX	Presidente Figueiredo (PRF)	02°02'31.95"S, 60°01'46.98"W
INPA-H041226	22467	XXX	Presidente Figueiredo (PRF)	02°02'31.95"S, 60°01'46.98"W
INPA-H041207	22468	XXX	Presidente Figueiredo (PRF)	02°02'31.95"S, 60°01'46.98"W

INPA-H041254	22469	XXX	Presidente Figueiredo (PRF)	02°02'31.95"S, 60°01'46.98"W
INPA-H041245	22470	XXX	Presidente Figueiredo (PRF)	02°02'31.95"S, 60°01'46.98"W
INPA-H041194	22471	XXX	Presidente Figueiredo (PRF)	02°02'31.95"S, 60°01'46.98"W
INPA-H041220	22472	XXX	Presidente Figueiredo (PRF)	02°02'31.95"S, 60°01'46.98"W
INPA-H041241	22473	XXX	Vila de Balbina (BAL)	01°54'03.47"S, 59°24'52.12"W
INPA-H041260	22474	XXX	Vila de Balbina (BAL)	01°54'03.47"S, 59°24'52.12"W
INPA-H041218	22475	XXX	Vila de Balbina (BAL)	01°54'03.47"S, 59°24'52.12"W
INPA-H041246	22476	XXX	Vila de Balbina (BAL)	01°54'03.47"S, 59°24'52.12"W
INPA-H041227	22477	XXX	Vila de Balbina (BAL)	01°54'03.47"S, 59°24'52.12"W
INPA-H041205	22478	XXX	Vila de Balbina (BAL)	01°54'03.47"S, 59°24'52.12"W
INPA-H041237	22479	XXX	Vila de Balbina (BAL)	01°54'03.47"S, 59°24'52.12"W
INPA-H041235	22480	XXX	Fazenda Experimental da UFAM (FUF)	02°38'51.36"S, 60°03'13.43"W
INPA-H041250	22481	XXX	Fazenda Experimental da UFAM (FUF)	02°38'51.36"S, 60°03'13.43"W
INPA-H041193	22482	XXX	Fazenda Experimental da UFAM (FUF)	02°38'51.36"S, 60°03'13.43"W
INPA-H041195	22483	XXX	Fazenda Experimental da UFAM (FUF)	02°38'51.36"S, 60°03'13.43"W
INPA-H041243	22484	XXX	Fazenda Experimental da UFAM (FUF)	02°38'51.36"S, 60°03'13.43"W
INPA-H041217	22485	XXX	Fazenda Experimental da UFAM (FUF)	02°38'51.36"S, 60°03'13.43"W
INPA-H041213	22486	XXX	Fazenda Experimental da UFAM (FUF)	02°38'51.36"S, 60°03'13.43"W
INPA-H041206	22487	XXX	Ramal Vila Nova - km 1062 (VLN)	01°29'36.64"S, 60°14'25.12"W
INPA-H041197	22488	XXX	Ramal Vila Nova - km 1062 (VLN)	01°29'36.64"S, 60°14'25.12"W
INPA-H041196	22489	XXX	Ramal Vila Nova - km 1062 (VLN)	01°29'36.64"S, 60°14'25.12"W
INPA-H041231	22490	XXX	Ramal Vila Nova - km 1062 (VLN)	01°29'36.64"S, 60°14'25.12"W
INPA-H041255	22491	XXX	Ramal Vila Nova - km 1062 (VLN)	01°29'36.64"S, 60°14'25.12"W
INPA-H041204	22492	XXX	Ramal Vila Nova - km 1062 (VLN)	01°29'36.64"S, 60°14'25.12"W
INPA-H041208	22493	XXX	Ramal Vila Nova - km 1062 (VLN)	01°29'36.64"S, 60°14'25.12"W

INPA-H041233	22494	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041259	22495	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041238	22496	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041228	22497	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041244	22498	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041225	22499	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041249	22500	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041221	22501	XXX	Vila de Jundiá (VLJ)	00°12'20.76"S, 60°41'46.54"W
INPA-H041199	22516	XXX	Vila do Equador (VLE)	00°07'24.93"N, 60°33'43.69"W
INPA-H041211	22517	XXX	Vila do Equador (VLE)	00°07'24.93"N, 60°33'43.69"W
INPA-H041257	22518	XXX	Vila do Equador (VLE)	00°07'24.93"N, 60°33'43.69"W
INPA-H041212	22519	XXX	Vila do Equador (VLE)	00°07'24.93"N, 60°33'43.69"W
INPA-H041203	22520	XXX	Vila do Equador (VLE)	00°07'24.93"N, 60°33'43.69"W
INPA-H041240	22521	XXX	Vila do Equador (VLE)	00°07'24.93"N, 60°33'43.69"W
INPA-H041234	22522	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H041236	22523	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H041230	22524	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H041210	22525	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H041251	22526	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H041247	22527	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H041201	22528	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H042215	22529	XXX	Nova Colina (NVC)	00°34'55.23"N, 60°27'53.01"W
INPA-H041232	22530	XXX	Vila do Equador (VLE)	00°07'24.93"N, 60°33'43.69"W
