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Research Article

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Phylogeography and ecological niche modelling reveal a geographical break in the genetic structure of *Boana crepitans* (Anura: Hylidae) during the Pleistocene in Eastern Brazil

ELVIS ALMEIDA PEREIRA[1,2], SARAH MÂNGIA[3,4], KAROLINE CERON[5], ERIC RAGALZI[6], ADRIAN ANTONIO GARDA[7], MARCELO FELGUEIRAS NAPOLI[8], HÉLIO RICARDO DA SILVA[9] & DIEGO JOSE SANTANA[3,4]

[1]Departamento de Biologia Animal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, São Paulo, 13083-862, Brazil
[2]Leibniz Institute for the Analysis of Biodiversity Change, Museum Koenig Bonn, Bonn, Germany 53113
[3]Instituto de Biociências, Universidade Federal de Mato Grosso do Sul, Campo Grande, 79070-900, Mato Grosso do Sul, Brazil
[4]Negaunee Integrative Research Center and Keller Science Action Center, The Field Museum of Natural History, 1400 S Shore Dr, 60605, Chicago, IL, USA
[5]Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, 60440-900, Ceará, Brazil
[6]Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil
[7]Departamento de Ecologia, Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74690-900, Goiânia, Goiás, Brazil
[8]Departamento de Zoologia, Instituto de Biologia, Universidade Federal da Bahia, 41170-115, Salvador, Bahia, Brazil
[9]Departamento de Biologia Animal, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, 23890-000, Brazil

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Integrating evolutionary and ecological approaches provides key insights into diversification processes in Neotropical biomes. Here, we combined phylogeographic analyses and ecological niche modelling (ENM) to investigate the diversification of the treefrog *Boana crepitans*, a widely distributed species associated with mesic habitats across the Atlantic Forest and Seasonally Dry Tropical Forests (SDTFs). We used mitochondrial and nuclear markers to estimate divergence times, reconstruct ancestral area, and assess population structure, genetic diversity and demographic history. ENMs were used to evaluated niche divergences between lineages, identify potential dispersal corridors, and compare palaeodistributions with historical demography. Our findings revealed two lineages, Eastern and Western, that diverged in the late Pleistocene (~0.0178 Ma). Both exhibited high haplotype sharing and demographic stability over the last 280,000 years, likely influenced by Quaternary climatic oscillations and the persistence of forest refugia across the Atlantic Forest, Caatinga and Cerrado. Spatial and temporal patterns suggest recurrent connections among mesic habitats during cooler periods, facilitating gene flow and maintaining population stability. Overall, these findings highlight the role of climatic dynamics and landscape connectivity in shaping diversification within *B. crepitans* and underscore the value of integrative evolutionary-ecological approaches for understanding biogeographic patterns in the Neotropics.

Key words: Atlantic forest, biogeography, coalescent methods, quaternary, seasonally dry tropical forest

Introduction

The Neotropics are the most diverse region on Earth (Myers et al., 2000; Rull, 2008), with a wide variety of morphoclimatic domains ranging from tropical forests to open and dry formations (Meseguer et al., 2022). The Atlantic Forest, one of the main morphoclimatic domains in this region, is bordered to the east by the Atlantic Ocean and to the west by the long continuous portion of open vegetation composed of Seasonally Dry Tropical Forests (hereafter SDTFs), the Cerrado savannas in central Brazil, and the Chaco in Argentina, Paraguay and Bolivia (Ledo & Colli, 2017). These interconnected domains extend diagonally across South America, from northeastern Brazil to northern Argentina and eastern Paraguay and Bolivia, forming what is

Correspondence to: Elvis Almeida Pereira, E-mail: elvisaps@hotmail.com

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known as the Diagonal of Open Formations (hereafter DOF) (Werneck et al., 2011).

The Cerrado and Caatinga regions harbour plateaus covered by savanna-like vegetation and mesic forests, which have remained relatively stable over time (Vanzolini, 1963; Werneck et al., 2011, 2012b). Between these plateaus, depressions are covered by mesic vegetation types in the Cerrado domain, ranging from savannas to dry forests with bands of gallery forests (Oliveira-Filho & Fontes, 2000; Oliveira-Filho & Ratter, 2010). In the mostly xerophytic Caatinga (de Oliveira et al., 1999; Kuhlmann, 1977), these depressions include riparian forests along intermittent rivers and extensive stretches of SDTFs (Prado, 2003). Near the Atlantic coast, mesic enclaves associated with the Atlantic Forest are embedded within a matrix of dry vegetation. At higher altitudes (>800 m), orographic rainfall supports moist forest enclaves whose biotas share affinities with either Amazonia or the Atlantic Forest (Melo Santos et al., 2007).

Geological, phytogeographic, palynological, and zoogeographic data suggest that the DOF and the Atlantic Forest share a long and dynamic landscape history (Batalha-Filho et al., 2013; de Oliveira et al., 1999; Ledru et al., 1998; Oliveira-Filho & Ratter, 2010). The DOF, which currently separates Amazonia from the Atlantic Forest, historically maintained dispersal routes shaped by climatic cycles (Ledo & Colli, 2017). Mesic enclaves and gallery forests within the Caatinga and Cerrado acted as corridors facilitating species exchange and long-term stability of tropical forest lineages (Carnaval & Bates, 2007; de Sá et al., 2019; Ledo et al., 2020). During glacial periods, the morphoclimatic domains of the DOF expanded, while forest biomes expanded during interglacial periods (Pennington et al., 2000). These Quaternary fluctuations reshaped the distribution of South American biomes and likely drove diversification across multiple taxa (de Oliveira et al., 1999; Pessenda et al., 2004, 2010).

The current distribution of Neotropical morphoclimatic domains reflects the combined influence of Quaternary climatic oscillations and the persistence of stable habitats (refugia) (Carnaval et al., 2009; Nores, 1994; Sérsic et al., 2011; Sobral-Souza et al., 2015). Recurrent cycles of expansion and retraction during the Pleistocene generated fragments of Cerrado and SDTFs within rainforests, and vice versa, reshaping current landscape configuration (Leite et al., 2016; Pennington et al., 2000; Prado & Gibbs, 1993). These dynamics influenced population demography and speciation (see de Oliveira et al., 2021; Nevado et al., 2018) by altering habitat connectivity and promoting isolation (Cabanne et al., 2007; Haffer & Prance, 2002; Pellegrino et al., 2005; Werneck, 2011).

In regions such as the DOF, topographic heterogeneity and rivers, including the São Francisco River, likely acted as partial barriers to gene flow while still allowing occasional dispersal (Oliveira et al., 2018; Vasconcellos et al., 2019). Environmental resistance from elevation, hydrology, and habitat suitability also shapes genetic differentiation in amphibians (Barratt et al., 2018; McCartney-Melstad & Shaffer, 2015), and the Cerrado's complex topography has contributed to divergence and speciation in several lizard and anuran lineages (Camurugi et al., 2021; Guarnizo et al., 2016; Mittan et al., 2022).

Widely distributed species, like *Boana crepitans* (Wied-Neuwied, 1824), present good opportunities to investigate diversification processes linked to these Quaternary dynamics and are useful models for testing the role of climate-driven habitat stability on genetic structure (Carnaval et al., 2009; Fitzpatrick et al., 2009). *Boana crepitans* is distributed from northeastern to southeastern Brazil, predominantly associated with forest and mesic habitats, but also found in adjacent xeric environments of the Caatinga and Cerrado (Orrico et al., 2017). Recent taxonomic revisions have restricted its range to central-eastern Brazil, following the recognition of *Boana xerophylla* (Duméril & Bibron, 1841) as a distinct species in northern South America (Dantas et al., 2019; Orrico et al., 2017; Silva et al., 2020).

The Pleistocene Climatic Fluctuation Hypothesis (Guillory et al., 2024) proposes that Pleistocene climatic oscillations triggered cycles of expansion and contraction of humid forests, leading to isolation in refugia and subsequent diversification of populations (Rull, 2011; Vanzolini & Williams, 1981). Under this framework, we predict that populations of *B. crepitans* were shaped by these Quaternary climatic cycles, resulting in genetic structuring associated with periods of forest contraction and expansion.

Climatic oscillations not only affected the spatial dynamics of populations but also influenced the ecological niches of Neotropical taxa (Peterson et al., 2011; Wiens & Graham, 2005). If climatic fluctuations promoted isolation and local adaptation, populations of *B. crepitans* would be expected to occupy distinct climatic spaces and show differences in habitat stability through time. Conversely, species with broad environmental tolerances and high dispersal capacity tend to maintain similar ecological niches and demographic stability despite past environmental changes (Carnaval et al., 2009; Fitzpatrick et al., 2009). Thus, comparing the climatic niches of *B. crepitans* lineages provides a framework to assess whether ecological differentiation can be

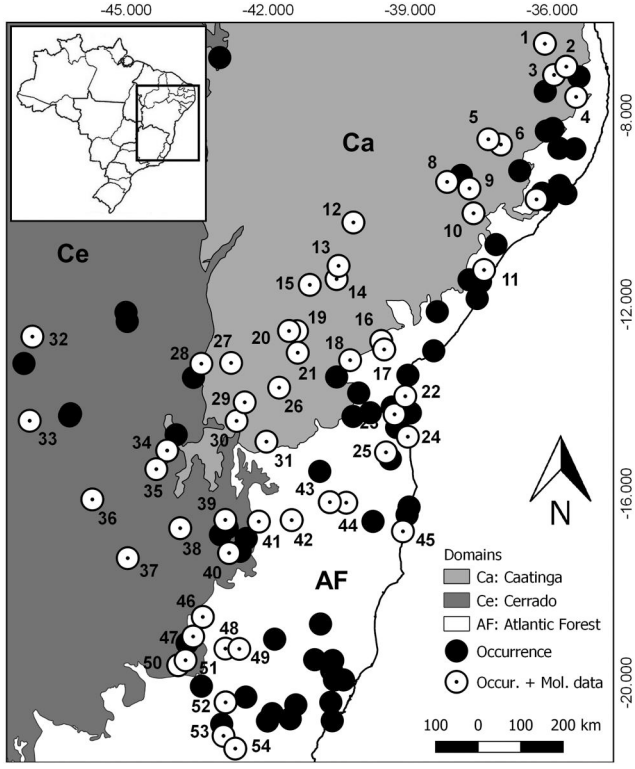

Fig. 1. Geographic distribution of *Boana crepitans*. The numbers correspond to 54 localities described in Supplemental Material 1.

responsible for genetic patterns shaped by Pleistocene dynamics.

Therefore, the main goals of this study were to (1) investigate the genetic structure of *B. crepitans* using multiloci phylogeographic data; (2) estimate divergence times and infer the demographic history of its lineages; and (3) evaluate whether Pleistocene climatic fluctuations, areas of climatic stability, and major geomorphological features such as the São Francisco River, correspond to the current genetic patterns inferred for this species. By integrating phylogeographic and ecological niche models, we aim to test whether Quaternary climatic dynamics and landscape heterogeneity shaped the diversification of *B. crepitans* across eastern Brazil.

Materials and methods

Sampling

Our sampling included 116 individuals of *B. crepitans* obtained from 40 localities across its distribution range. Tissue samples were collected during field expeditions conducted by the authors and complemented with donations from several Brazilian herpetological collections (see Supplemental Material 1). To increase geographic coverage, we also incorporated 36 mitochondrial sequences from GenBank (Sayers et al., 2019) (15 of the 12S gene, 16 of the COI gene, and five of the 16S gene) representing 14 additional localities (see Supplemental Material 1). In total, this dataset covers most of the species's known distribution (Fig. 1). *Boana xerophylla* was used as outgroup in subsequent phylogenetic analyses. This comprehensive sampling allowed us to capture most of the genetic and geographic variation within the species.

Sequencing

We extracted genomic DNA from muscle and/or liver samples using the phenol-chloroform protocol from Sambrook et al. (1989) and amplified the DNA using the Polymerase Chain Reaction (PCR) protocol of Costa et al. (2016). Amplifications were performed using gene-specific primers under optimized conditions (Supplemental Material 2). PCR products were purified using the acetate-ammonium technique (Hirata & Head-Gordon, 1999) and the purified products were sequenced according to the Sanger method using an ABI 3500 Genetic Analyzer (Applied Biosystems, USA).

First, we sequenced a fragment of the mitochondrial 16S gene (mtDNA) for all individuals. Next, we identified different haplotypes using the DnaSP 5.10.1

program (Librado & Rozas, 2009) and selected individuals of each haplotype to sequence two nuclear genes (nuDNA): proopiomelanocortin (POMC) and recombination activators 1 (RAG1). GenBank sequences were not used in this step. In cases where the same haplotype was found in two or more localities, we selected two or more individuals to encompass samples from the furthest points within the geographic distribution of that haplotype, totalling 52 samples for nuclear loci, following the methodology established in prior studies (Mângia et al., 2020; Oliveira et al., 2015; Ruane et al., 2014).

All sequences obtained were aligned using the MUSCLE 3.8.31 algorithm (Edgar, 2004) implemented in Geneious 9.1.2 (Biomatters Ltd), and the alignments were visually inspected and manually adjusted when necessary. We removed gaps in 12S and 16S genes using Gblocks 0.91b (Castresana, 2000; Talavera & Castresana, 2007), which eliminates ambiguously aligned sites and standardizes sequence length.

We used PHASE 2.1.1 (Stephens et al., 2001) in DnaSP 5.10.1 (Librado & Rozas, 2009) with default options to determine the most likely allele pairs for each nuclear sequence in heterozygosity. We considered in subsequent analyses only allele pairs with a probability greater than 80%. All sequences obtained in this study were deposited in GenBank (accession numbers not yet available) (see Supplemental Material 1).

Population structure

We used a genotypic matrix containing one mtDNA (16S) and two nuDNA genes (POMC and RAG1) together with spatial coordinates to investigate population structure using GENELAND 4.0.3 (Guillot et al., 2005a, 2005b), implemented in R 3.6.1 (R Core Team, 2019). GenBank sequences were not used in this step. This analysis assesses the presence of population structure in a set of georeferenced genetic data by explicitly identifying and inferring genetic discontinuities across the landscape. The most likely number of lineages (k) was determined by the Monte Carlo method *via* Markov Chain (MCMC), with three repetitions (1 x 10^6 iterations each) from $k = 1$ to 4. The number and spatial configuration of genetic clusters (lineages) were determined a *posteriori* based on GENELAND results (see Results section).

Molecular diversity and haplotype network

Mitochondrial fragments (16S, 12S and COI genes) and phased nuclear sequences (POMC and RAG1 genes) of each lineage identified in GENELAND were used to calculate the number of polymorphic sites (S), number of haplotypes (h), diversity of haplotypes (Hd) and nucleotide diversity (π), for each locus in DnaSP 5.10.1 (Librado & Rozas, 2009). We quantified genetic differentiation between and within lineages, and between lineages left and right of the São Francisco River (using only the 16S, POMC and RAG1 genes) through an Analysis of Molecular Variance (AMOVA), in Arlequin 3.5 using 10,000 permutations (Excoffier & Lischer, 2010).

We calculated sequence divergences based on the 16S gene (uncorrected *p*-distance) between lineages using MEGA 6.06 (Tamura et al., 2013), with pairwise deletion of gaps and uniform rates among sites. Furthermore, we also estimated haplotype networks for mtDNA and nuDNA genes (phased) using the median-joining (MJ) method (Bandelt et al., 1999) in POPART 1.7 (Leigh & Bryant, 2015) with default settings. For this analysis, individuals were assigned to populations following the GENELAND results.

Species tree

We estimated the most appropriate substitution model for each gene, using the Bayesian Information Criterion (BIC, Sullivan & Joyce, 2005) implemented in the jModelTest 2.1.4 program (Darriba et al., 2012) (Supplemental Material 3). The species tree was estimated in BEAST 2.7.6 (Bouckaert et al., 2019) using mtDNA and nuDNA (phased) genes, with populations assignments defined according to GENELAND results.

Due to the lack of fossil calibrations for this group, we used the 16S gene mutation rate proposed by Lemmon et al. (2007) (0.735% per lineage per million years) under a Birth-and-Death process. Then, analyses in BEAST 2.7.6 (Bouckaert et al., 2019) were performed for 3×10^8 generations and sampled every 3 x 10^4 generations (more details in Supplemental Material 3). We evaluated the convergence of MCMC executions and ESS values ($\geq$200) using TRACER 1.6 (Rambaut & Drummond, 2007). The first 20% of the sampled genealogies were discarded as *burn-in* and the maximum clade credibility tree was annotated with TreeAnnotator 2.7.6 (Bouckaert et al., 2019).

Historical demography

We calculated Tajima's D to assess deviations from neutrality that may reflect historical demographic events. This statistic is expected to be close to zero if effective population sizes did not change over time, negative if it increased, and positive if it shrank (Tajima, 1989). We

estimated Tajima's *D* with 1,000 simulations for each locus in the DnaSP 5.10.1 (Librado & Rozas, 2009).

To investigate demographic changes in effective population sizes of *B. crepitans* lineages, we used a non-parametric single-locus model that estimates the effective population sizes (Ne) over time, the Bayesian Skyline Plot (Drummond et al., 2005; 2012; Drummond & Rambaut, 2007). Each lineage was analysed separately in BEAST 1.8 (Drummond et al., 2012). The parameters used in the analyses are shown in Supplementary Material 3. We calculated Ne values for 50-time intervals (grid points) over the last 500,000 years (cutoff). We based the time calibration on the estimated substitution rate for the 16S gene (2.8×10^{-3} replacement/site/year) and evaluated convergence, stationarity and ESS ($\geq$200) visually in TRACER 1.6 (Rambaut & Drummond, 2007). Then, using the Skyline Reconstruction function, we generated graphs that illustrate the historical demographic dynamics of the populations.

Phylogeographic reconstruction and centre of origin

We used the Homogeneous Brownian Diffusion model to generate phylogeographic reconstructions of the 16S gene for Eastern and Western lineages in BEAST 1.8 (Lemey et al., 2010), using geographic coordinates of samples as traits and the nucleotide sequences. Some coordinates of the samples were duplicated, so we used a jitter option of 0.05. As the software estimates the geographic location of internal nodes in each genealogy, the analysis also uses Bayesian inference to reconstruct ancestral continuous characters.

We loaded the final 16S gene tree, inferred in BEAST 1.8 (Drummond et al., 2012), into SPREAD 1.0.4 (Bielejec et al., 2011) to create a keyhole markup language file (.kml) containing the reconstruction of the phylogeographic history of the gene. The .kml file can be opened in Google Earth Pro (Google LLC, 2023) to display the representation of the area where each node of the phylogenetic tree possibly originated and the gradual emergence of polygons (based on 80% highest posterior density interval (HPD)). The possible colonization routes are suggested in the order in which polygons appear.

The probable centre of origin of each lineage of *B. crepitans* was recognized by the area in which there is an overlap of polygons of the most ancestral nodes of the gene tree used. After identifying genetic lineages and their demographic patterns, we used ecological niche modelling to explore whether their distributions and potential refugia were consistent with the phylogeographic results.

Ecological niche model

We used 120 georeferenced records to build ecological niche models (ENM): 21 for the Western lineage and 99 for the Eastern lineage (Supplemental Material 1). We modelled ecological niches for the species and separately for each lineage recovered in population analysis to allow estimation of the potential distribution areas for the species and its lineages, as well as for additional comparisons of ecological niches between lineages.

We designed ENMs using paleoclimate data, Last Interglacial (LIG; ~120 to 140 kya), Last Glacial Maximum (LGM; ~21 kya) and mid-Holocene (~6 kya), to detect patterns of expansion and contraction of the distributions that were compatible with expansions and contractions of populations detected by genetic data, indicative of a role as habitat refuges or expansion to wet and dry forests, respectively. We downloaded high-resolution bioclimatic layers (5 arc-min resolution) from the WorldClim project 1.4 (Fick & Hijmans, 2017). To avoid overprediction and low specificity, we cropped bioclimatic layers to span from latitude −40 to 10 and longitude −80 to −30 (values in decimal degrees) (Werneck et al., 2011).

To select the most important variables, we conducted a Principal Component Analysis (PCA) using ade4 1.7-13 package in R 4.2.1 (R Core Team, 2019) and visualized the correlation between variables. Four bioclimatic variables, Annual Mean Temperature (Bio1), Temperature Annual Range (Bio7), Mean Temperature of Wettest Quarter (Bio8), and Precipitation of Driest Quarter (Bio17), were selected based on their non-collinearity and contribution to overall environmental variation.

We generated ecological niche models (ENMs) for *B. crepitans* using nine algorithms available in the *biomod2* 3.3–7.1 package (Thuiller et al., 2016) in R 4.2.1 (R Core Team, 2019). These included three regression-based methods: GAM (generalized additive model; Hastie, 2017), GLM (general linear model; McCullagh, 2019), and MARS (multivariate adaptive regression splines; Friedman, 1991); three machine learning approaches: GBM (generalized boosting model; Ridgeway, 1999), MAXENT (maximum entropy; Phillips et al., 2006), and RF (random forest; Breiman, 2001); two classification methods: CTA (classification tree analysis; Breiman et al., 1984) and FDA (flexible discriminant analysis; Hastie et al., 1994); and one environmental envelope model, SRE (surface range envelope; Busby, 1991). For models requiring absence data

(all except SRE), we generated two sets of random pseudo-absence points, equal in number to the presence records and distributed across the modeling background. We calibrated the models using 70% of randomly selected data, retaining the remaining 30% for evaluation.

We evaluated the performance of each individual model using two metrics: the True Skill Statistic (TSS) and the area under the receiver operating characteristic curve (ROC), as implemented in the biomod2 3.3–7.1 package (Thuiller et al., 2016). TSS was calculated as sensitivity + specificity −1, ranging from −1 to +1, where +1 indicates perfect agreement, 0 corresponds to random performance, and values below 0 indicate worse-than-random predictions (Allouche et al., 2006). Only models demonstrating high predictive accuracy (TSS $\geq$0.8 and ROC $\geq$0.8) were retained for ensemble modeling.

We then generated ensemble maps by taking the median of two runs of all selected models, considering only those with TSS values equal to or greater than 0.8 (Supplemental Material 1). Ensemble models are continuous probability maps that were transformed into a predicted bivariate map of potential presence versus absence of the species using a threshold approach. Finally, regions of climatic stability through time were identified by stacking and averaging the current and two projected paleoclimate ENMs. Regions highlighted in these stacked projections were inferred to represent potential climate refugia through time for *B. crepitans*.

Niche comparisons

We calculated ecological niche characteristics to assess the degree of shared environmental niche space between *B. crepitans* lineages. The assessment of niche overlap quantifies the niche shared between lineages. Initially, we included all bioclimatic variables, as tests of niche equivalence and similarity are most reliable when performed using the PCA-env approach (Broennimann et al., 2012). This method constructs a principal component analysis (PCA) based on the full environmental background, reducing the multidimensional climatic data to the first two principal axes. The resulting environmental space is then compared with the locations actually occupied by each lineage. In this study, niche overlap between pairs of *B. crepitans* lineages was computed by means of the Schoener's D statistic directly from ecological niche space (Schoener, 1968; Warren et al., 2008). The value of D ranges from 0, when two lineages have no overlap in the environmental space, to 1, when two lineages share the same environmental space (Schoener, 1968; Warren et al., 2008).

We used the niche equivalence tests to assess whether the ecological niches of pairs of *B. crepitans* lineages were significantly different from each other and if the two niche spaces were interchangeable. We assessed whether ecological niches of *B. crepitans* lineages are equivalent or more similar than expected by chance using two complementary approaches. First, a niche equivalence test was applied by comparing observed Schoener's D values between lineage pairs to a null distribution of 100 randomly generated D values (Hanley & McNeil, 1982). This test evaluates whether two lineages occupy the same environmental space based on their observed occurrences, ignoring the broader available environmental background. Non-equivalence was inferred when the observed overlap was significantly lower than the null distribution ($p \leq 0.05$).

Second, a niche similarity test was conducted to determine whether niche overlap between lineages is greater or smaller than expected given the environmental conditions available in their respective ranges (Warren et al., 2010). This approach accounts for differences in the geographic distribution of habitats and evaluates whether the niches of one lineage can predict occurrences of another better than expected by chance. Significant results indicate that observed niche differences are not simply a consequence of geographic variation in environmental availability. These analyses were performed in R 4.2.1 (R Core Team, 2019) using the *ecospat* package (Di Cola et al., 2017).

Results

Sequencing and population structure

A total of 116 individuals, representing 40 localities, were successfully sequenced for the 16S gene (285 bp). From these, 52 samples had both the mtDNA gene and at least one nuDNA marker successfully amplified. For the nuclear loci, 51 samples (102 phased sequences) of the POMC gene (354 bp) and 52 samples (104 phased sequences) of the RAG1 gene (1,038 bp) were obtained (Supplemental Material 1). In addition, sequences available on GenBank included 15 of the 12S gene (718 bp), 16 of the COI gene (497 bp), and five of the 16S gene (285 bp). The 16S sequences from GenBank were incorporated into our alignment, resulting in a final dataset of 121 samples.

Population structure analyses in GENELAND detected two distinct populations (Fig. 2). The first lineage is distributed from northeastern to southeastern Brazil, primarily along the Atlantic Forest, occupying a large part of this domain, extending into adjacent areas of the Caatinga and Cerrado regions (=Eastern lineage).

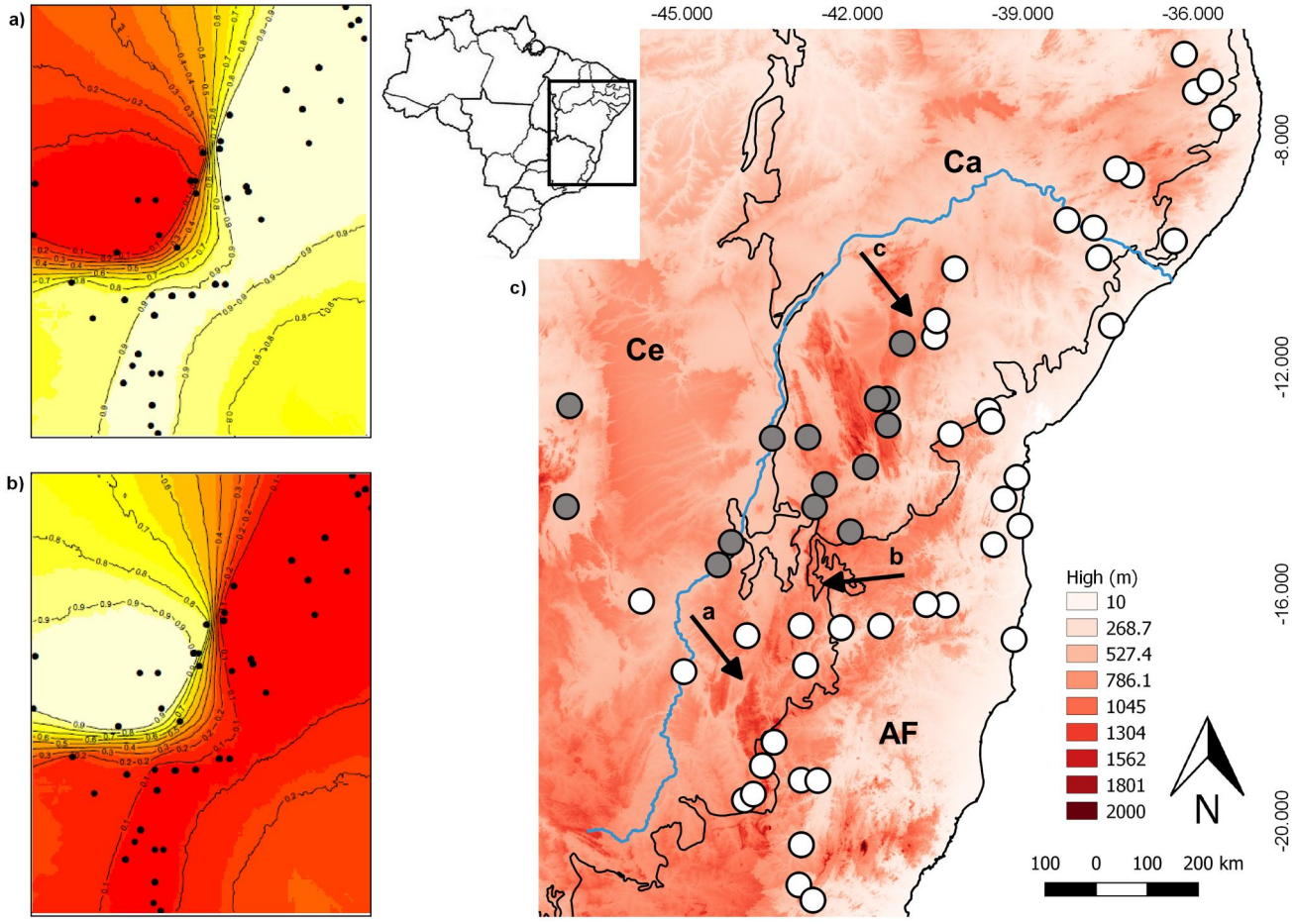

Fig. 2. GENELAND analysis (a and b) with posterior probability isoclines, which indicate extensions of the genetic populations found (black lines with inclusion probabilities). Coloured zones on each map indicate the groups of localities most likely to belong to the same genetic unit. Black dots indicate the points of the 54 locations analysed. The map on the right (c) shows the distribution of the Eastern lineage (white circles) and the Western lineage (gray circles) over the morphoclimatic domains Caatinga (Ca), Cerrado (Ce) and Atlantic Forest (AF). Red represents higher altitudes, and white represents lower altitudes. The arrows represent: (a) Southern limit of the Meridional Espinhaço Mountain Range, (b) Northern limit of the Central Espinhaço Mountain Range, and (c) Northern limit of the Septentrional Espinhaço Mountain Range. The blue line represents São Francisco River.

The second lineage is predominantly found in the southern Caatinga and the southeastern to central areas of the Cerrado domain (=Western lineage).

The Western lineage presents higher diversity of haplotypes and nucleotides in the mtDNA genes in comparison to the Eastern lineage (Table 1). Conversely, for the nuDNA genes, the Eastern lineage exhibits a greater diversity of haplotypes and nucleotides (Table 1). AMOVA results based on GENELAND clusters showed low F_{ST} values (1–9%) for all genes. In all five genes, more than 90% of the genetic variation was explained by differences within lineages and only a small portion of the differences were found between lineages, indicating lack of population structure (Table 2). Similarly, AMOVA comparing populations on opposite sides of the São Francisco River also showed low F_{ST} values for the 16S, POMC and RAG1 genes, with more than 85% of the genetic variation explained by differences within lineages, also indicating lack of population structure (Table 3).

The *p*-distances of the mtDNA 16S gene indicate up to 2.2% difference between Eastern and Western lineages. Additionally, there is internal differentiation of up to 1.8% in the Eastern lineage and up to 2.2% within the Western lineage (Supplemental Material 4). The population structure is not readily discernible through haplotype networks constructed from both mtDNA and nuDNA, as several haplotypes are shared between the Eastern and Western lineages across all genes (Fig. 3).

Species tree and divergence time

The BEAST species tree inferred from five loci recovered the east and west lineages as sister clades with

Table 1. Genetic statistics for each locus for Eastern and Western lineages of *Boana crepitans* from its distribution throughout Brazil.

Locus	Population	*L* (bp)	*N*	*S*	*H*	H_d	π	Tajima's *D*	*P*-value
12S	Eastern	718	11	5	4	0.745	0.00274	−0.31446	>0.10
	Western	718	4	6	4	1.000	0.00384	1.22875	>0.10
16S	Eastern	285	101	13	13	0.782	0.01016	0.67051	>0.10
	Western	285	21	15	11	0.838	0.01027	−0.86487	>0.10
COI	Eastern	497	13	11	4	0.756	0.00922	1.68877	>0.10
	Western	497	4	10	3	0.833	0.00934	−0.52807	>0.10
POMC	Eastern	354	*68	33	12	0.772	0.02647	1.15819	>0.10
	Western	354	*34	26	10	0.731	0.01376	−0.81691	>0.10
RAG1	Eastern	1038	*70	12	17	0.906	0.00229	−0.13227	>0.10
	Western	1038	*34	10	12	0.891	0.00214	−0.28094	>0.10

Test Tajima's *D* and *P-values*. *phased sequences. **Abbreviations:** ***L***, base pairs length; ***N***, sample size; ***S***, number of polymorphic sites; ***H***, number of haplotypes; ***Hd***, haplotypic diversity; **π**, nucleotide diversity.

Table 2. Variance percentages for components of analysis of molecular variance (AMOVAs) performed with different genes in the Eastern and Western lineages of *Boana crepitans.*

	Variation sources		
Locus	Between lineages	Within lineages	F_{st}
12S	1.07	98.93	0.01068
16S	9.38	90.62	0.09384
COI	5.47	94.53	0.05472
POMC	3.27	96.73	0.03272
RAG1	5.08	94.92	0.05077

All *P*-values < 0.0001.

Table 3. Variance percentages for components of analysis of molecular variance (AMOVAs) performed with different genes in the lineages on the left and right sides of the São Francisco River of *Boana crepitans.*

	Variation sources		
Locus	Between lineages	Within lineages	F_{st}
16S	14.87	85.13	0.14874
POMC	8.99	91.01	0.08991
RAG1	−0.29	100.29	−0.00291

All *P*-values < 0.0001.

moderate support (PP = 0.85; Fig. 4). Their divergence was dated to 0.0178 Ma (95% HPD = 0.0064 to 0.032 Ma), consistent with the late Pleistocene. The split between the ingroup (East + West) and the outgroup was strongly supported (PP = 1.0) and estimated at 0.1532 Ma (95% HPD = 0.0034 to 0.4878 Ma), also within the late Pleistocene.

Historical demography

Skyline analyses indicate that both Eastern and Western lineages maintained relatively stable effective population sizes throughout most of the Late Pleistocene, followed by a modest demographic expansion beginning approximately 50 thousand years ago (Fig. 5). Although uncertainty increases toward deeper time, the median estimates consistently suggest recent growth in both lineages. These patterns are consistent with the haplotype networks (Fig. 3), which do not display the pronounced star-like configurations typically associated with rapid, large-scale demographic expansions. Furthermore, Tajima's *D* values were non-significant for all genes in both lineages (Table 1), supporting a scenario of demographic stability through time.

Phylogeographic reconstruction and centre of origin

The dispersal centre analyses indicate that the most ancient common ancestor of the Eastern and Western lineages likely originated in southern Bahia State, east of the Espinhaço Mountain Range (Fig. 6). The polygons corresponding to ancestral clades overlapped the Central and Septentrional Espinhaço Mountain Range region, indicating these areas as the probable ancestral centre of origin.

According to the phylogeographic reconstruction of the 16S gene, populations extending from northwestern Bahia State toward Paraíba State and southeastern Minas Gerais State appear to be the result of more recent colonization events. Subsequently, haplotypes underwent modifications and expanded toward the northeast, west, east, and south of the Serra do Espinhaço, covering the entire region during the late Pleistocene (Fig. 6).

Ecological niche model

The ensemble model had a TSS of 0.88 and ROC of 0.98, indicating that the final model was reasonably good (Supplemental Material 5; Supplemental Table S2). Annual Mean Temperature (Bio1) was the most important variable in all models (49% of contribution), followed by Precipitation of the Driest Quarter (Bio17, 38%), and Temperature Annual Range (Bio7, 26%). The ENM predicted extensive suitable area across northeastern and southeastern Brazil (Fig. 7).

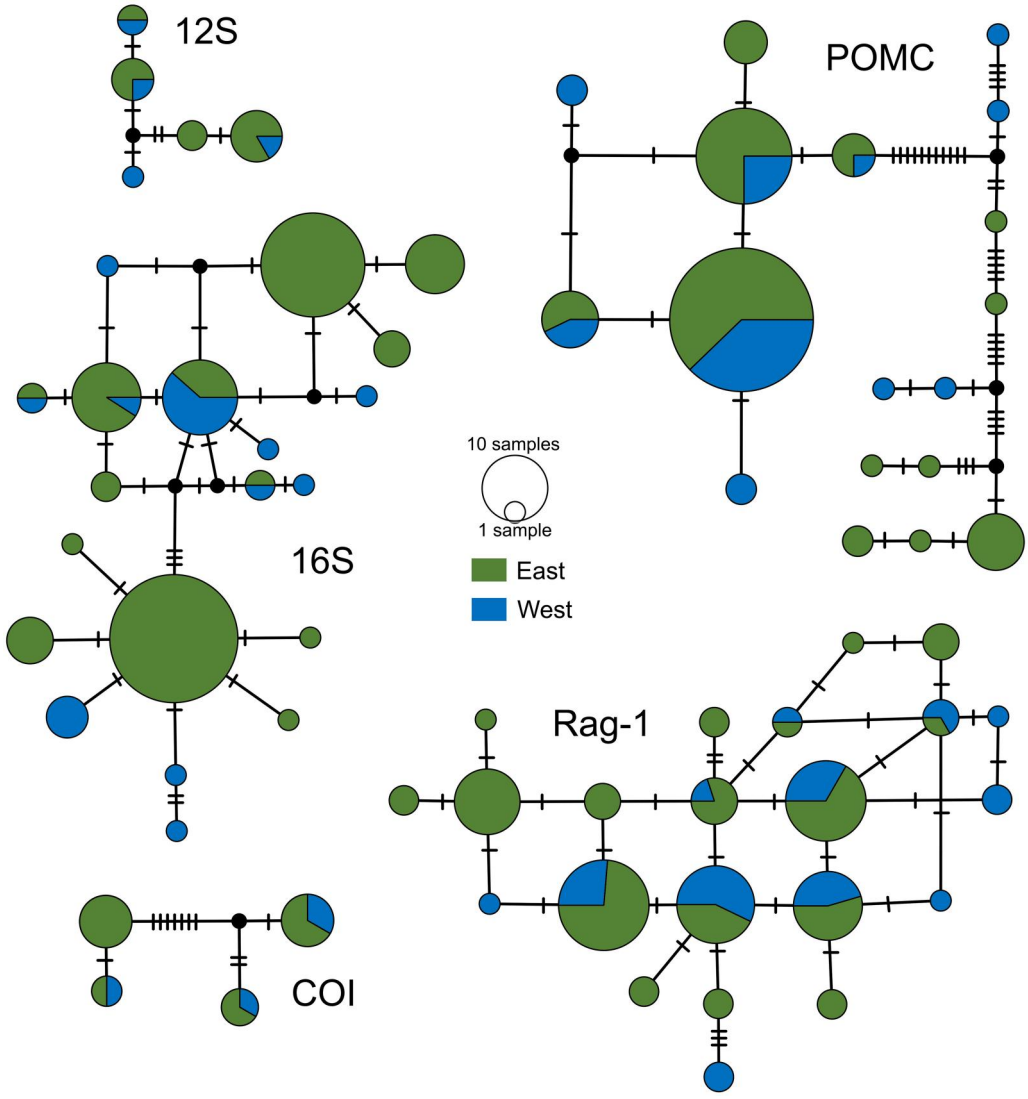

Fig. 3. Haplotype network for mtDNA genes 12S, 16S, and COI, and nuDNA genes POMC and RAG1 using the median joining method. Each haplotype is represented by a circle whose area is proportional to its frequency. Black dots, median vectors (unsampled or extinct haplotypes).

Lineage-specific models also showed high predictive accuracy: Eastern lineage (TSS = 0.99; ROC = 0.99) and Western lineage (TSS = 0.92; ROC = 0.94), indicating that final models were good for both lineages. For the Western lineage, the model predicted high adequacy over a large area in the centre-northern Bahia State, where the lineage occurs, reaching some areas within the Caatinga in northeastern Brazil and other areas west of the Espinhaço Mountain Range (Fig. 7). For the Eastern lineage, the model predicts a potential distribution area along northeastern and southeastern Brazil, with some overprediction into Cerrado in São Paulo State and Atlantic Forest in Rio de Janeiro State, where the species is not currently recorded (Fig. 7).

Palaeodistribution models

Palaeoclimatic projections for the LGM (~21 kya) indicated that the potential area suitable for the Eastern lineage was fragmented during this period, occurring in only a few refugial areas in northeastern and southeastern Atlantic Forest and in areas in the Amazon region (Fig. 7).

The palaeodistribution models for both lineages revealed broader suitable area during this period compared to the present. The climatic conditions suitable for the occurrence of the Western lineage were broader during the LGM (~21kya) than today (Fig. 7). The palaeodistribution models for the two lineages during the LIG (~120 to 140 kya) indicated a slightly larger distribution than for the mid-Holocene (~6 kya) and smaller than

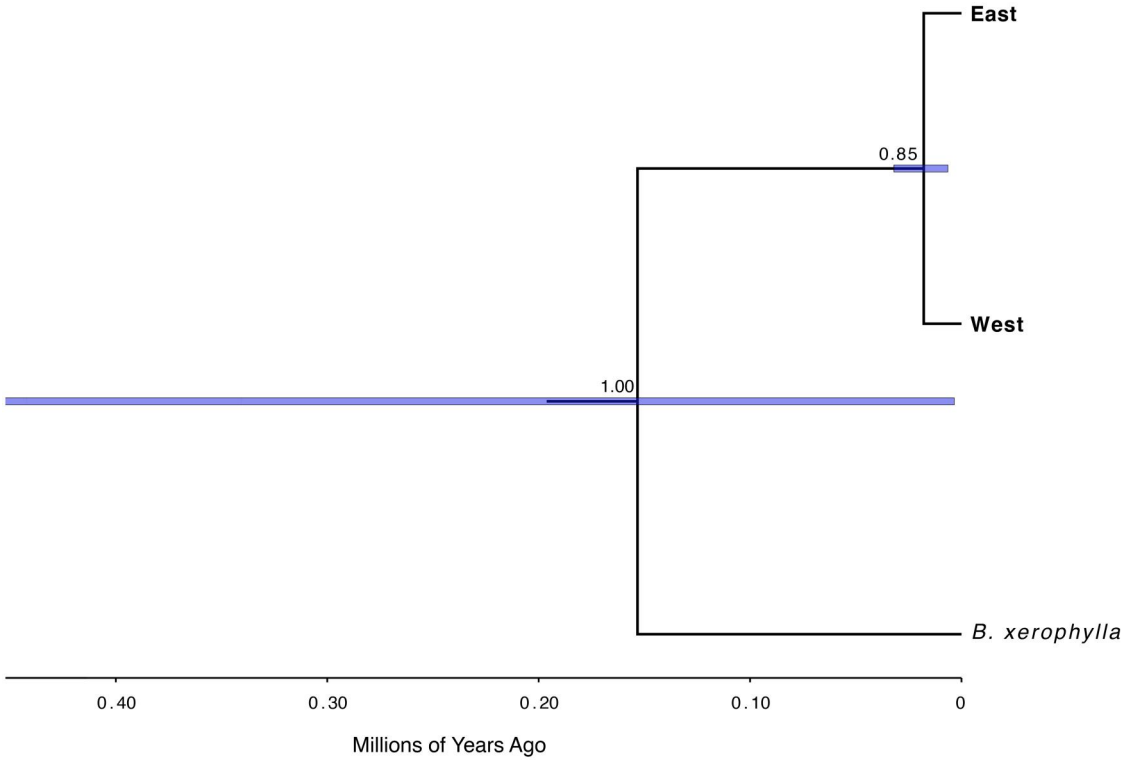

Fig. 4. Species tree between Eastern and Western lineages of the *Boana crepitans*, based on Bayesian analysis of mtDNA genes 12S, 16S, and COI, and nuDNA genes POMC and RAG1. The value above the branch indicates posterior probability.

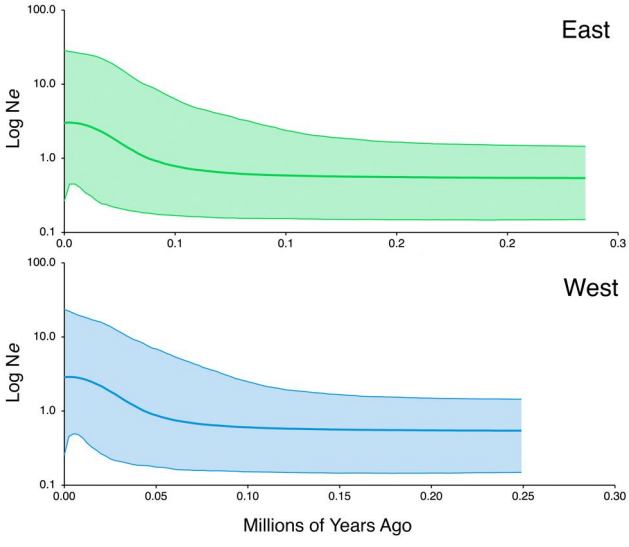

Fig. 5. Bayesian Skyline plots illustrating effective population sizes (Ne) over time (in years) of the Eastern (a) and Western (b) lineages. The coloured lines represent the median estimates of Ne, and the shaded areas indicate the 95% highest posterior density (HPD) intervals.

the LGM and the present (Fig. 7). By overlapping maps of all models, several stable areas (refuges) were found (Fig. 8).

Niche comparisons

Niche overlap results suggest a low overlap in the climatic space inhabited by *B. crepitans* lineages ($D = 0.37$). The hypothesis of equivalence of ecological niches for both lineages was rejected ($D = 0.37$, $p = 0.23$), likewise for the pairwise of the Western lineage with the Eastern lineage ($p = 0.28$) in relation to niche similarity. Niche similarities involving the background of the Eastern lineage with the Western lineage were more similar than expected by chance ($p = 0.01$; Fig. 9), indicating a niche similarity between lineages.

Discussion

Our results support the Pleistocene Climatic Fluctuation Hypothesis (PCFH, *sensu* Guillory et al., 2024), as evidenced by the shallow genetic divergence between lineages, extensive haplotype sharing, low Fst values, and demographic stability inferred over the last ~280,000 years. During this period, repeated cycles of expansion and contraction of morphoclimatic domains led to the isolation and reconnection of xeric forest patches, which were intermittently isolated by mesic corridors connecting the Atlantic Forest and Amazonia across SDTFs (Werneck, 2011). Species associated

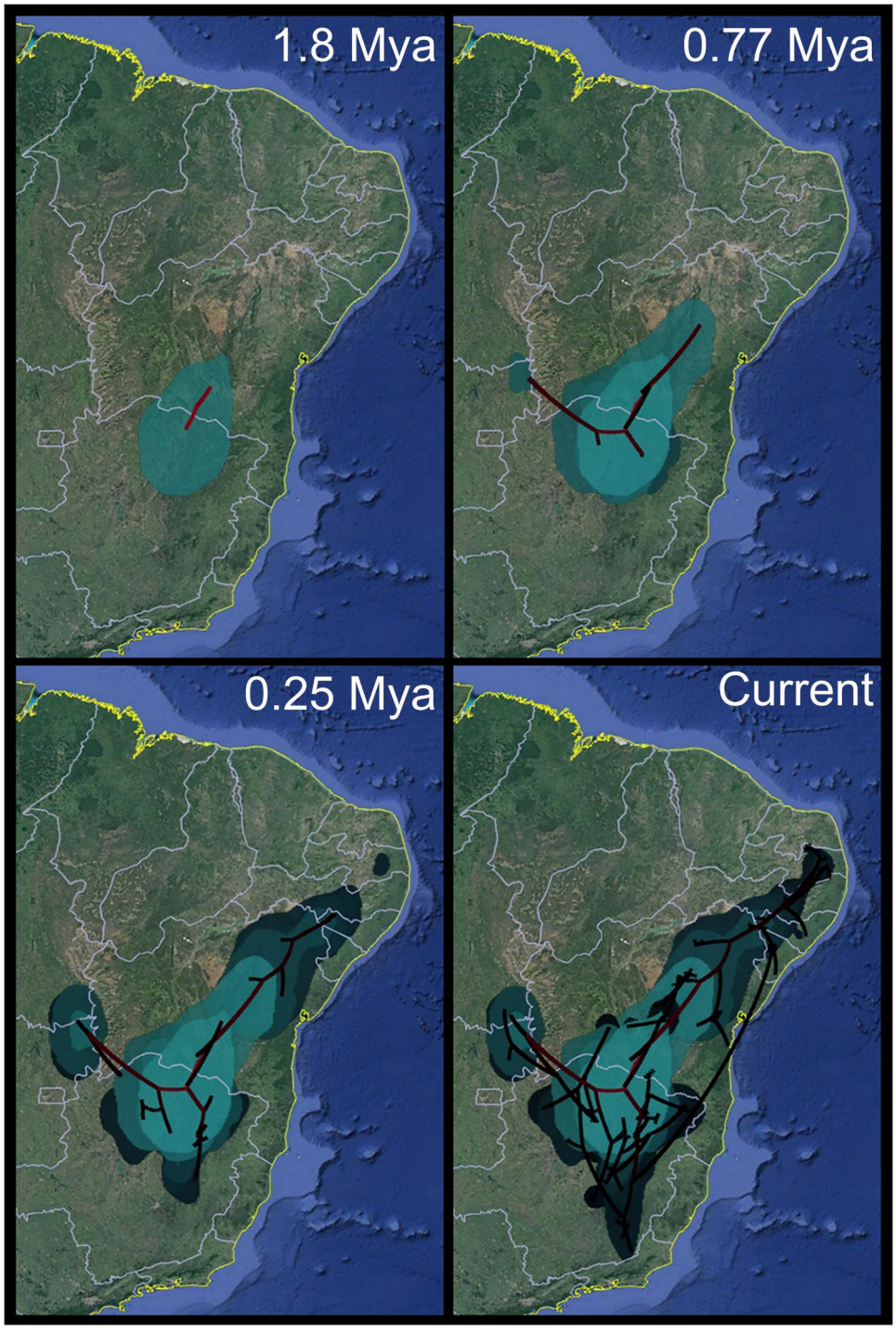

Fig. 6. Phylogeographic reconstructions of the *Boana crepitans*. The spatiotemporal dynamics of the mtDNA 16S gene is shown in four different time periods from its origin to the present. Light polygons represent basal clades, and dark polygons represent recent clades. The lines represent the tree branches with maximum credibility of the clade projected onto the surface. The maps are based on satellite images viewed on Google Earth.

with mesic habitats that experience relatively stable microclimatic conditions, such as *Boana crepitans*, may therefore persist in climatically buffered microhabitats during unfavourable phases and expand during wetter intervals, particularly when suitable habitats persist in refugial areas or along riparian and forested corridors (Carnaval et al., 2009; Sobral-Souza et al., 2015; Wiens, 2011).

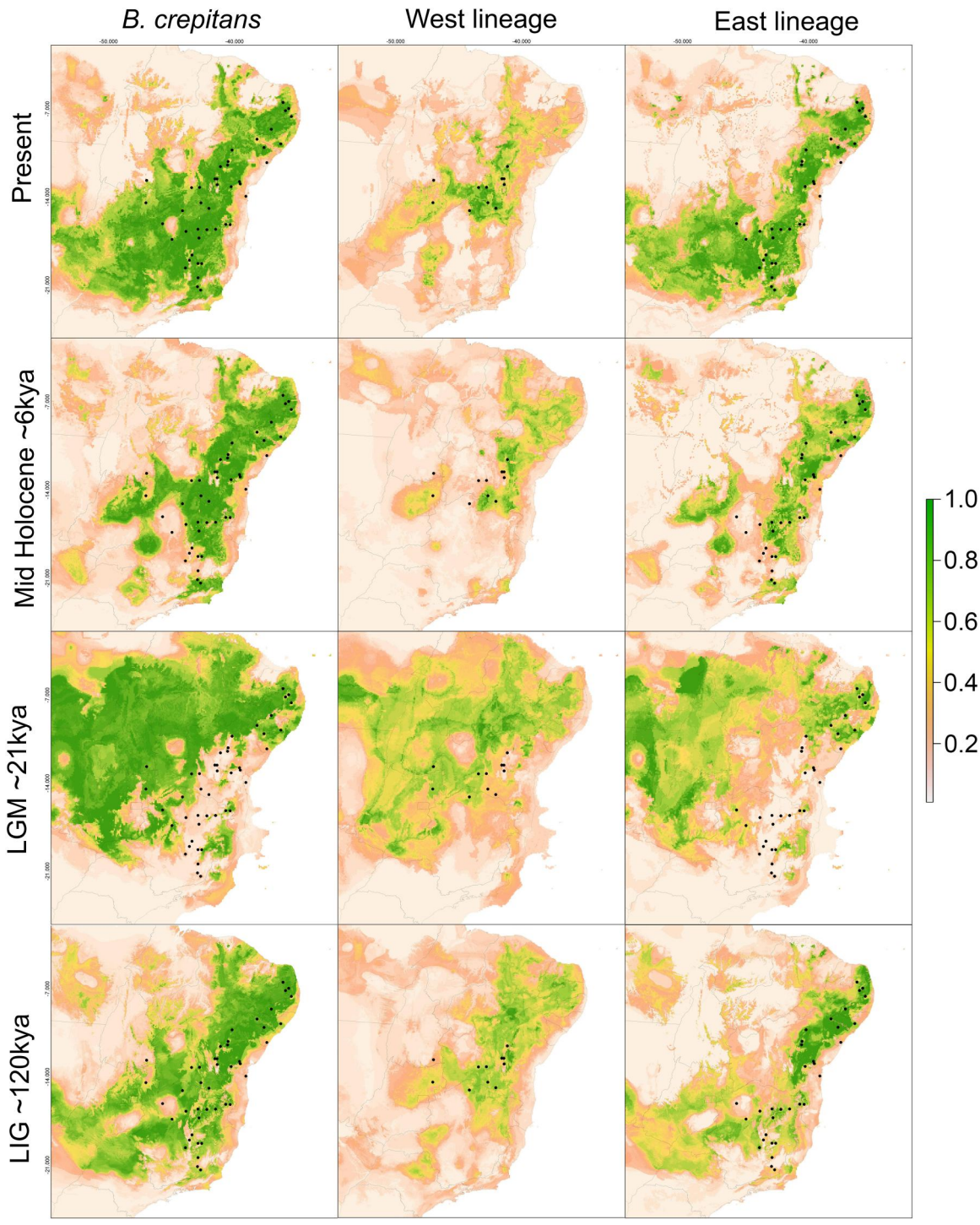

Fig. 7. Ecological niche models (ENMs) for *Boana crepitans* (using all occurrence records) and for the Eastern and Western lineages of *B. crepitans* projected for four climate scenarios (present, 6kya, 21kya and 120kya). Colours indicate predicted suitability, ranging from inadequate (white), low (orange), medium (yellow) and high (green). Black dots represent occurrence records.

The estimated divergence between *B. crepitans* and the outgroup species occurred around 0.153 mya, during the Pleistocene. This period was characterized by pronounced climatic fluctuations, with glacial cycles (Antonelli et al., 2018; Hoorn et al., 2010; Prado & Gibbs, 1993; Sobral-Souza et al., 2015). Such conditions likely promoted the ecological differentiation between Amazonian (*B. xerophylla*) and Atlantic Forest (*B.*

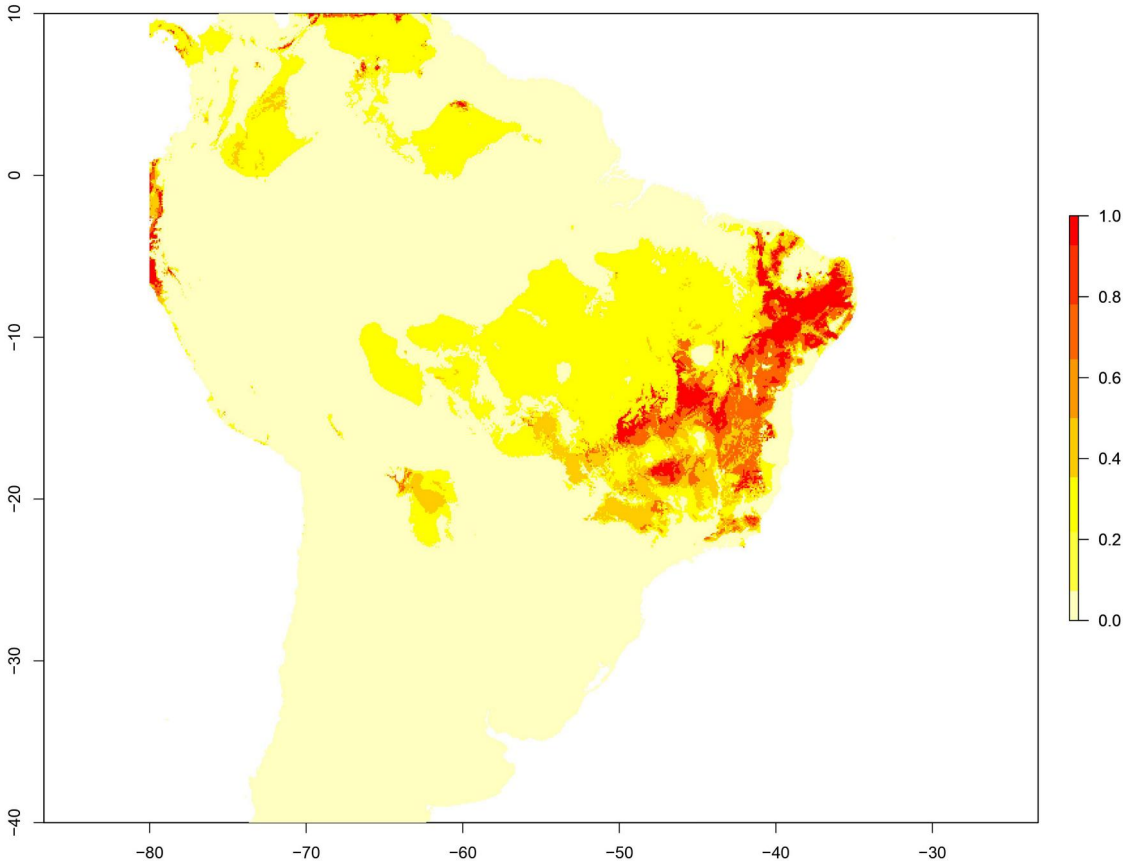

Fig. 8. Stable areas (refuges) considering the four projected climate scenarios for *Boana crepitans* (present, 6kya, 21kya and 120kya). Colours indicate areas of predicted stability, ranging from inadequate (white), low (yellow), medium (orange), and high (red).

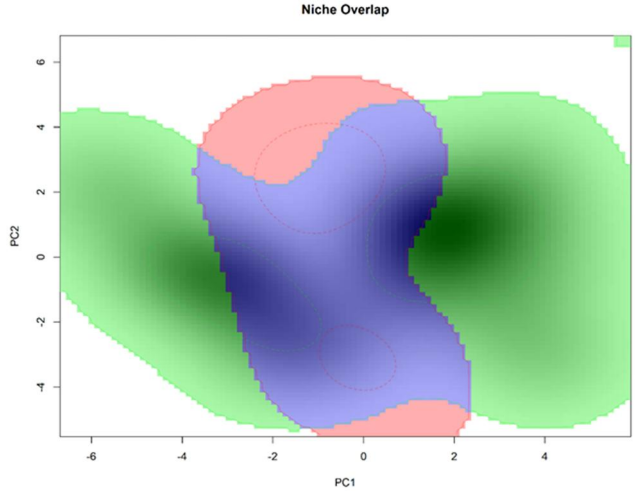

Fig. 9. Niche overlap between the two lineages of *Boana crepitans*. The Eastern lineage is represented in red, the niche overlap in blue, and the Western lineage in green.

crepitans) lineages, leading to their current disjunct distributions. Following this earlier, interspecific divergence, Pleistocene climatic oscillations further structured populations within *B. crepitans*, generating the Eastern and Western lineages identified in this study. The low divergence between lineages and extensive haplotype sharing suggest limited historical differentiation. This shallow divergence may result from stochastic

evolutionary processes such as incomplete lineage sorting or recurrent gene flow during periods of population connectivity (Leaché et al., 2014; Maddison & Knowles, 2006).

Rather than being primarily structured by topographic gradients, the spatial distribution of the Eastern and Western lineages appears more consistent with climate-driven processes. Specifically, lineages' distributions suggest the persistence of at least two refugial areas during drier climatic phases of the Pleistocene, one associated with mesic environments in the Atlantic Forest and another in the Chapada Diamantina region (Thomé et al., 2010). These refugia may have maintained some connectivity within lineages, followed by post-glacial range expansions that promoted secondary contact and genetic admixture, thereby preventing the formation of deep phylogenetic structure. Similar climate-mediated patterns of shallow divergence and haplotype sharing have been reported for other Neotropical taxa (Camurugi et al., 2021; Oliveira et al., 2018; Werneck et al., 2012a).

Our spatial-temporal reconstruction suggests that the most recent common ancestor of the extant sampled sequences was likely located in the southern portion of the Espinhaço Mountain Range in Bahia State. From this reconstructed ancestral location, a westward dispersal scenario is inferred for the Western lineage. The inferred diffusion routes largely coincide with ecotonal regions between xeric and mesic environments (Thomé et al., 2010), supporting the idea that climatic suitability and habitat continuity, rather than sharp topographic or hydrographic barriers, played a primary role in shaping lineages' distributions. Despite the recognition of two geographic populations, the low F_{ST} values and the extensive haplotype sharing indicate weak population structure, a pattern consistent with historical connectivity and/or recurrent gene flow during climatically favourable periods (Oliveira et al., 2015).

The São Francisco River does not appear to constitute a strict barrier to gene flow for *B. crepitans*, as no abrupt genetic discontinuities were detected between populations on opposite riverbanks. Similar patterns of phylogeographic continuity across the river have been reported for several Caatinga-associated taxa (Caetano et al., 2008; Magalhães et al., 2014; Oliveira et al., 2018; Recoder et al., 2014), suggesting that the barrier effect of the São Francisco River is taxon-specific and context-dependent (e.g., Coelho et al., 2022). For *B. crepitans*, our results indicate that Pleistocene climatic dynamics and habitat connectivity outweighed the potential isolating effect of the river in structuring genetic variation.

Our results are consistent with previous evidence indicating that riparian forests and mesic enclaves facilitated connectivity across South American dry regions during humid phases of the Quaternary, forming intermittent dispersal networks for forest-associated organisms (de Oliveira et al., 1999; Mares & Ernest, 1995; Oliveira-Filho & Ratter, 2010). Rather than permanent landscape features, climatic oscillations appear to have played a dominant role in shaping dispersal opportunities and evolutionary histories across the South American Dry Diagonal.

These dynamics are broadly aligned with the Refuge Theory (Haffer, 1969; Vanzolini & Williams, 1981), which proposes that Quaternary climatic fluctuations promoted the fragmentation of mesic environments into isolated refugia during glacial periods, followed by expansion and reconnection during interglacial periods. However, empirical evidence suggests that species responses to these dynamics are highly variable. While some taxa conform to this classic model (e.g., de Ré et al., 2014), others exhibit demographic stability or pre-LGM expansions, highlighting the importance of species-specific ecological traits and landscape context in mediating responses to climatic change (Batalha-Filho et al., 2012; Peres et al., 2015).

In *Boana crepitans*, Quaternary climatic fluctuations appear to have produced similar demographic responses in both the Eastern and Western lineages. Coalescent-based analyses indicate long-term demographic stability over the last ~280,000 years, consistent with the persistence of climatically stable areas predicted by ecological niche models. The absence of deep genetic divergence, together with low Fst values and extensive haplotype sharing, suggests that lineage differentiation was shaped by episodes of partial isolation within mesic refugia during drier phases, followed by recurrent connectivity during wetter intervals, rather than by prolonged geographic isolation.

Palaeodistribution models further indicate that suitable habitats for *B. crepitans* were reduced during the Last Glacial Maximum, with climatically stable areas persisting in central-western and northeastern Brazil. These regions correspond to endemism centres previously proposed for the Cerrado and Atlantic Forest (Carnaval & Moritz, 2008; Werneck et al., 2012b) and have been implicated in shaping genetic patterns in multiple Neotropical taxa (Carnaval et al., 2009; Menezes et al., 2016). For a mesic-associated species such as *B. crepitans*, contraction of humid environments likely restricted populations to these refugial regions, while subsequent interglacial expansions promoted secondary contact and episodic gene flow between lineages.

Overall, the persistence of suitable environments through time, combined with the ecological flexibility of

B. crepitans and its broad geographic distribution across distinct morphoclimatic domains, likely contributed to long-term population persistence and limited genetic divergence. Rather than strong niche divergence or complete isolation, the observed genetic structure is best explained by a history of partial isolation within mesic refugia interspersed with recurrent connectivity during favourable climatic periods. This scenario underscores the importance of climatic dynamics and species-specific ecological traits in shaping phylogeographic patterns across South American dry forests.

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Authors' contributions

E.A.P. and D.J.S. conceived the ideas; A.A.G., M.F.N. and D.J.S. contributed with tissue samples; E.A.P., K.C., E.R. and D.J.S. analysed the data; and E.A.P. led the writing with assistance from S.M., K.C., A.A.G. and D.J.S; and S.M., K.C., E.R., A.A.G., M.F.N., H.R.S. and D.J.S. revised the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Supplemental material

Supplemental material for this article can be accessed online here: https://doi.org/10.1080/14772000.2026.2676069.

Data accessibility statement

DNA sequence data are available in GenBank under accession numbers PZ509333-PZ509448, PZ508190-PZ508240 and PZ508241-PZ508292 and in the NCBI database under SUB16244429, SUB16237407 and SUB16237122. Climate data used in this study are publicly accessible *via* WorldClim (*Bioclim* dataset): https://www.worldclim.org/data/bioclim.html

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ORCID

Elvis Almeida Pereira http://orcid.org/0000-0002-8810-7878
Sarah Mângia http://orcid.org/0000-0003-0038-123X
Eric Ragalzi http://orcid.org/0009-0009-6230-3211
Adrian Antonio Garda http://orcid.org/0000-0002-1178-1207
Marcelo Felgueiras Napoli http://orcid.org/0000-0003-3843-0543
Diego Jose Santana http://orcid.org/0000-0002-8789-3061

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