Herpetologica, 81(2), 2025, 104–116
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Mitochondrial Phylogeography of *Anomaloglossus stepheni* (Anura: Aromobatidae) Reveals Low Genetic Diversity Despite Wide Guiana Shield Distribution

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Abstract: Historical climate and landscape features are important mechanisms that can drive genetic differentiation of lineages. Habitat shifts between forested and open environments during the Quaternary Period contributed substantially to regional diversity in Amazonia and other South American environments. Herein, we used a widely distributed frog species in the Guiana Shield to assess whether the species' spatiotemporal dynamics are related to historical climate changes and other regional landscape features. For that, we used ecological niche models (ENMs) and phylogeographical analyses with a single-locus approach (mitochondrial DNA). We found two genetic clusters spatially structured along the Guiana Shield, with some degree of overlap and low genetic diversity. Part of the genetic differentiation was attributed to spatial distances. Population structure did not match to any strong vicariant barrier that could completely preclude gene flow. However, ENMs detected large unsuitable climatic areas for current and historical periods that likely restricted the dispersion of populations. The divergence time of clusters was relatively recent, dating from the middle-to-late Pleistocene, a period of intense climate oscillations. Although such historical changes seem to have influenced the spatial distribution of the species genealogies, demographic analyses suggested that populations were stable from the Late Pleistocene. Climate shifts may have shaped the patterns of gene flow by reducing or increasing connectivity of populations, depending on the period.

Key words: Amazon rainforest; Genetic differentiation; Isolation by distance; Lowlands; Quaternary

Historical climate and geomorphological changes are the main drivers of organismal diversification (Finarelli and Badgley 2010; Hoorn et al. 2017; Antonelli et al. 2018). Given the plethora of possible evolutionary mechanisms involved in the diversification of species-rich regions, understanding the origins of the extraordinary Neotropical biodiversity is still a main challenge for biogeographers (Wallace 1852; Ayres and Clutton-Brock 1992; Antonelli et al. 2018; Thom et al. 2020). In Amazonia, the largest rainforest in the world, several landscape changes have shaped the diversification of lineages. The effects of geologic forces (e.g., tepuis sky island formation and Andes uplift), marine incursions, and climate change (e.g., Quaternary Period climate shifts) are the main drivers of Amazonian diversification (Hoorn et al. 2010; Rull 2011; Oliveira et al. 2017). Using different disciplines and multiple sources of evidence is crucial to reveal the main patterns and processes of diversification and the relative contributions of climate changes and geological events to species evolutionary histories (Carnaval and Moritz 2008; Collevatti et al. 2015; Oliveira et al. 2017). Moreover, the observed geographic patterns in species genealogies can provide clues on how environmental differences and species biology contributed to lineage differentiation (Avise et al. 1987; Carnaval et al. 2009; Turchetto-Zolet et al. 2013).

The Amazonian landscape experienced severe and continuous changes during the Tertiary and Quaternary periods with deep impacts on species diversification that shaped the present biodiversity patterns (Rull 2008; Antonelli et al. 2009; Hoorn et al. 2010; Cooke et al. 2012). The flooding of lowland Amazonia by Miocene marine incursions (from 23 to 5 Ma), for example, could have affected the evolutionary dynamics in at least two ways: 1) by extinguishing populations and by vicariant effects and 2) by having isolated the Guiana Shield (Lovejoy et al. 2006; Wesselingh and Salo 2006; Bloom and Lovejoy 2011). Several predictions on the patterns of diversification can be derived as possible results of the marine incursions (Bloom and Lovejoy 2011). If marine incursions affected a given taxon, it is expected that there would be higher levels of endemism in upland areas (acting as refugia), whereas lowland lineages would be younger than the incursion periods (Nores 1999; Hubert and Renno 2006; Solomon et al. 2008; Bloom and Lovejoy 2011).

The classical refugia hypothesis posits that during the dry and cold periods in the Pleistocene, moist environments such as the Amazonian forest shrank and fragmented into climatically stable refugia (Haffer 1969). These refugia could have ultimately promoted diversification through vicariance of populations, creating high phylogeographic structure among refuges (Carnaval et al. 2009). Some phylogeographic studies conducted on Amazonian terrestrial vertebrates suggest that the Pleistocene climatic changes contributed to the spatial genetic structure and demographical changes for pan-Amazonian lineages (Lynch Alfaro et al. 2015; Ferreira et al. 2017; Capurucho et al. 2018; Ruiz-García et al. 2018). In spite of the effects of climatic changes at large scales, covering several areas of endemism, some lineages, or even species have a restricted distribution in one of these areas.

Several vertebrate groups present a well-documented regionalization within the Amazon, and many regions are known for their endemism (Cracraft 1985; Haffer 2008; Morrone 2014; Godinho and da Silva 2018). One of the most inventoried areas of endemism in the Amazon is the Guiana Shield. Located in the Guyana Craton, the Guiana Shield is delimited by the Venezuelan and Colombian Llanos and the Amazon and Orinoco basins and is characterized by the presence of summits separated by surrounding lowlands (Hammond 2005; Rull and Nogué 2007). The Guiana

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Shield has a complex landscape with elevations up to 3000 m a.s.l, tabletop mountains (tepuis), highland and lowland moist forests, flooded forests, shrublands, and savannas (Hammond 2005; Gond et al. 2011; Naka et al. 2012). In the eastern region of the Guiana Shield, several lineages of anurans with different life-history traits share refugial areas, suggesting a strong role of past climatic shifts in species diversification (Fouquet et al. 2012).

Frogs of the genus *Anomaloglossus* are highly diversified and endemic to the Guiana Shield (Fouquet et al. 2015a, 2018; Vacher et al. 2017). Cases of cryptic diversity coupled with discordant molecular and phenotypic divergences and different species occupying contrasting elevational gradients (e.g., lowland vs. highlands) make this genus an interesting group to test the diversification hypotheses associated with the Amazonia and specifically the Guiana Shield (Vacher et al. 2017; Fouquet et al. 2019). Among these, the Rocket Frog (*Anomaloglossus stepheni*) differs from most species in the genus by its larger geographic distribution, mainly associated with lowlands. It occurs both north and south of the Guiana Shield, with a distribution gap in savanna areas along the Guiana area of endemism. Its taxonomy has been evaluated in recent years, with molecular sampling along its distribution (Vences et al. 2003; Grant et al. 2006; Fouquet et al. 2012; Kok et al. 2012; Lyra et al. 2016; Vacher et al. 2017).

Anomaloglossus stepheni is a widespread species on the Guiana Shield (Vacher et al. 2017). We were interested in assessing whether the species is a single evolutionary unit and evaluating the mechanisms that could generate the present patterns of genetic diversity within this species. We explored the patterns of spatial genetic boundaries of populations and the related processes that drove the species' diversification history. Using single-locus and geospatial data in a phylogeographic approach, we tested whether genetic variation and structure of the species could be explained by 1) spatial distances according to the isolation by distance hypothesis and 2) Quaternary climatic shifts in which both distribution ranges and demographic history were influenced by climatic instability on the Guiana Shield.

MATERIALS AND METHODS

Sampling and Molecular Methods

To assess how the genetic diversity of *A. stepheni* is spatially distributed, we used sequences of a short 16S ribosomal RNA (rRNA) mitochondrial fragment available in GenBank (http://www.ncbi.nlm.nih.gov/genbank). This fragment has been extensively used as a DNA barcode for amphibians (Vences et al. 2005; da Silva et al. 2020; Vacher et al. 2020; Moraes et al. 2022) and in phylogeographic studies (Gehara et al. 2014; Arteaga et al. 2016). We downloaded 39 sequences of *A. stepheni* available in GenBank, adding two newly collected specimens to our dataset totaling 41 from 18 localities (Supplemental Table S1, available online). The final 16S rRNA alignment used in genetic analyses comprised 364 base pairs (bp).

We also collected two specimens from a southern location to add to our analysis. We conducted visual surveys and used pitfall traps at Óbidos municipality, Pará state, Brazil, in January–February 2015 (Cechin and Martins 2000). We collected two specimens that were captured manually and killed using 5% lidocaine, fixed in 10% formalin, and transferred to 70% ethanol for permanent storage (following Conselho Federal de Biologia-CFBio no. 148/2012, 2012). Voucher specimens are housed at the Coleção Zoológica da Universidade Federal de Mato Grosso do Sul (ZUFMS-AMP; Table S1), Campo Grande, Brazil. Appropriate protocols for the collection and handling of the individuals were followed for the present research according to Brazilian federal law (System of Authorization and Information in Biodiversity [SISBIO], Ministry of the Environment, license 45889).

We used polymerase chain reaction (PCR) to amplify fragments of the 16S rRNA mitochondrial gene by using the primers 16Sa (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16Sb (5′-CCG GTC TGA ACT CAG ATC ACG T-3′; Palumbi et al. 1991). We edited electropherograms and built sequence alignments by using the MAFFT algorithm (Katoh and Standley 2013) in Geneious v9.1.6 (Kearse et al. 2012) with default settings.

Spatial and Population Structure Analyses

Because isolation by distance can affect the results of population structure analysis and potentially bias genetic clusters (Perez et al. 2018), we tested the degree of correlation between spatial and genetic distances. To evaluate whether genetic distances were predicted by the geographic distance, we used redundancy analysis (RDA). The analysis was used to calculate the amount of genetic variation that can be explained by geographic distance among localities. An interindividual genetic distance matrix was obtained using the dist.dna function and TN93 model in ape R package (Paradis et al. 2004). The matrix was then transformed using a principal coordinate analysis and used as a response variable, whereas geographic distances was used as a predictor variable. RDA was performed with the rda function and significance ($P \leq 0.05$) was accessed with the anova.cca function in the vegan R package (Dixon 2003), by using 1000 random permutations.

To assess whether *A. stepheni* comprises a single panmictic population, or alternatively whether there is spatial genetic structure, we applied a Bayesian analysis of population structure (performed by BAPS v6.0 software; Corander et al. 2008). To estimate the optimal number of populations (K) we ran a spatial clustering method in BAPS with the following configurations: mixture model with spatial clustering of individuals and maximal number of K set to 10. We also explored the spatial genetic boundaries of populations (associated with genetic discontinuities) and the geographic location of potential barriers to gene flow by using Monmonier's maximum difference algorithm (Manni et al. 2004; Monmonier 2010) in the adegenet R package (Jombart 2008).

Samples were assigned according to clusters recovered with BAPS and used for further genetic population statistics. We used DnaSP v5.10 (Librado and Rozas 2009) to estimate the following population genetic metrics: number of haplotypes, haplotype diversity, number of polymorphic sites, and nucleotide diversity. We estimated the genetic divergence between BAPS clusters and among localities via uncorrected pairwise *p*-distances, with 1000 bootstrap replicates in MEGA X (Kumar et al. 2018). To explore the amount of population differentiation at different levels (intra- and interpopulational levels), we assessed the genetic variation partitioning of BAPS clusters and localities with hierarchical analysis of

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molecular variance (AMOVA) in Arlequin v3.5.2 software (Excoffier and Lischer 2010). We also built a haplotype network by using the median-joining algorithm in PopArt v1.7 software (Leigh and Bryant 2015) to visualize the relationships of individuals of the sampling localities. For comparisons at the localities level, we discarded localities with single individuals, and AMOVA and uncorrected *p*-distances were estimated for 10 from the 18 localities.

Phylogenetic Analysis and Divergence Times

To evaluate the genealogical relationships among individuals of *A. stepheni* ($n = 41$) and divergence among populations, we used Bayesian inference in BEAST v1.10.4 software (Suchard et al. 2018) with three species from the *A. stepheni* group as outgroups (*A. apiau*, *A. baeobatrachus*, and *A. leopardus*; Vacher et al. 2017). To generate the 16S gene tree, we first estimated the best substitution model based on the Bayesian information criterion in jModelTest v2.1.7 software (Darriba et al. 2012), with the TrN+I as the chosen model. Next, we used a constant size coalescent tree prior with a strict clock model and calibrated the tree by using a mean substitution rate of 0.0055 site/million yr, following Fouquet et al. (2012) for *Anomaloglossus* genus, with a SD of 0.0009. We performed three independent runs with 20,000,000 generations sampled at every 2000 generations, each. The results of runs were combined with LogCombiner v1.10.4 (Suchard et al. 2018) after discarding the initial 20% generations as burn-in of each run. The convergence of the Markov chain Monte Carlo runs were checked in Tracer v1.6 (ESS > 200; Rambaut and Drummond 2007). A maximum clade credibility tree was generated in TreeAnnotator v1.10.4 (Suchard et al. 2018) and visualized in FigTree v1.4.3 (Rambaut 2016). Voucher, GenBank number, and sample localities are presented in Table S1.

Demographic History

To infer possible signs of the demographic events, we implemented neutrality tests (Tajima's *D* and Fu's F_S) by using 10,000 coalescent simulations to assess the significance in DnaSP v5.10 (Librado and Rozas 2009). We also assessed the population demographic history of the recovered clusters using Bayesian Skyline plots (BSPs; Drummond 2005). The BSPs allow the visualization of changes in population size (Ne) through time and were performed in BEAST v1.10.4 (Suchard et al. 2018). We used a mean substitution rate of 5.5×10^{-9} site/yr, with a SD of 9×10^{-10} (Fouquet et al. 2012) and a strict clock, with independent runs of 20,000,000 generations sampled at every 2000 generations and a 20% burn-in. Plots were generated in Tracer.

Ecological Niche Models

To predict present-day and historically suitable areas for *A. stepheni* and to explore the possible effects of environmental differences during Quaternary climatic changes (from Middle Pleistocene to the current period), we used ecological niche models (ENMs). Thus, we assessed suitable areas with ENMs by using species' occurrence data and bioclimatic data extracted from PaleoClim (http://paleoclim.org; Brown et al. 2018). First, we obtained 42 occurrence points (all coordinates in decimal degrees; datum = WGS84) for the species from scientific collections and bibliographies (Vences et al. 2003; Grant et al. 2006; Fouquet et al. 2012; Kok et al. 2012; Lyra et al. 2016; Motta et al. 2018; Vacher et al. 2017; Supplemental Table S2, available online). Next, to decrease sampling bias effects, we used spThin package (Aiello-Lammens et al. 2015), filtering occurrences ≤5 km between each other, resulting in 36 points used to build ENMs. Bioclimatic data were downloaded at a spatial resolution of 2.5 arc-minutes grid (~5-km^2 resolution), for the following periods: current (1979–2013 ya; Karger et al. 2017), Mid-Holocene (Holocene; 8.326–4.2 thousand years ago [kya]; Fordham et al. 2017), Last Glacial Maximum (LGM; 21 kya; Karger et al. 2017), Last Interglacial (LIG; 120 kya; Otto-Bliesner 2006), and Marine Isotope Stage 19 in the Pleistocene (MIS19; 787 kya; Brown et al. 2018). Because five PaleoClim variables (BIO2, BIO3, BIO5, BIO6, and BIO7) for the MIS19 period were not available, we excluded them for other periods and used 14 bioclimatic as potential predictors to build the ENMs. We clipped all layers to an extent higher than the known distribution of species that could allow the transition of suitable areas in time (longitude = −68 to −49, latitude = −9 to 11°). Given the possible effects of high collinearity among bioclimatic variables and overparameterization, we removed highly correlated variables ($r > 0.8$). The retained variables used to generate ENMs were as follows: temp. seasonality (BIO4), mean temp. of wettest quarter (BIO8), mean temp. of driest quarter (BIO9), annual precip. (BIO12), precip. of wettest month (BIO13), precip. of Driest Month (BIO14), precip. of warmest quarter (BIO18), and precip. of coldest quarter (BIO19).

We generated models by using the maximum entropy algorithm MaxEnt (Phillips et al. 2017). MaxEnt models were implemented with dismo package (Hijmans et al. 2017), and ENMeval v2.0 package (Kass et al. 2021) was used for tuning and evaluating models. We produced different model configurations and selected the best model based on the Akaike's information criterion corrected for small sample sizes to avoid overfitting (Muscarella et al. 2014). The competing models were built with 10,000 background points, checkerboard1 method, and regularization multipliers (rm) and feature classes (fc) were set to default, resulting in 48 alternative models. Models were projected with cloglog transformation. The model performances were estimated by the area under the curve (AUC) that varies from 0.5 to 1, with AUC values of 1.0 making a perfect prediction, $0.9 \leq$ AUC < 1.0 considered as very good, and $0.8 \leq$ AUC < 0.9 considered as good (Swets 1988). We also used an algorithm (Boyce index) designed for presence-only models to evaluate model performance (Boyce et al. 2002). The Boyce index varies from −1 to 1, with 0 meaning models are no different than a random model and 1 indicating a perfect model performance (Hirzel et al. 2006), and performance was assessed with ecospat package (Di Cola et al. 2017). We also projected the present-day ENM (current) into the four historical scenarios (Holocene, LGM, LIG, and MIS19).

Results

Population Structure and Divergence Times

The results of the RDA indicate that spatial distance is an important predictor of the observed genetic distance ($R^2_{adj} = 40\%$, $P = 0.001$), supporting the isolation by distance model. In addition, we found two genetic clusters spatially structured

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with BAPS with some degree of overlap: one mainly distributed in the Guiana area of endemism (sensu da Silva et al. 2005; hereafter, the northern cluster), and the other spanning the Inambari and Guiana areas (hereafter, the southern cluster; Fig. 1A). Although potentially co-occurring in the edge of Guiana area (one locality having both clusters), Monmonier's algorithm detected a geographic barrier that supports a genetic discontinuity similar to BAPS results. Genetic differences between clusters (uncorrected *p*-distance) were low (0.9%), and the haplotype genealogy showed low levels of haplotype sharing, separated by a few mutational steps. However, the most common haplotype was widely distributed and predominant in the northern cluster (Hap1; Fig. 1A, B). Most genetic variation detected by AMOVA was observed between populations (61.03%, $P < 0.001$), and the remaining (38.97%) within populations. Both species and clusters had relatively low-to-moderate levels of genetic diversity (Table 1). Haplotype diversity and nucleotide diversity of the northern cluster were lower than the those of the southern cluster, even with the number of samples almost three times higher. Uncorrected *p*-distances between pairs of localities showed in general low levels of genetic distances, ranging from 0 to 1.2% (Table 2).

Based on the mitochondrial DNA (mtDNA) gene tree, the divergence of *A. stepheni* clusters was estimated during the Pleistocene (Fig. 1C). The coalescent time estimated for the divergence of clusters for the mtDNA sequences was ~1.30 Ma (95% highest posterior density [HPD] = 0.54–2.21 Ma). Nevertheless, the relationship between the recovered clusters was weakly supported, with one sample of the northern cluster being grouped with the southern cluster. Besides, considering the low posterior probability (<0.5) for reciprocally monophyletic groups, and given the large confidence intervals of divergence times (95% HPD) of internal branches, we could not estimate the divergence times between clusters.

Historical Demography

Neutrality tests were nonsignificant for all groups, suggesting population stability through time (Table 1). Accordingly, Skyline plots of both clusters showed population stability through time, back to 120,000 yr (Fig. 2). For both clusters, the oldest coalescent event happened in the Late Pleistocene. However, for the northern cluster the coalescence (in the past 60,000 yr) was two times faster than for the southern cluster. As observed in ENMs (see below), the wider climatic suitability areas in the south distribution of the species in the LIG period (120,000 yr ago) can explain the older demographic history of the southern cluster. Besides, it is possible that the recent expansion of suitability in the northern distribution of the species has not yet restored genetic diversity (at least for this gene), accounting for the low diversity and more recent coalescence events for the northern cluster.

Ecological Niche Models

Models for all climatic scenarios showed good model performance, with AUC values higher than 0.87 (Fig. 3) and a Boyce index of 0.83. In the current climatic scenario (Fig. 3A, B), an unsuitable climatic area was predicted between two major areas: one in the north, comprising the Guiana center of endemism, and the other at the southernmost portion. Most of these unsuitable areas coincide with a higher topographic complex region with elevations that varies from 300 to 900 m (Fig. 4). The climatic variables with highest contribution for models were as follows: BIO14 (62.45%), BIO9 (18.31%), and BIO18 (14.25%). Five localities (~2° of latitude and −56° of longitude) at the south of Suriname occurred in areas with low climatic suitability.

For both Holocene and LIG periods (Fig. 3C, E), the climatic suitable areas coincided with regions found for the current period, but were much reduced in area. During the LGM period (Fig. 3D) there was a strong and apparent shrinkage in the suitable areas, suggesting that populations may also have reduced during this period. The extension of suitable areas in the MIS19 period was similar to the current ENM.

Discussion

The genetic variation of the Rocket Frog is moderately geographically structured, with two genetic clusters and a low genetic diversity. The geographic distance of samples was an important predictor of the genetic distances, supporting the isolation by distance model. However, climatic shifts during the Quaternary Period in the species range of distribution (mainly the Guiana area of endemism) seem to have influenced the spatial distribution of the species, with impacts on genetic diversity and differentiation. The species is mainly distributed in the Guiana area of endemism, but it is also found in the Inambari and Rondonia areas of endemism. The northern cluster is more widely distributed, comprising the Guiana area, whereas the southern cluster occurs in the edge of the Guiana area and in the transition region including the Inambari area of endemism. The species occurs in lowland areas of the Guiana Shield, generally below 300 m a.s.l. In addition to the impact of climatic shifts, its current distribution from west to east in the Guiana Shield could be affected by landscape features such as the highlands of the tepuis (>700 m) and mountains and steeper slopes at the border of Suriname, French Guiana, and Pará and Amapá Brazilian states (inselbergs can reach 700 m a.s.l). Considering the landscape features, we could expect that higher elevations in the center of the species distribution could drive genetic differentiation by reducing the gene flow of populations and splitting the Suriname populations from those in Brazil. However, there are no clear physical barriers (e.g., rivers or highlands) coinciding with the genetic break of the two clusters, and one locality includes haplotypes representing both northern and southern clusters.

In contrast to the microendemic *Anomaloglossus* species (Vacher et al. 2024), *A. stepheni* is broadly distributed; consequently, it could be a case of cryptic diversification. Nevertheless, the low genetic divergence of clusters, differing in <1%, suggests that it is a single species broadly distributed in Guiana Shield (Vacher et al. 2017). Future studies using highly variable nuclear data (e.g., single-nucleotide polymorphisms [SNPs]) and coalescent simulations could test different diversification scenarios. Some of them can assess whether genetic structure remains similar when accounting for biparental data and if lineages share haplotypes due to incomplete lineage sorting or are better explained by isolation with migration models with/without founder effect, among others. The genetic structure of species is strongly related to life-history traits (e.g., dispersal

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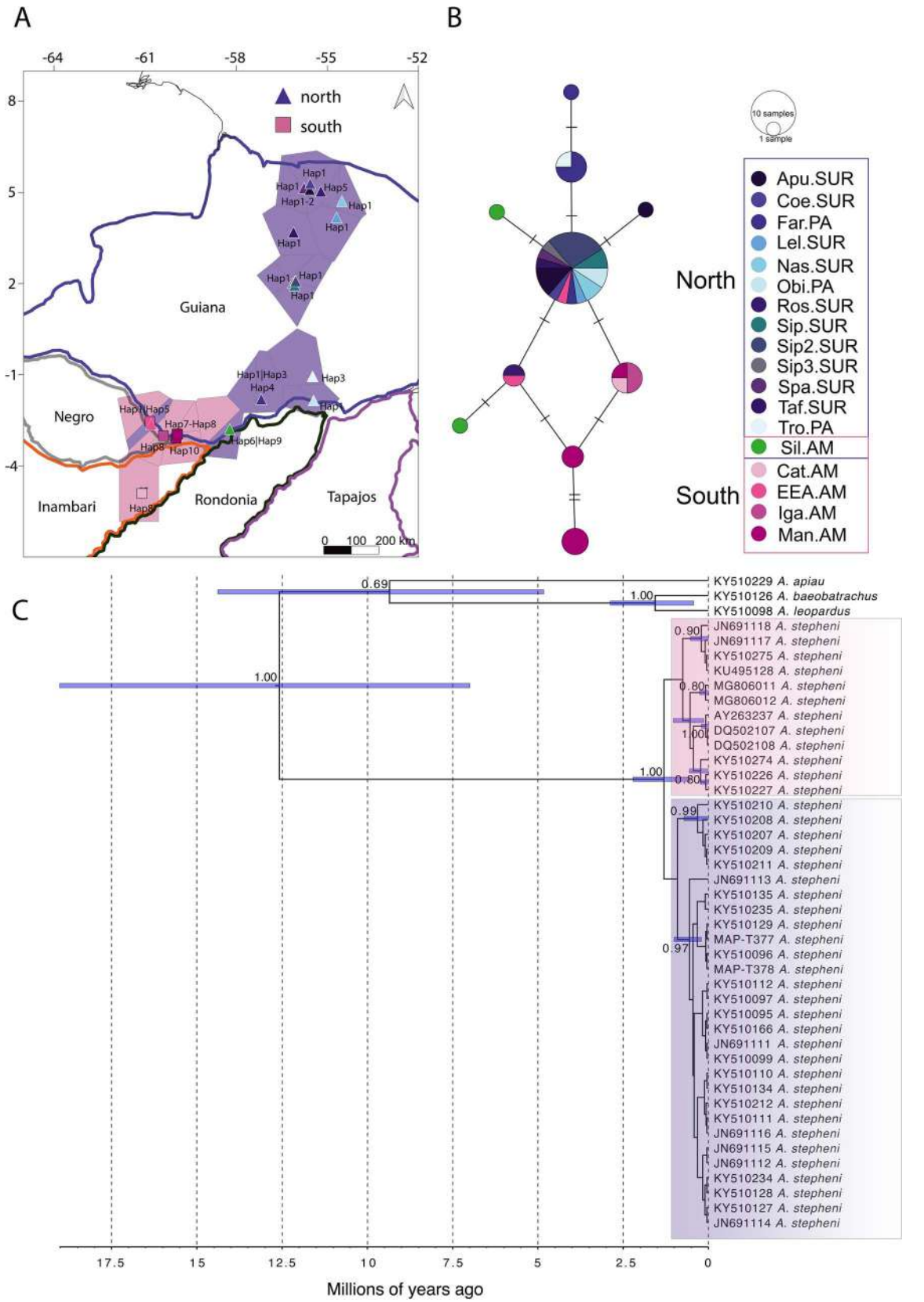

FIG. 1.—Population structure and intraspecific relationships of *Anomaloglossus stepheni*. (A) Distribution of the two recovered clusters (north and south) in the areas of endemism based on 16S rRNA from BAPS analysis. (B) Relationships among haplotypes considering the sampling sites and BAPS assignment, where the circle size represents the proportion a given haplotype and the mutational steps are characterized by crossed lines. (C) Genealogical relationships of samples dated by Bayesian 16S rRNA tree (maximum clade credibility). The divergence times is given in millions of years (*x*-axis) and the interval of divergences is given by the graphic bars (95% HPD).

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TABLE 1.—Genetic population statistics for *Anomaloglossus stepheni*. Shown are the number of samples (N), number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), and neutrality tests (Tajima's D and Fu's F_S). Neutrality tests were nonsignificant for all groups.

Group	N	S	H	Hd	π	Tajima's D	Fu's F_S
All	41	9	10	0.698	0.005	−0.079	−0.001
Southern cluster	11	5	5	0.818	0.005	−0.054	−0.230
Northern cluster	30	5	6	0.455	0.002	−0.018	0.131

capability, body size, and reproductive mode) environmental preferences, and landscape features (Paz et al. 2015; Zamudio et al. 2016). Small anuran species, for example, are expected to have a higher genetic divergence given its low dispersal rates (Pabijan et al. 2012; Paz et al. 2015; Wollenberg Valero 2015). Limited dispersal also could lead to patterns of isolation by distance, in which genetic divergence increases with geographic distances, because limited movement favors gene flow across closer populations (Wright 1943; Slatkin 1985). In fact, isolation by distance explained a substantial amount of genetic differentiation in *A. stepheni*. Given the small size of *A. stepheni* and the expected low rates of dispersal, the effects of distance among populations on genetic divergence was expected. In addition, landscape heterogeneity can reinforce genetic differentiation by constraining the movement of individuals, in which the connectivity of populations decreases as landscape environmental resistance increases (Manel et al. 2003; McRae 2006). For example, heterogeneous landscapes, with complex topography, climatic unsuitable areas, and presence of large rivers, are significant barriers for gene flow and are responsible for genetic differentiation of several amphibians (Guarnizo and Cannatella 2013; Fouquet et al. 2015b; Wollenberg Valero 2015; García-Rodríguez et al. 2021). However, the most common haplotype was found throughout the range of the species, implying either that the genetic clusters were more widely overlapping previously, or the species dispersal rate is higher than expected.

Deeper phylogeographic structure in anurans is observed in topographically complex regions and in rainforest species (Rodríguez et al. 2015). In the Amazonia–Guiana system, regions with higher elevations present higher endemism and lower richness (Señaris and Rojas-Runjaic 2020; Vacher et al. 2024). Also, lowlands tend to be occupied by widespread lineages (Señaris and Rojas-Runjaic 2020; Vacher et al. 2024). For Amazonian birds, for example, lowland regions harbor higher species richness, but the differentiation is at a slower rate (Weir et al. 2015). In a similar manner, a lower genetic divergence in frogs is expected in regions with less complex topography (Guarnizo and Cannatella 2013). Still, most of the lowland frog species in the Guiana Shield showed phylogeographic structure (Fouquet et al. 2012). *Anomaloglossus stepheni* occurs in a topographically less complex region in the Guiana Shield, and despite the genetic divergence of clusters, the species showed low levels of genetic diversity, presenting just 10 haplotypes. The southern cluster had higher haplotype diversity than the northern cluster, despite a lower sample size. This result raises the hypothesis that the center of species dispersion was in the south of the species range and/or this region was more stable than in the north, favoring the increase of mutations through time. In this scenario, colonization first occurred from the southern part to the northern part of the Guiana Shield, leading to a widespread distribution. Next, the posterior disjunction of southern and northern populations during unfavorable climatic periods, and a subsequent colonization from the north to the southern region, contributed to the current distribution of the genetic variation.

No endemic species of the Guiana Shield have had their diversification processes thoroughly evaluated. However, species that are characteristic of this region, such as *Boana diabolica*, *Rhinella alata*, and *Synapturanus mirandaribeiroi* (Fouquet et al. 2016, 2024; Vacher et al. 2024), which are sympatric with *A. stepheni*, could be analyzed to confirm and support the observed pattern in the southern part of the Guiana Shield. This region, which is more climatically stable, could serve as a source of dispersal and recolonization for areas to the north, reinforcing its importance in the evolutionary history of the region's species. Our results also suggest that climatic shifts could have an important role in the current spatial distribution of the genetic diversity, through local extinctions during climatically unsuitable periods. Repetitive extinctions in unsuitable climatic periods could explain the low levels of genetic diversity in *A. stepheni*. The species' intraspecific divergence dated from the middle Pleistocene, ~1.3 million yr ago. Across the middle-to-late Pleistocene, between 770,000 and 11,700 yr ago, the global temperature decreased and there were marked glacial periods (Lisiecki and Raymo 2005; Clark et al. 2006; Hughes et al. 2013). In the past 420,000 yr, several glacial–interglacial cycles with

TABLE 2.—Comparison matrix of ϕst values and average p-distance among localities based on the 16S rRNA mtDNA fragment of *Anomaloglossus stepheni*. The lower left matrix shows ϕst values; significant values ($P \leq 0.05$) are bolded. The upper right matrix represents the average p-distance. Localities are abbreviated as follows: Apu = Apura, Suriname; EEAM = Estação Ecológica Anavilhanas, AM, Brazil; Far = Faro, PA, Brazil; Iga = Igarape-Araras, AM, Brazil; Man = Manaus, AM, Brazil; Nas = Nassau, Suriname; Obi = Óbidos, PA, Brazil; Sil = Silves, AM, Brazil; and Sip and Sip2 = Sipaliwini, Suriname.

	Apu	EEAM	Far	Iga	Man	Nas	Obi	Sil	Sip	Sip2
Apu	—	0.0034	0.0056	0.0062	0.0112	0.0007	0.0008	0.0062	0.0007	0.0007
EEAM	0.2113	—	0.0055	0.0055	0.0082	0.0027	0.0025	0.0055	0.0027	0.0027
Far	**0.5673**	0.2857	—	0.0060	0.0111	0.0049	0.0047	0.0082	0.0049	0.0049
Iga	**0.8367**	0.5	**0.5904**	—	0.0050	0.0055	0.0050	0.0082	0.0055	0.0055
Man	**0.7248**	0.4511	**0.6576**	0.3756	—	0.0105	0.0112	0.0110	0.0105	0.0105
Nas	−0.2632	0	0.5062	1	**0.6842**	—	0.0000	0.0055	0.0000	0.0000
Obi	−0.2632	0	0.5062	1	**0.6842**	0	—	0.0059	0.0000	0.0000
Sil	0.2846	−0.5	0.3393	0.3333	**0.4684**	0	0	—	0.0055	0.0055
Sip	−0.2632	0	0.5062	1	**0.6842**	0	0	0	—	0.0000
Sip2	0.1111	0.5385	**0.6962**	**1**	**0.8**	0	0	**0.5385**	0	—

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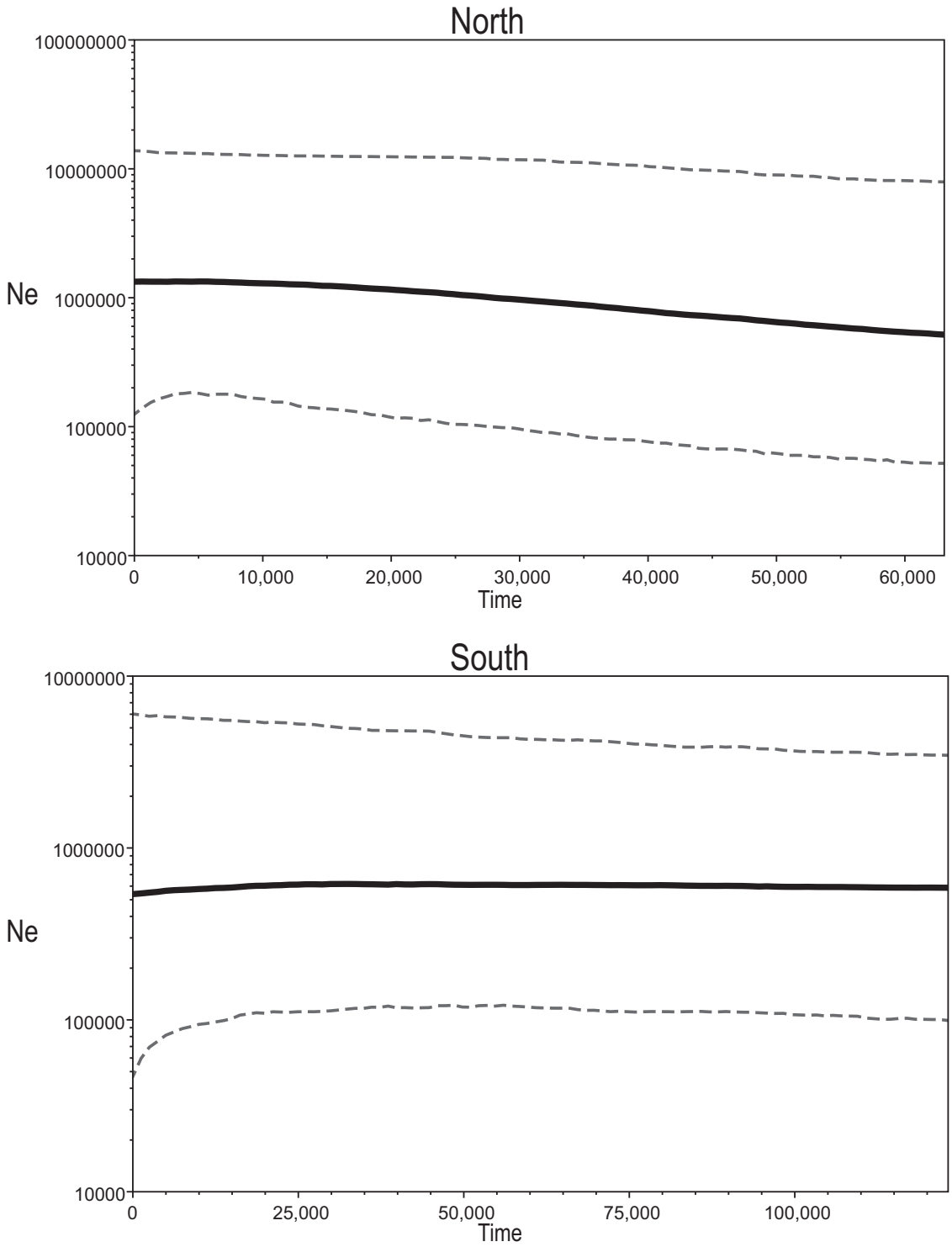

FIG. 2.—Bayesian skyline plots of the two recovered clusters of *Anomaloglossus stepheni* (north and south). The black line represents the mean of the effective population size (Ne) through time, with the 95% higher posterior probability represented with the dashed lines.

intervals of ~100,000 yr (Petit et al. 1999) probably reshaped the patterns of distribution of several lineages. As in *A. stepheni*, for several leaf-litter frog species distributed in lowlands of the eastern Guiana Shield, intraspecific divergence predated the middle-to-late Pleistocene Period, from 0.89 to 1.3 Ma (Fouquet et al. 2012). In addition, major rivers and Quaternary refugia were likely the drivers of genetic structure for these species (Fouquet et al. 2012). For other Amazonian species, refugia do not explain the observed genetic structure (Naka et al. 2012). Given that *A. stepheni* is distributed in lowlands and cluster boundaries do not match with major rivers, we hypothesize that other evolutionary scenarios, such as the formation of historical unsuitable areas during Pleistocene, played a role in intraspecific genetic differentiation,

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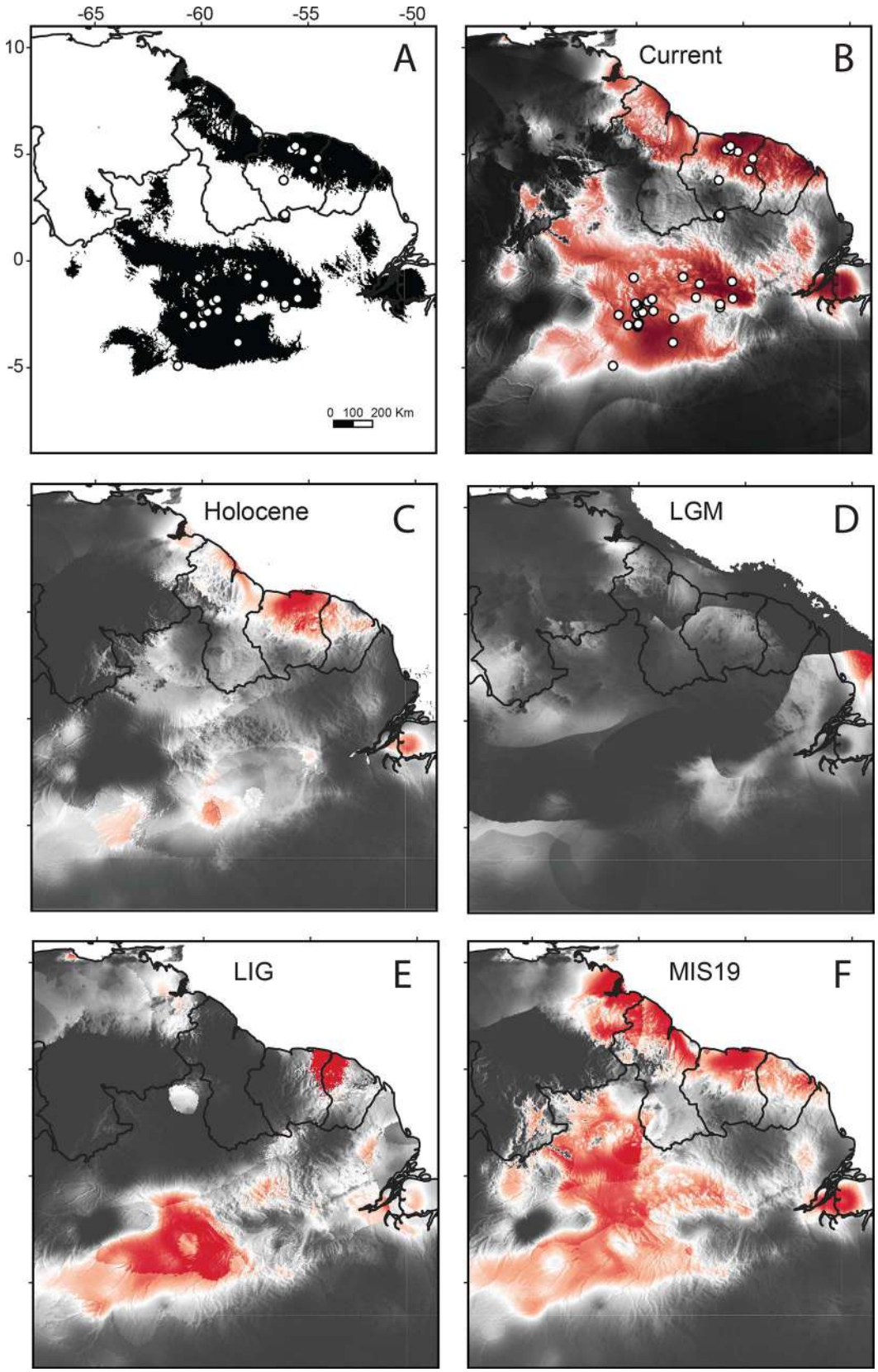

FIG. 3.—Current and past suitable areas for *Anomaloglossus stepheni* predicted by ENMs. The binary map (A) represents the current model scenario (B) by using equal training sensitivity and specificity logistic threshold. The projected paleoclimatic models were (C) Holocene (8 to 4 kya), (D) LGM (~21 kya), (E) LIG (~120 kya), and (F) MIS19 (~787 kya). Warmer colors mean higher habitat suitability.

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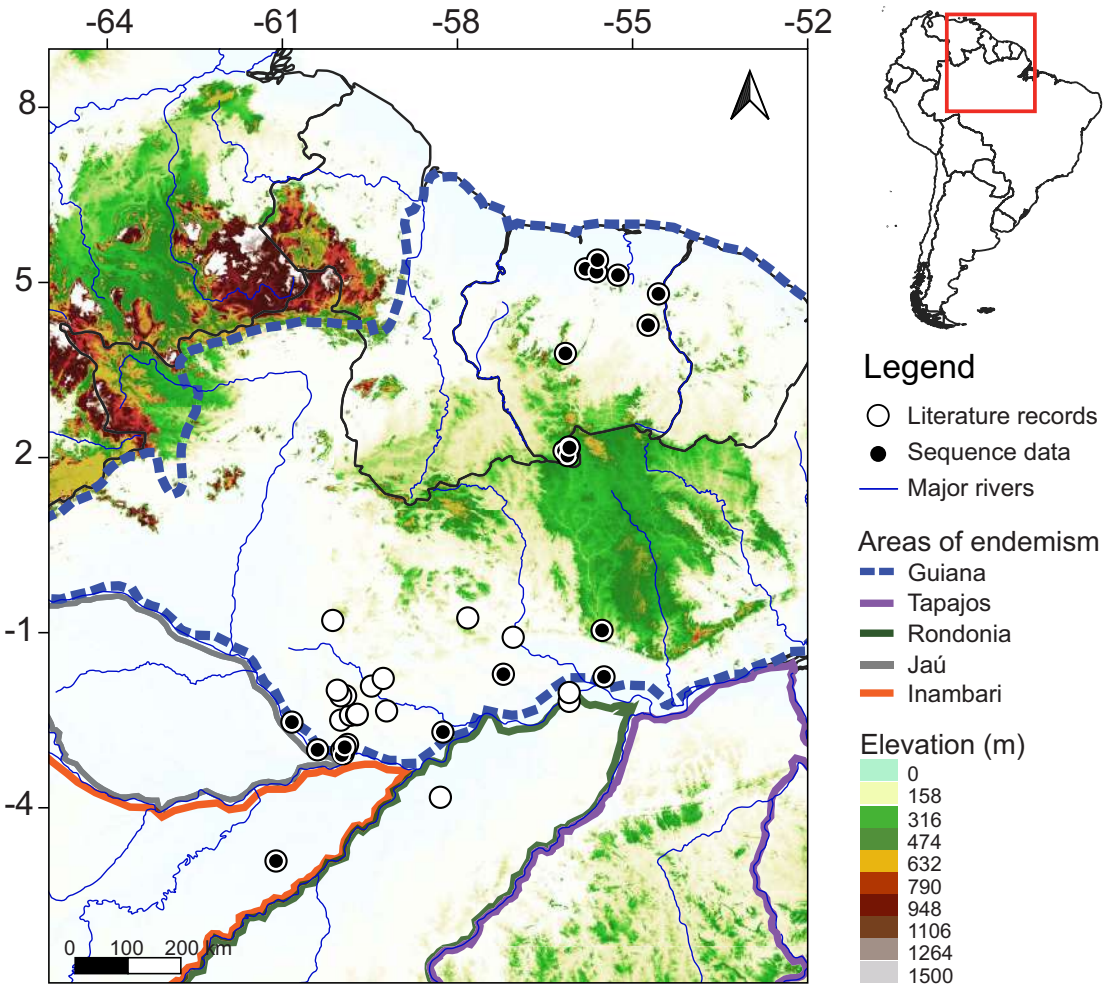

FIG. 4.—Distribution of *Anomaloglossus stepheni* in the areas of endemism of Amazonia, showing occurrence points (based on literature records) and localities with 16S rRNA sequence data. The elevation gradient is presented in the background. The red box in the South America map shows the area of the present study. The boundaries of the areas of endemism were based on da Silva et al. (2005) and Borges and Silva (2012).

although refuges were not detected in our niche analyses. By contrast, surprisingly, demographic analyses detected no significant changes through time, suggesting that climatic shifts drove genetic differentiation and distribution of clusters through time, but with minor effects on species demographic history. The lack of correspondence between BSP profiles and range size changes can be related, for example, to local population densities (Miller et al. 2021).

ENMs projected for present and past climatic scenarios suggest that historical climate influenced the *A. stepheni*'s distribution patterns, in which Quaternary climatic shifts promoted successively population expansions and shrinkages through time. During dry and cold periods of the Pleistocene, the aridity probably allowed the expansion of the savannas and other nonforested habitats over parts of the Amazonian forest, promoting its fragmentation during such periods (Van Der Hammen and Hooghiemstra 2000). Currently, open vegetated areas are found disjointly in the Guiana Shield (Pennington et al. 2000; Olson et al. 2001). Nonetheless, paleoclimatic models predicted that during drier/colder periods, such as LGM, the climate favored the expansion of these vegetated areas in the northern Amazon (Werneck et al. 2012). Conversely, there are controversies on the role of dry/cold periods driving lowland Amazonia fragmentation, as some studies point out that even in these periods Amazon remained forested (Colinvaux et al. 1996; Bush et al. 2004). Based on global vegetation models, the biome probably experienced vegetation shifts in the past 140,000 yr, mainly at the north and south Amazon (Allen et al. 2020). Considering that different parts of Amazonia likely responded differently to the climatic oscillations, these alternative models are not necessarily contradictory if we treat them as different units (Leite and Rogers 2013). Indeed, predicted biome distributions models for South America suggest an expansion and stability of tropical forests since the LGM (Costa et al. 2018). However, *A. stepheni* occurs in a region of higher instability in the Amazon (see Supplemental Fig. S1, available online; Costa et al. 2018, fig. 3a).

The current climatic model indicated that two suitable areas in the species distribution are separated by a large unsuitable area, one in the north portion (Guiana, Suriname, and French Guiana) and another in the south (Roraima, Amazonas, and Pará Brazilian states). However, five localities located in the south of Suriname are distributed in climatically unsuitable areas that may be explained as the population remnant of past wider distribution that got

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fragmented with the quaternary climatic oscillations and the models did not manage to identify them. Conversely, among others, the different weights and contributions of the selected variables can have impacted the omission errors with these known presences predicted as absences. For past periods, from LIG to Holocene (from ~120,000 to 6000 yr), there was a substantial decrease in climatic suitable areas for the species, with special reduction during LGM (~21,000 yr ago). Corridors of suitable areas connecting north and south can be observed during LGM, with lower probability, but also during MIS19 (~790,000 yr ago). Stability areas through time were not detected for the species probably due to LGM climate effects (Fig. 3). Climatic unsuitability of some periods during the Pleistocene, such as in LGM, seems to have played a major role in the species evolutionary history. Thus, we hypothesized that unsuitable climatic periods should dramatically have reduced the populations and species' geographic distribution, followed by recolonization from north to south. Also, our models may help researchers to focus on sampling areas with higher occurrence probabilities and conduct fieldwork in areas less sampled or even in areas that may have isolated populations that were once widely suitable for the species occurrence.

The Rocket Frog is spatially structured in two clusters with divergence time dating from Pleistocene. Climate changes during this period likely impacted the levels of genetic diversity and differentiation in the species. The Guiana Shield region harbors an incredible diversity and the instability of the region during the Quaternary should leave genetic signatures in the amphibian community, a group known for its lower tolerance to habitat fragmentation and sensitivity to climate changes. Thus, species traits such as environmental tolerances and dispersion capabilities should be reflected in the species' genealogical histories. Using more variable nuclear data such as SNPs, further studies may focus, for example, on studying landscape genetics/genomics, assessing the routes of connections or barriers to gene flow, and testing concomitantly different landscape features that shaped the genetic differentiation of *A. stepheni* and other codistributed taxa.

Acknowledgments.—FC thanks Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support and his post doc fellowship (processes 71/700.083/2020 and 302162/2020-8). PSC thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (finance code 001). DJS thanks CNPq for productivity funding (311284/2023-0). The samplings were authorized by the SISBIO (license 45889), Ministry of the Environment.

Supplemental Material

Supplemental material associated with this article can be found online at https://doi.org/10.1655/Herpetologica-D-23-00013.S1; https://doi.org/10.1655/Herpetologica-D-23-00013.S2 and https://doi.org/10.1655/Herpetologica-D-23-00013.S3.

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Accepted on 8 January 2025
Published on 28 March 2025
Associate Editor: Christopher John Raxworthy

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