



Pleistocene Climatic Changes Drove Genetic Variation in Southern Atlantic Forest Treefrogs *Boana polytaenia* (Anura, Hylidae)

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Abstract

Mountainous regions play a crucial role in shaping genetic differentiation among organisms. Climate change and topographical complexity are considered the most important processes influencing the diversification of these areas. In this study, we used *Boana polytaenia* treefrog species to assess how such biogeographical factors shaped its evolutionary history in the highly biodiverse Brazilian Atlantic Forest Mountain range, Serra do Mar and Serra da Mantiqueira. We investigated whether the patterns of genetic diversity of *B. polytaenia* along the mountains regions of the Southern Atlantic Forest were related to Pleistocene climate changes. Our dataset included mitochondrial and nuclear loci sequences from *B. polytaenia*, which we used to assess the genealogical relationships of lineages, population structure, lineages validation, changes in effective population sizes over time, time of divergence of lineages, climatic suitability through time with ecological niche modeling and whether niches of lineages are more or less similar than expected. The species exhibited two well-structured clades in each of the mountain chains, Serra da Mantiqueira and Serra do Mar. However, there was a lack of niche similarity and non-reciprocal geographic predictions for species distribution between the clades. Divergence between the clades occurred during the Pliocene/Pleistocene transition. We found contrasting responses to cooler periods on climatic suitability of lineages, with the expansion of suitable areas for *B. polytaenia* Serra da Mantiqueira Clade and retractions of climatically suitable areas for *B. polytaenia* Serra do Mar Clade. Despite clear differentiation, the sharing of nuclear haplotypes indicates retention of ancestral polymorphism, complicating the resolution of phylogenetic relationships in this species. Therefore, it is necessary to investigate the presence of more than one evolutionary unit in this dataset. Finally, Pleistocene climate changes influenced spatial distribution, leading to genetic variation in *B. polytaenia* species.

Keywords Phylogeography · Anuran · Mountain range · Climate · Glacial periods

Introduction

The rugged relief and the past climate change are implicit elements that drove the diversification processes of mountain biota in the neotropical region (Graham et al., 2014;

Antonelli, 2015; Rangel et al., 2018; Muellner-Riehl et al., 2019). Species with a preference for habitats located in higher elevation regions or mountain ranges may present deeper phylogeographic structures and more divergent lineages (Guarnizo & Cannatella, 2013; Rodríguez et al., 2015; Zamudio et al., 2016). After tectonics and climate, mountains are considered the third most important mechanism that acts at all levels of biogeographic differentiation (Antonelli, 2017). Variations in climatic conditions, environmental heterogeneity, topographical complexity, and elevational gradients are the main factors contributing to the high levels of diversity and endemism recorded in mountains around the world (Fjeldså et al., 2012; Luebert & Muller, 2015; Badgley et al., 2017; Antonelli et al., 2018). For this reason, mountains are usually considered biodiversity hotspots and priority areas for conservation

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(Stattersfield et al., 1998), harboring several organisms that have demonstrated evidence of genetic divergence among their populations (e.g. Moritz et al., 2000; Wollenberg et al., 2008; Kosciński et al., 2009).

The topographic complexity of habitats, associated with reduced mobility of organisms, can cause isolation and decrease in populations connectivity, driving genetic divergence and strong phylogeographic structure (Guarnizo & Cannatella, 2013, 2014; Rodríguez et al., 2015). These patterns of genetic differentiation in mountainous regions are even more marked due to higher environmental heterogeneity, topographic complexity, and climatic variation along elevation gradients, mainly on smaller geographic scales (Guarnizo & Cannatella, 2013; Rodríguez et al., 2015). Some amphibians, particularly those inhabiting mountainous regions, may have limited distribution due to specialized, isolated habitats on mountaintops (e.g., cloud forests and upland grasslands; Safford, 1999). These higher elevation habitats are more susceptible to fragmentation by climate change when compared to lowland habitats (Hewitt, 2004). As a consequence, organisms with restricted geographic distribution, particularly in the highlands habitats, are more likely to reveal phylogeographic patterns, as seen in the Atlantic Forest (AF), where distinct bioclimatic regions shape genetic differentiation between populations (i.e. North and South bioclimate areas; details in Carnaval et al., 2014).

The AF biome comprises regions with a wide elevational gradient, areas of greater topographical complexity, and an extensive latitudinal range that extends over almost the entire east coast of Brazil, which promotes a high heterogeneity of habitats and biological diversity (Ribeiro et al., 2009, 2011; Rodríguez et al., 2015). The highlands of the AF form mountain complexes with great importance for the biome (Costa et al., 2000). The AF is characterized by two distinct bioclimatic domains, delineated by patterns of fauna, flora, and climate distribution, dividing the biome into North and South regions around the Rio Doce (Carnaval et al., 2014). The southern region of AF, located south to the Rio Doce, contains some of these mountain complexes, located around the Continental Rift of Southern Brazil (Riccomini et al., 2004), composed of Serra da Mantiqueira and Serra do Mar. These two mountain ranges originated from tectonic events initiated during the Paleocene, around 30 to 40 million years ago (de Almeida & Carneiro, 1998). Both mountain ranges can reach an elevation of 2000 m above sea level. In the Paraíba do Sul River valley between the mountainous complexes, is the Paraíba do Sul River. Phylogeographic breaks in the AF are associated with this river for the diversification of lizards and frogs (Pellegrino et al., 2005; Pirani et al., 2022). The complexity of habitats along the elevational gradient of these regions directly contributes

to the high diversity and levels of endemism of frogs (Cruz & Feio, 2007; Silva et al., 2018a; Guedes et al., 2020). The anuran distribution patterns can vary due to environmental requirements and complex biogeographic history (Valdujo et al., 2012; Vasconcelos et al., 2014). In particular, frogs from mountainous regions of AF, generally have their distribution restricted to smaller areas (Villalobos et al., 2013).

In general, due to the characteristics of their life history, anurans show a high degree of population genetic structure and are good models for investigating historical changes in the environment (Monsen & Blouin, 2004; Zeisset & Beebe, 2008; Pabijan et al., 2020). In the AF, evolutionary history of anuran species has a strong relationship with the past climate changes (Carnaval et al., 2009, 2014; Fitzpatrick et al., 2009; Sabbag et al., 2018). The Pleistocene Refuge Hypothesis is widely proposed to explain the diversification of AF taxa (Carnaval et al., 2014; Cabanne et al., 2016; Cardoso et al., 2015; Menezes et al., 2016) and assumes that, during the Pleistocene, climate shifts would have caused successive contractions and expansions of forest environments, and areas that remained climatically stable over time acted as refuges (Haffer, 1969; Vanzolini & Williams, 1981). The result of these vicariance events during periods of forest shrinkages can better explain the processes of genetic variation, population structure, demography, and speciation of different organisms (Avice, 2009; He & Jiang, 2014; Feng et al., 2016; Ye et al., 2016). Furthermore, it is important to highlight that the Refuge Hypothesis can act in a contrasting way between mountainous organisms and forest organisms. In lowland forest areas, glacial periods, such as the Last Glacial Maximum (LGM), resulted in population contractions of some taxa (Carnaval et al., 2009; de Mello Martins, 2011). In contrast, some organisms from mountainous areas experienced population expansion during this colder period (Amaro et al., 2012; Cabanne et al., 2016; Leite et al., 2016). These climatic dynamics are part of a broader context where various processes contribute to diversification. Climatic variations along elevational gradients, complex topographies, and the role of rivers as biogeographical barriers are also factors contributing to diversification (Pellegrino et al., 2005; Guarnizo & Cannatella, 2013; Graham et al., 2014; Rodríguez et al., 2015; Santos et al., 2020). Therefore, to understand the diversification of AF, it is necessary to test several different mechanisms and historical events, since the diversity of this biome is complex and can be explained by the interaction of multiple processes (Batalha-Filho et al., 2012; Brunet et al., 2015; Sotelo-Muñoz et al., 2020).

To explore the biogeographic processes in the evolutionary history of the southern AF mountain ranges, we used a species that are typically associated with higher-altitude environments, *Boana polytaenia* (Cope, 1870), from the Mantiqueira mountain range and Serra do Mar Mountain

range. The species occurs in open and forested areas, in topographically complex regions (Cruz et al., 2009; Caramaschi & Cruz, 2013). *B. polytaenia* is part of the *B. polytaenia* Clade, which is also composed by *B. bandeirantes*, *B. beckeri*, *B. botumirim*, *B. buriti*, *B. cipoensis*, *B. jaguari-aivensis*, *B. latistriata*, *B. leptolineata* and *B. stenocephala* species. (Faivovich et al., 2021). However, recent phylogenetic analyses (Faivovich et al., 2021) revised the taxonomic status of this species complex and propose *B. beckeri*, *B. latistriata*, from Serra da Mantiqueira, and *B. bandeirantes*, from Serra do Mar, as junior synonyms of *B. polytaenia*. Based on this classification, we selected samples representing populations from each of the mountain ranges, which are currently assigned to *B. polytaenia*, to investigate the evolutionary relationships associates with the AF mountain ranges, since the species presents a separation into two clades located in the Serra do Mar and Serra da Mantiqueira (Faivovich et al., 2021). Thus, we expect that our study will advance the understanding of evolutionary history in part of the *B. polytaenia* species complex.

Given their distribution and association with high elevation environments, we expect to find phylogeographic structures associated with mountain ranges and that Pleistocene climate cycles may have influenced the species' evolutionary history. Specifically, by a vicariance/dispersal model during the cyclical Pleistocene climatic changes, through reduction/expansion of climatically suitable areas affecting the demographic history of lineages. Thus, we investigate whether the genetic diversity of *B. polytaenia* along the mountainous regions of the Southern AF are related to Pleistocene climate changes dynamics. Furthermore, we tested whether there is climatic niche variation for both clades in southern AF mountain ranges. For this, we inferred the phylogenetic relationships of lineages, divergence time between lineages, changes in the effective population size of lineages over time and demographic history using distribution data and mitochondrial and nuclear DNA sequences. We also modelled the areas of better climatic suitability for the present and different past scenarios, in order to verify the influences of the climate on the species distribution through time. Finally, we conducted a test for niche overlap between the clades. These approaches will allow us to better understand the mechanisms that shaped the genetic diversity of this species and their responses to climate change over time, contributing to phylogeographic knowledge of topographically complex regions of the Atlantic Forest.

Methods

Sampling and Sequencing

We obtained DNA sequences from 44 individuals of *Boana polytaenia*, 26 from the Serra da Mantiqueira and 18 from the Serra do Mar (Fig. 1, and Locality in Table S1 in Supplementary Information). The sequences are the same as those presented in the study by Faivovich et al. (2021), representing Serra do Mar Clade and Serra da Mantiqueira Clade of *B. polytaenia* species. As outgroups, we used one sample of each of the following species: *B. cordobae*, which belongs to the *B. prasina* clade, and the species *B. buriti* (Caramaschi & Cruz, 1999) and *B. stenocephala* (Caramaschi & Cruz, 1999), which belong to the *B. polytaenia* clade, in the phylogeny of *Boana pulchella* group (outgroups information in Table S1, Supplementary information). All mitochondrial and nuclear DNA sequences were produced by Faivovich et al. (2004, 2005, 2013, 2021) and are available on Genbank (accession numbers in Table S1, Supplementary Information). We also verified available localities in online databases (GBIF, iNaturalist, and iDigBio) of *B. polytaenia* occurrences for ecological niche modeling and for the niche overlap test.

We used segments of mitochondrial (mtDNA) and nuclear (nuDNA) gene sequences as our genetic data set. The mitochondrial gene sequences used include partial fragments of 12S 16S ribosomal, Cytochrome c oxidase subunit 1 (COI), NADH dehydrogenase subunit 1 (ND1), tRNA^{ile}, tRNA^{leu}, and tRNA^{val}. Nuclear genes sequences include the single Recombination Activation exon 1 (Rag-1) and Rhodopsin exon 1 (RHO). We use GENEIOUS v9.0.5 (Biomatters Ltd.) to verify the sequences and edit, if necessary. The sequences were aligned for each gene locus using the Muscle algorithm v3.8.425 with default configuration (Edgar, 2004) (module implemented in GENEIOUS v 9.0.5). In DnaSP v5.10 (Librado & Rozas, 2009), we determined the most probable pair of alleles for nuDNA genes in heterozygosity, using the PHASE algorithm v2.1.1 (Stephens et al., 2001). We estimated the most appropriate nucleotide substitution models and partition schemes for the data based on the Bayesian Information Criterion (BIC), using Partition-Finder 2 software (Lanfear et al., 2016), under the linked branch-length model (Supplementary Information Table S2).

Phylogenetic Trees and Haplotype Network

To investigate the phylogenetic relationships between the *B. polytaenia* clades, we used the Bayesian inference method using BEAST v2.6.6 (Bouckaert et al., 2019), for concatenated mitochondrial genes 12S, 16S, COI, ND1, tRNA^{ile},

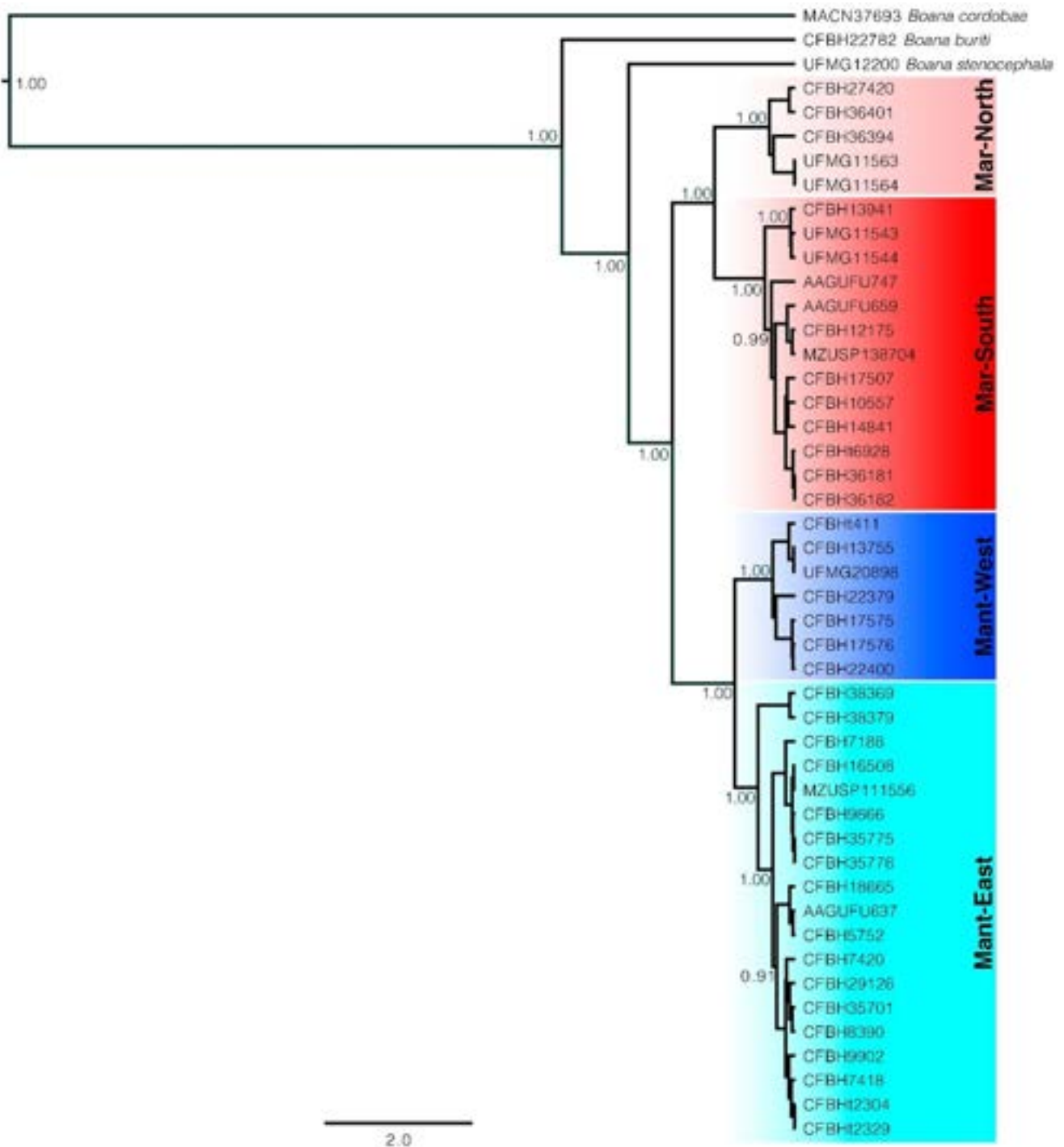


Fig. 1 mtDNA Gene tree inferred using Bayesian inference in the program BEAST. The colors represent each lineage and their respective geographic divisions. Mant-East and Mant-West represent lineages of *B. polytaenia* Serra da Mantiqueira clade, and Mar-South and Mar-

North represent lineages of *B. polytaenia* Serra do Mar clade. Posterior probabilities are indicated by numbers at the nodes. Terminal names of samples are available in Table S1 (Supplementary information)

tRNA^{leu}, and tRNA^{val}. We ran a Markov Chain Monte Carlo (MCMC) with 1×10^8 generations. We performed this analysis using the Strict Clock model, and we calibrated the concatenated gene tree according to the estimated values for Hylidae 16S fragment substitution rate proposed by Gehara

et al. (2014) (7.35×10^{-3} mutation/site/million years) and for COI (1.47×10^{-2} mutation/site/million years), as it proportionally accumulates nucleotide mutations twice times faster than 16S (Vences et al., 2005; Lyra et al., 2017). We used the coalescent constant population size tree prior and the

substitution rate of partitions was changed from ‘gamma’ to ‘log normal’. We discard the initial 20% of the generations as burn-in. The convergence of parameters was verified based on the effective sample size ($ESS \geq 200$) in Tracer v1.7.1 software (Rambaut et al., 2018). A maximum clade credibility tree was generated with TreeAnnotator v2.6.6 (Bouckaert et al., 2019). The consensus tree was visualized using Figtree v1.4.4 (Rambaut, 2018).

We use the DnaSP v5.10 software (Librado & Rozas, 2009) to create a matrix of haplotypes for all mtDNA and nuDNA loci. We also investigate the relationship between haplotypes using the Median-Joining (MJ) method implemented in PopArt v1.7 (Leigh & Bryant, 2015). We estimate haplotype networks for mitochondrial loci 12S, 16S, COI, ND1, and for nuclear loci Rag-1 and RHO (phased sequences), considering each lineage identified based on the BAPS clusters assignments (see below).

Species Delimitation

We employed the Bayesian Phylogenetics and Phylogeography (BPP) species delimitation method (Flouri et al., 2018; Yang, 2015) to validate the genetic breaks identified by BAPS and the mtDNA gene tree. BPP allows the use of multilocus data to classify incomplete lineages and validate uncertainties in gene trees. It uses the Bayesian species coalescence model to compare different phylogeny models and delimit species and/or lineage structure (Yang & Rannala, 2010, 2014; Rannala & Yang, 2013; Sukumaran & Knowles, 2017). Considering that, recent taxonomic revisions (Faivovich et al., 2021) treat the sampled populations from Serra da Mantiqueira and Serra do Mar as belonging to a single species, *B. polytaenia*, we run BPP to test whether the observed genetic structure supports potential lineage diversification within this single taxonomic entity. Thus, BPP was applied to assess the validity of the identified genetic partitions, ensuring that they reflect significant lineage differentiation within *B. polytaenia*. We used the Bayesian Markov chain Monte Carlo method implemented in the program BPP v4.2.9 (Rannala & Yang, 2013; Yang, 2015) to generate the posterior probabilities of different methods of species delimitation. We used concatenated mitochondrial genes (12S, 16S, COI, ND1, tRNA^{ile}, tRNA^{leu}, and tRNA^{val}) and nuclear genes (Rag-1 and RHO) together.

We ran BPP with four different prior combinations for population size ($\theta = \theta$) and divergence time ($\tau = \tau$) parameters as proposed by Leaché and Fujita (2010): (a) large ancestral population sizes and deep divergences [θ and $\tau \sim \text{IG}(3, 0.2)$]; (b) large ancestral population sizes and recent divergences [$\theta \sim \text{IG}(3, 0.2)$ and $\tau \sim \text{IG}(3, 0.002)$]; (c) small ancestral population sizes and recent divergences [θ and $\tau \sim \text{IG}(3, 0.002)$]; and (d) small ancestral population sizes

and deep divergences [$\theta \sim \text{IG}(3, 0.002)$ and $\tau \sim \text{IG}(3, 0.2)$]. We performed the A11 analysis, which jointly delimit species and infer a species tree (Yang & Rannala, 2014), using the species tree recovered by BEAST and congruent with BAPS as a guide tree. We run all previous priors at least twice using algorithms 0 and 1 available in BPP and the finetuning was adjusted to assure acceptance proportions between 0.30 and 0.70. Each run consisted of a burn-in phase of 10,000 iterations followed by a sampling phase of 400,000 iterations, with a frequency of two iterations per sampling. Posterior probabilities ≥ 0.95 were considered highly supported.

Genetic Diversity and Lineages Structure

The lineage structure was estimated using all mitochondrial genes (12S, 16S, COI, ND1, tRNA^{ile}, tRNA^{leu}, and tRNA^{val}), and RHO and Rag-1 nuclear genes sequences for all individuals. We estimated the most probable number of clusters (k) with the Bayesian Analysis of Population Structure (BAPS) through BAPS v6.0 software (Corander et al., 2008) using the occurrence points as a priori information, in a spatially explicit model, to generate the a posteriori distribution model through the Markov Chain Monte Carlo. We analyzed mitochondrial and nuclear loci combined, first in a mixture analysis in the clustering with linked loci module, and then in the spatial clustering of individuals. The mixture model was run using a k range of 1–10. Then, we use the results of the mixture models to run the admixture model using values indicated by Corander et al. (2013): 100 interactions, 200 reference individuals and 20 interactions per individual. Finally, we assume the significance of Admixed individuals $P < 0.05$. In the phylogenetic, diversity, and demographic analyses, each cluster estimated by BAPS was considered a genetic lineage.

We estimate the differentiation between the lineages identified with BAPS analysis using mitochondrial and nuclear loci through the uncorrected pairwise genetic distance (p -distance) using Mega X v6.06 (Tamura et al., 2013), with default options. To estimate genetic differentiation within and among lineages (variation distribution), we used the Hierarchical Analysis of Molecular Variance (AMOVA), implemented in the Arlequin v3.5 program (Excoffier & Lischer, 2010), with 10,000 permutations. We also calculated statistics of genetic diversity through the number of polymorphic sites (S), the number of haplotypes (h), the haplotypes diversity (H_d), and the nucleotide diversity (π) for each locus using DnaSP v5.10 (Librado & Rozas, 2009).

Demographic Analysis

We applied the neutrality tests of Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) using 10,000 coalescent simulation replicates in DnaSP v5.10 (Librado & Rozas, 2009). These tests are conducted for mitochondrial and nuclear loci to detect significant deviations in the neutral evolution model and variations in the constant population size of lineages. Additionally, neutrality tests can indicate the occurrence of evolutionary events, such as expansion, retraction, or stability (Nielsen, 2001).

We also generated Bayesian Skyline Plots (BSP) (Drummond et al., 2005), implemented in BEAST v2.6.6 (Bouckaert et al., 2019) to infer historical changes in effective population size (N_e). This test was applied only to two lineages due to the available number of individuals (Mant-East [$n=19$] and Mar-South [$n=13$]) and we used all mitochondrial loci. We focused on these two lineages separately, as BSP models assume that dataset represents a single panmictic population, whether with singles or multilocus data, and the samples adequately capture the underlying genetic diversity, which improves demographic estimates of past population size (Heled & Drummond, 2008). Including lineages with a limited number of individuals and low genetic variation may violate BSP model assumptions and result in unreliable demographic inference. It has been demonstrated that increasing the number of loci contributed more significantly to demographic inference than expanding the number of samples per locus (Ho & Shapiro, 2011). We set the Clock calibrations as 16S mean substitution rate as in the previous analysis. We ran 2×10^7 MCMC simulations sampled every 2,000 chains. Finally, we verify the run convergence using Tracer v1.7 (Rambaut et al., 2018) checking the effective sample size (ESS > 200).

Species Tree and Divergence Times

To estimate the period of lineage divergence, we constructed a dated species tree under the multispecies coalescent method, using mitochondrial and nuclear (phased) loci, in StarBEAST2 v0.15.13 (Ogilvie et al., 2017) in the BEAST v2.6.6 software packages (Bouckaert et al., 2019). Firstly, we assigned our samples to the four genetic clusters recovered by BAPS as lineages. We calibrated the species tree using 16S and COI substitution rates, as in our gene tree (Gehara et al., 2014; Vences et al., 2005). Additionally, we used the substitution rate of nuDNA RHO (2.5×10^{-4} mutation/site/million years) (Yokoyama & Tada, 2010) and RAG-1 (1.25×10^{-3} mutation/site/million years) (Gehara et al., 2017; Hugall et al., 2007).

We assumed a strict clock model just for RHO nuDNA locus and relaxed clock model with uncorrelated lognormal

for all other loci and a birth-death model of diversification as tree prior (Substitution models in Table S2). We used the Linear with Constant Root Population as a model prior because we do not have prior information on ancestral effective lineages sizes. We execute the MCMC chain for 5×10^7 generations, sampling every 5,000 and discarding the initial 20% as burn-in. We performed three independent analyses to check for convergence among runs and combined log files and trees with LogCombiner (Drummond et al., 2012) and checked chain mixing and stationarity using Tracer v1.7 (ESS > 200) (Rambaut et al., 2018). We generated a maximum clade credibility tree with TreeAnnotator and visualized the final tree in FigTree v1.4.4 (Rambaut, 2018).

Ecological Niche Modeling

We used ecological niche models (ENM) to assess the potential current and past distribution of environmentally suitable areas of *B. polytaenia* clades across different climate scenarios. To perform the ENMs, we used georeferenced collection and/or observation points of *B. polytaenia* specimens previously analyzed, as well as those available in biodiversity databases, totaling 645 records. All georeferenced points were examined to find possible misidentifications in the records of biodiversity databases. Thus, we removed duplicate and inconsistent records and points outside the geographic range of the species according to Faivovich et al. (2021). We also used the spThin package (Aiello-Lammens et al., 2015), in R Statistical Software v4.3.3 (R Core Team, 2024), to do a spatial thinning of the occurrence points, so that there were no points less than 5 km from each other ensuring that the density of close records and sampling bias was reduced (Aiello-Lammens et al., 2015). For this analysis, we divided the dataset into two subsets: (1) only with records from Serra da Mantiqueira Mountain range (*B. polytaenia* Serra da Mantiqueira Clade); (2) only with records from Serra do Mar Mountain range (*B. polytaenia* Serra do Mar Clade).

We downloaded 19 bioclimatic variables at a spatial resolution of 2.5 arc-minutes (~5km²) from CHELSA climatic database (<https://www.chelsa-climate.org/>) (Karger et al., 2017). These variables unite the characteristics of precipitation and temperature. The variables bio02, bio03, bio05, bio06, and bio7 were excluded from the analysis due to their unavailability for the Middle Pleistocene (ca. 787 kya), mid-Pliocene (ca. 3.2 mya) and for Late Pliocene (ca. 3.3 mya) paleoclimates scenarios, as reported by Brown et al. (2018). To delimit a reasonable extent, the raster layers used were cut considering a distribution of *B. polytaenia* species (circumscribed within -50, -40, -26, -20).

We checked which variables were correlated and discarded them to avoid overparameterization of the model

(Rissler & Apodaca, 2007). To remove correlated variables, we used variance inflation factor (VIF; threshold > 10) with the `usdm` package (Naimi et al., 2014) in R v4.3.3 (R Core Team, 2024). This resulted in five bioclimatic variables: temperature seasonality (bio04), mean temperature of warmest quarter (bio10), precipitation of wettest month (bio13), precipitation of driest month (bio14), precipitation of warmest quarter (bio18).

To generate the ENMs, we used the maximum entropy algorithm Maxent v3.3 (Phillips et al., 2006) and the ENMeval R package (Muscarella et al., 2014) to adjust the Maxent models. We used 10,000 random points for each species across their distribution as background. To identify the optimal model parameters for ENM, we used default parameters of Feature Classes (fc=“L”, “LQ”, “H”, “LQH”, “LQHP”, “LQHPT”) and regularization multipliers (rm) with values between 0.5 and 4 at 0.5 intervals in ENMeval, as in Muscarella et al. (2014). Model performance was evaluated using the area under the curve (AUC) and the Akaike’s information criterion corrected (AICc). Variables contributions, AUC training and test values, fc and rm combinations of models are in Table S7 (see Supplementary Information).

Finally, we designed ENMs for the past climatic conditions of the Mid-Holocene (MH, ca. 6 Kya) (Fordham et al., 2017), the Last Glacial Maximum (LGM, ca. 21 Kya) (Karger et al., 2021), the Last Interglacial (LIG, ca. 130 Kya) (Otto-Bliesner et al., 2006), Marine Isotope Stage 19, in the Pleistocene (MIS19, ca. 787 Kya) (Brown et al., 2018), mid-Pliocene Warm Period (mPWP, ca. 3.2 Mya) (Hill, 2015) and to the Marine Isotope Stable in the Late Pliocene (M2, ca. 3.3 Mya) (Dolan et al., 2015). Numerous global climate changes events mark these periods Thornalley et al., 2010, 2011, 2013). To access possible areas of climate stability during Pleistocene, or refugia (Carnaval et al., 2014), we first produced binary maps for each scenario projected for the Pleistocene (Current; MH; LGM; LIG), using input data obtained from the “10th percentile presence Equal training sensitivity and specificity logistic threshold”, and subsequently, we superimposed and summed the maps to generate the climate stability scenario.

Climatic Niche Overlap

To estimate the differentiation of *B. polytaenia* clades niches, we employed the Principal Component Analysis (PCA-env), as proposed by Broennimann et al. (2012), which is capable of quantifying niche overlap. We used the `ecospat` package (Di Cola et al., 2017), in R (R Core Team, 2024), together with the binary maps generated by the ENM as background areas for each species. This method tests equivalence, checking whether the compared niches exhibit distinctions between them, and similarity between niches,

aiming to determine whether the observed niche overlaps differ from the simulated niche overlaps and to compare whether clades niches are more or less similar to each other than expected by chance (Warren et al., 2008). For the equivalence and similarity tests, the PCA-env employs an ordination-based approach, which calculates the density of occurrences and density of environmental variables tested along the axes of the PCA for both compared species using the Kernel smoothing method and uses these densities to calculate the niche overlap along the axes (Broennimann et al., 2012; Hu & Jiang, 2018). We used D Schoener’s metrics to estimate niche overlap, which ranges from 0 to 1, representing the absence of niche overlap to complete niche overlap, respectively (Warren et al., 2008; Broennimann et al., 2012). Statistical confidence in niche overlap values was evaluated using a bidirectional similarity test (*B. polytaenia* Serra da Mantiqueira Clade → *B. polytaenia* Serra do Mar Clade; *B. polytaenia* Serra do Mar Clade → *B. polytaenia* Serra da Mantiqueira Clade) (Broennimann et al., 2012). Finally, we compared the observed overlap with a null distribution generated from 100 random points to assess the statistical significance of niche overlap. The statistical significance was determined using the equivalence and similarity tests of the niche with the settings of alternative = “higher” and `rand.type` = 1, considering a significance level of $p \leq 0.05$.

Results

After editing the sequences as needed, the mitochondrial gene alignments include 919 bp of 12S (21 haplotypes), 1431 bp of 16S (28 haplotypes); 459 bp of COI (19 haplotypes), 111 bp to tRNA^{ile}, 73 bp to tRNA^{leu}, 69 bp to tRNA^{val}, 922 bp of ND1 (28 haplotypes). The nuclear genes include 427 bp of Rag-1 (17 haplotypes), and 304 bp of RHO (three haplotypes) (Table S2 in Supplementary Information).

Phylogenetic Trees and Haplotype Network

The concatenated mtDNA gene tree topology of *B. polytaenia* recovered two well-supported major clades (Fig. 1). These clades correspond to distinct geographic regions, one clade distributed along the Serra da Mantiqueira region (Mant) and other at the Serra do Mar region (Mar). Each main clade is subdivided into two additional lineages, well-supported by high posterior probability values in each branch and with a clear geographic definition.

We classify the lineages of the Mant clade as Mantiqueira West (Mant-West) and Mantiqueira East (Mant-East) and for the lineages of the Serra do Mar in Serra do Mar south (Mar-South) and Serra do Mar north (Mar-North).

Haplotype networks for mitochondrial loci showed a high diversity of haplotypes for each lineage and they are separated by several mutations, mainly for 16S and ND1. While the haplotype network for nuclear loci demonstrated allele sharing between the different lineages. The highest haplotype diversity was observed for the Mant-East and Mar-South lineages (Fig. 2).

Species Delimitation

Two prior combinations that assume large ancestral population sizes (prior a and b, with θ IG (3,0.2)) support the existence of four well-structured lineages corresponding to Mar-North, Mar-South, Mant-East, and Mant-West. These results were supported by high posterior probability ($pp > 0.95$) and were consistent with the branches of the gene tree and clusters identified by BAPS. However, the prior combination with small ancestral population sizes and deep divergences (prior d, θ IG (3,0.002) and τ IG (3,0.2)), grouped the two Serra do Mar lineages into a single one (MNMS), and indicated low probability to validate the Serra da Mantiqueira lineages. The prior combinations with small ancestral population sizes and recent divergences (prior c, θ

and τ IG (3, 0.002)), also resulted in low posterior probability values to validate three lineages ($pp < 0.95$). pattern was consistent across both BPP algorithms settings (0 and 1).

Therefore, we consider that the BPP analyses recognized four lineages with high posterior probability, ($pp > 0.95$), particularly under the prior settings assuming large ancestral population sizes (see Table S8 in Supplementary information). These results reflect the strong structuring of the four lineages, as we found consistent patterns across different scenarios (using only mitochondrial loci in the gene tree, multilocus data in the species tree and BAPS). The convergence of evidence from mtDNA and nuDNA data support the recognition of the four lineages within the current taxonomic framework of *B. polytaenia* species.

Genetic Diversity and Lineages Structure

BAPS results indicated an optimal partition into four distinct genetic clusters: two clusters located in Serra da Mantiqueira (hereafter Mant-East and Mant-West, respectively), and two clusters located in Serra do Mar (hereafter Mar-North and Mar-South, respectively) (Fig. 3). These partitions are geographically and phylogenetically congruent

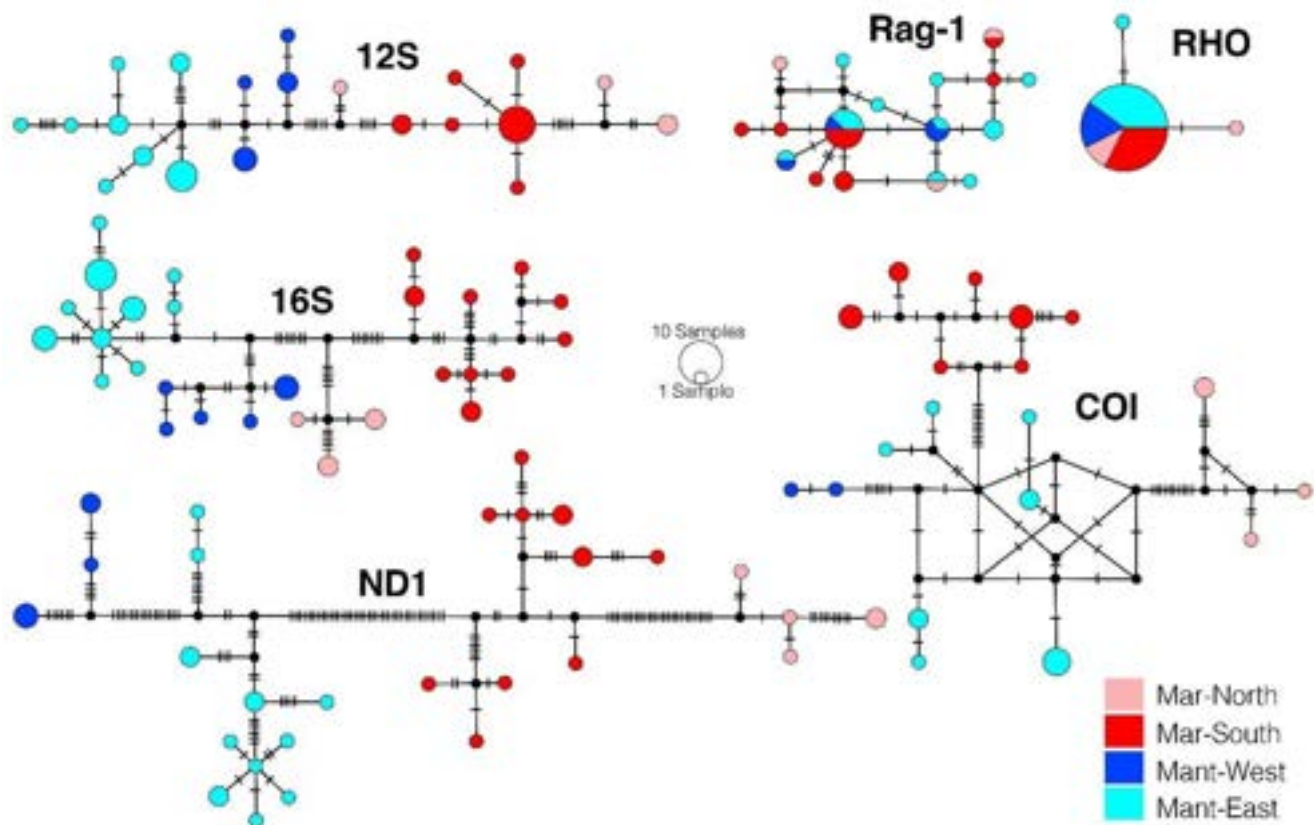


Fig. 2 Median-joining networks among mtDNA and nuDNA haplotypes constructed for *Boana polytaenia* lineages from Serra da Mantiqueira clade (light and dark blue points) and lineages from Serra do

Mar clade (red and pink points). Each color represents a genetic group determined by BAPS. The number of mutational steps is represented by bars along branches that connect haplotypes

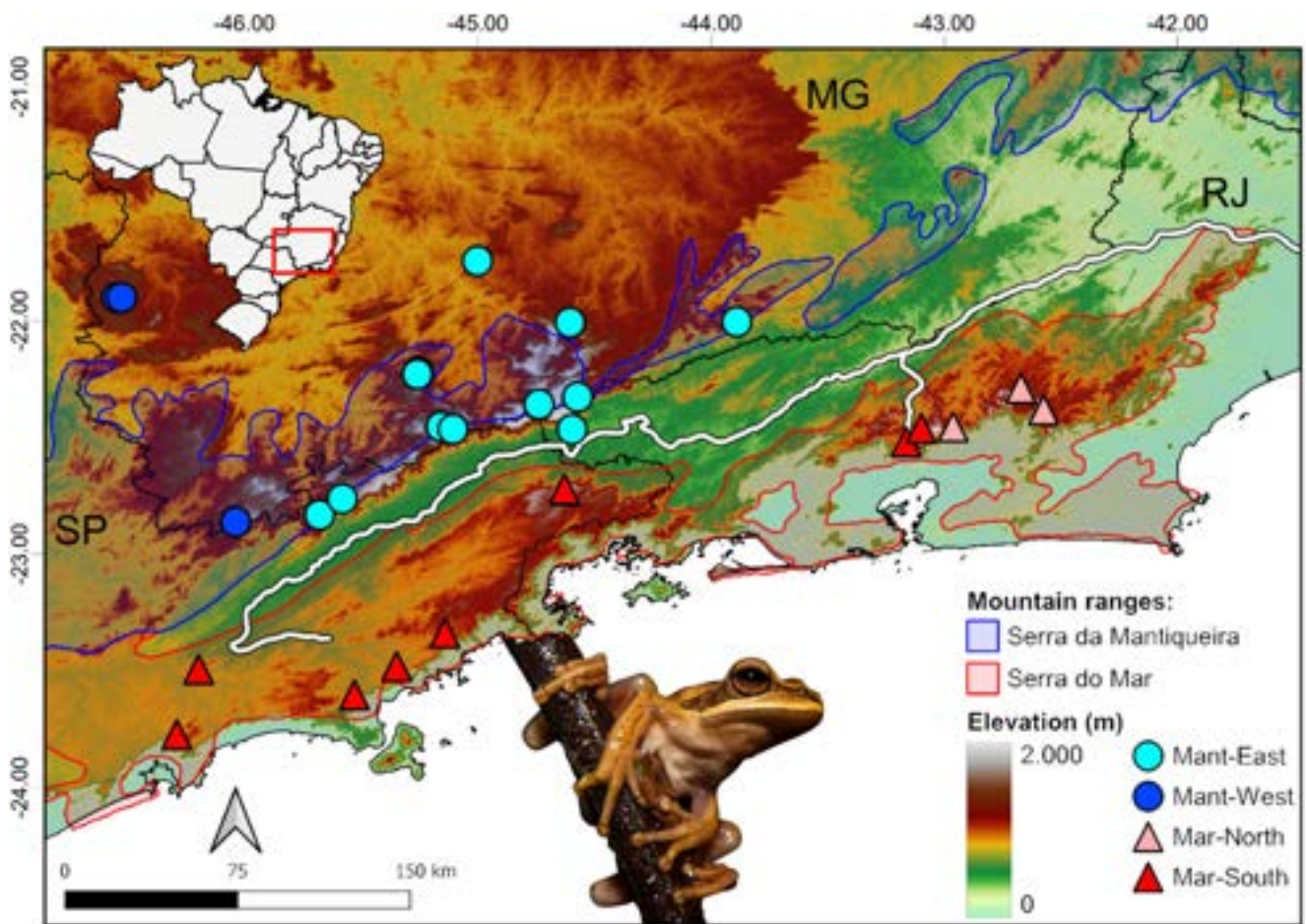


Fig. 3 Map of the distribution of the four lineages of *Boana polytaenia* from Serra da Mantiqueira clade (circles) and from Serra do Mar clade (triangles) in Southern Atlantic Forest. Light and dark blue represent lineages of *B. polytaenia* from Serra da Mantiqueira, while red and pink triangles indicate lineages of *B. polytaenia* of Serra do Mar. Each

color corresponds to a different lineage. The white line with black borders indicates the location of the Paraíba do Sul River, which separates the mountain ranges. Brazilian states: Minas Gerais (MG), Rio de Janeiro (RJ), São Paulo (SP). Photo by: Rodrigo Tinoco

with the four lineages recovered by the mitochondrial gene tree and by validated lineages from BPP. Four individuals were assigned to more than one genetic cluster. We opted to keep these individuals in the cluster to which they had the highest assignment probability.

Pairwise genetic distance (F_{ST}) values between clusters identified by BAPS were significant and consistently high for all mitochondrial loci (from ~ 0.76 to ~ 0.80 , Table S3), and reveal the majority of the genetic variation observed between lineages and strong lineage structure. For nuclear loci, the variation was greater within lineages, with significant F_{ST} values with low variation between different nuclear loci (from ~ 0.10 to ~ 0.11 , Table S3). The uncorrected p-distance values were generally smaller among lineages from the same mountain range (Table S4). Summary statistics showed that the nucleotide and haplotype diversities (total and intra-lineages) are high for the entire set of

mitochondrial loci and nuclear locus Rag-1, mainly for 16S and ND1 (Table S5). RHO presented lower values for the statistics.

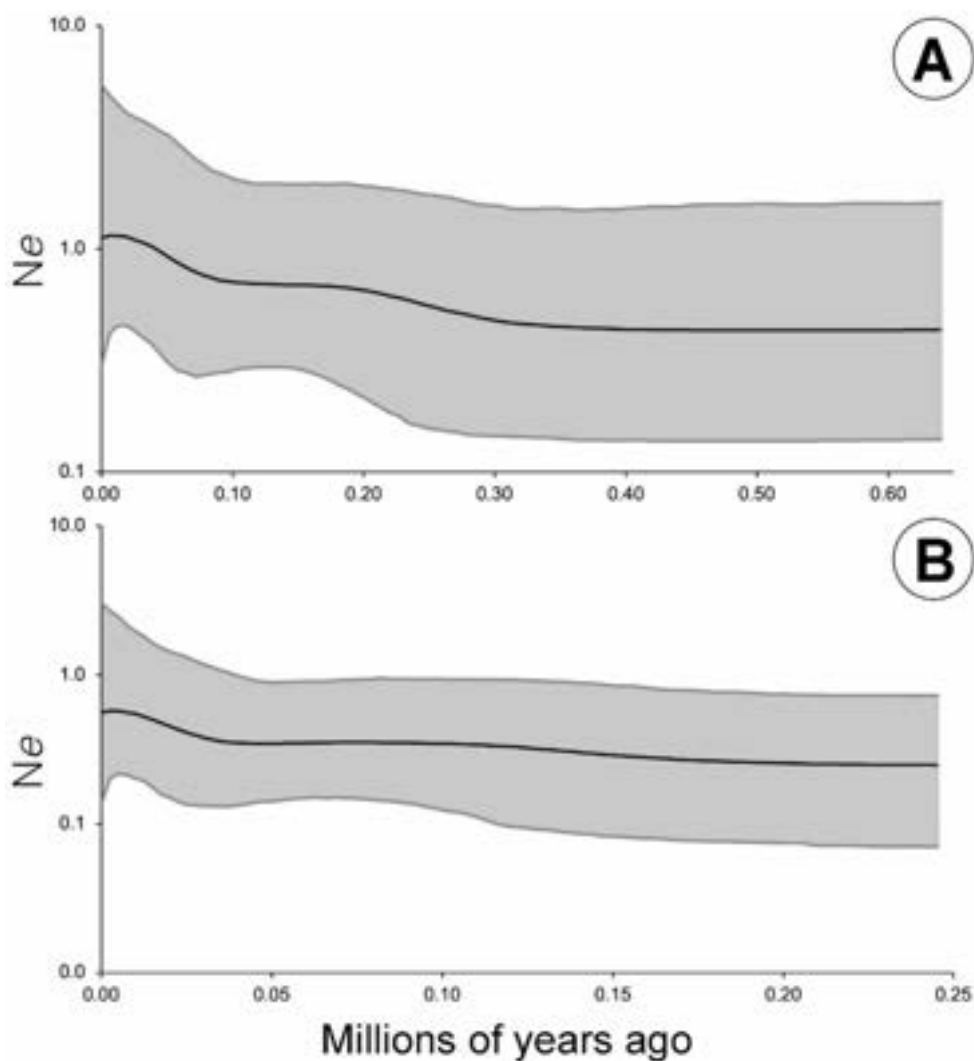
Demographic Analysis

The values of the Tajima's D and Fu's F_s neutrality tests were not significant for any gene or lineage, demonstrating that there is no sign of demographic expansion for any tested lineage (Table S5). There is no significant deviation from the neutral evolution model. BSP analysis indicated a tendency for stability in effective population size (N_e) through time for both tested lineages (Fig. 4).

Species Tree and Divergence Times

The species tree recovered the same topology found by the mtDNA gene tree (Fig. 5). The node between the two

Fig. 4 Reconstructions of demographic changes over time with Bayesian Skyline Plots (BSP) performed with all mtDNA loci for each *B. polytaenia* lineage. N_e means effective population size through time (in years). (A) *B. polytaenia* Mant-East lineage and (B) *B. polytaenia* Mar-South lineage. Black line shows the median population size of lineages with 95% higher posterior probability in grey lines



main clades (*Serra da Mantiqueira and Serra do Mar*) is well-supported ($pp=1.0$) and the divergence time started between the Plio-Pleistocene boundaries around 3.16 Mya (95% HPD=1.54–4.93 Mya). However, the phylogenetic relationships between the lineages of each clade have not been clearly resolved, as the posterior probabilities for both were low ($pp<0.81$). The divergence time for the division of the *B. polytaenia* Serra do Mar clade into two lineages is estimated for the Early Pleistocene, around 2.44 Mya (95% HPD=0.89–3.64 Mya) and for the *B. polytaenia* Serra da Mantiqueira clade, the division into two lineages was estimated at 1.79 Mya (95% HPD=0.8–2.64 Mya). All estimated divergence times in *B. polytaenia* lineages diversification dated between the late Pliocene and Early Pleistocene.

Ecological Niche Modeling

After selecting the occurrence points and thinning locality data, we retained a total of 70 localities for ENM, 32 for Serra da Mantiqueira clade and 38 for Serra do Mar clade and the mean AUC values were similar for both clades (>0.92), indicating better than random predictions (Table S6 and S7, in Supplementary Information). The Maxent ENMs predicted cyclic contractions and expansions in suitable areas during past climates for both clades, with these changes occurring in different ways between each mountain range (Fig. 6). The predicted suitable areas for each clade are distinct, with models for one clade not recovering the distribution of the other, and vice versa.

The models showed a considerable expansion northward of suitable areas for *B. polytaenia* Serra da Mantiqueira clade, most notable during the LGM, and marginally during M2 period. In LGM, suitable areas for Serra da Mantiqueira clade, came into contact with the mountain range

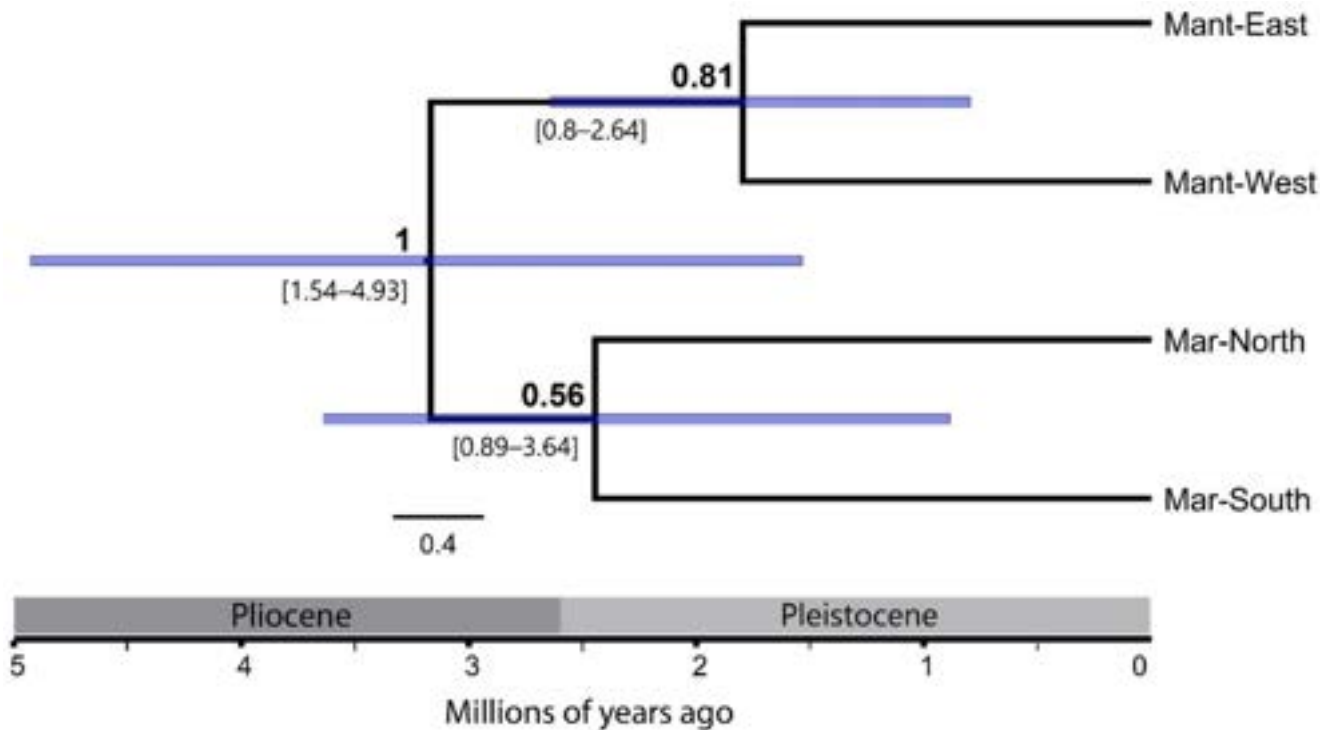


Fig. 5 Dated mtDNA and nuDNA Bayesian tree for the different *Boana polytaenia* lineages. Values on branches correspond to the posterior probability (pp) support and grey bars the divergence times' 95%

highest posterior density intervals (95% HPD, range values present between the brackets). The average time in a million years is represented in a scale below

of the Serra do Mar region, resulting in a large expansion that extended into the Atlantic Forest Coast. This expansion also occurred in the opposite direction, encompassing interior areas of the Southeast Cerrado of Brazil, specifically in state of Minas Gerais, and São Paulo. However, during the LGM, and in the mPWP period, suitable areas for *B. polytaenia* Serra do Mar clade, drastically retracted and were concentrated on the edge of the exposed continental shelves.

The changes in climatically suitable areas occurred in opposite ways for each main clade of *B. polytaenia*. During colder periods, the Serra da Mantiqueira clade possibly underwent expansions of climatically suitable areas while the Serra do Mar clade experienced contractions, and the opposite occurred during warmer periods. During the LIG and MIS 19 scenario, there was a drastic reduction in suitable areas for the Serra da Mantiqueira clade. These two same climatic scenarios suggest a slight expansion of suitable areas for the Serra do Mar clade, towards the southwest area. The mPWP period maintained the potential occurrence areas of Serra da Mantiqueira clade similar those of the current period, while drastically reducing suitable areas for Serra do Mar clade. Additionally, the climate stability model, which presents stable areas over the past 130 Kya on Pleistocene scenarios projections, indicated just a small climatically stable area at the northernmost of the Serra do

Mar clade distribution. No stable areas were identified with four congruent models for the Serra da Mantiqueira clade.

Climatic Niche Overlap

The clades of the *B. polytaenia* species showed niche divergence evidenced by the PCA-env ordering analysis, with each species occupying distinct environmental niches (Fig. 7). We observed low niche overlap between clades (Schoener's $D=0.135$), which rejects the hypothesis of niche conservatism between them. The niche of the two clades is not equivalent ($p>0.05$), as well as the bidirectional similarity test, showed non-significant values for both directions, *B. polytaenia* Serra da Mantiqueira clade \rightarrow *B. polytaenia* Serra do Mar clade ($p>0.05$), and *B. polytaenia* Serra do Mar clade \rightarrow *B. polytaenia* Serra da Mantiqueira clade ($p>0.05$). The first two axes of the PCA explained 81.09% of the total variance of the climatic variables.

Discussion

We recovered two clades of *B. polytaenia* phylogeographically structured in sister clades, which geographically correspond to the Serra da Mantiqueira and Serra do Mar Mountain range. Each clade presents a division into

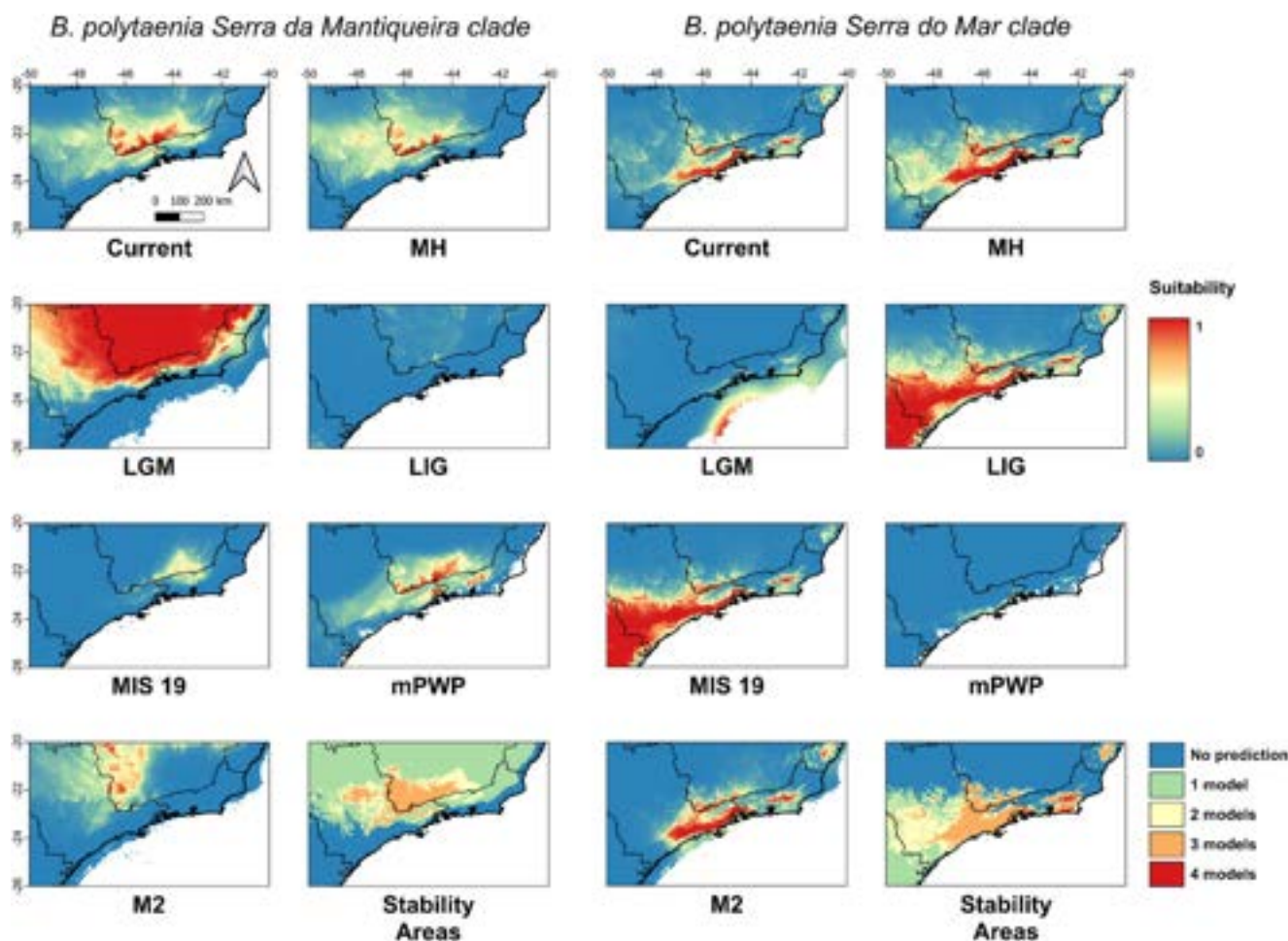


Fig. 6 Ecological Niche Modeling built with Maxent for both clades of *Boana polytaenia* species in southern AF mountain ranges. Climatically suitable areas were projected for *B. polytaenia* Serra da Mantiqueira clade and *B. polytaenia* Serra do Mar clade, respectively, under different scenarios: Current scenario; Mid-Holocene (MH, ca. 6 Kya); Last Glacial Maximum (LGM, ca. 21 Kya); Last Interglacial Maximum (LIG, ca. 130 Kya); Pleistocene-Marine Isotope Stage 19

(MIS, ca. 787 Kya); mid-Pliocene Warm Period (mPWP, ca. 3.2 Mya); Marine Isotope stable in the Late Pliocene (M2, ca. 3.3 Mya); and areas of climate stability through the past 130 Kya. Warmer colors on Stability scale indicate higher probabilities of occurrence of the species according to bioclimatic variables. The color scale of Stability areas maps represents the number of models from different periods that suggest local climate stability

two well-structured lineages, congruent across the results obtained from the mtDNA gene tree, the mtDNA and nuDNA species tree and BAPS partitions. The BPP species delimitation analyses suggest that the observed genetic structure reflects a strong differentiation of lineages within the taxonomic structure of *B. polytaenia*. The haplotype network also reveals the separation of these four lineages based on mtDNA. The split of the sister clades is dated to the Plio-Pleistocene transition period, around 3.16 Mya, and their population sizes have remained stable over time. The Serra do Mar clade was divided into two lineages around 2.44 Mya and the Serra da Mantiqueira clade around 1.79 Mya. The mitochondrial loci revealed a strong lineage structure for each *B. polytaenia* clade within each mountain range, whereas nuclear loci showed lower differentiation, indicating greater genetic variation within lineages.

The species delimitation analyses, used to validate the genetic structure of the four lineages, reinforced the results recovered by gene tree and BAPS. The monophyly of *B. polytaenia* is strongly supported by posterior probability values, and each clade is also structured and well-supported. Furthermore, the ENM analysis detected the expansion of suitable areas for *B. polytaenia* Serra da Mantiqueira clade during glacial periods, while expansions of suitable areas for *B. polytaenia* Serra do Mar clade occurred during warmer periods. Regarding climatic refugia, each clade responded differently. The Serra da Mantiqueira clade did not retain stability areas over time, while Serra do Mar clade showed some stable areas over time, particularly located in the state of Rio de Janeiro. The predicted suitable areas for each clade are distinct, with models for one clade not recovering the distribution of the other, and vice versa. Additionally,

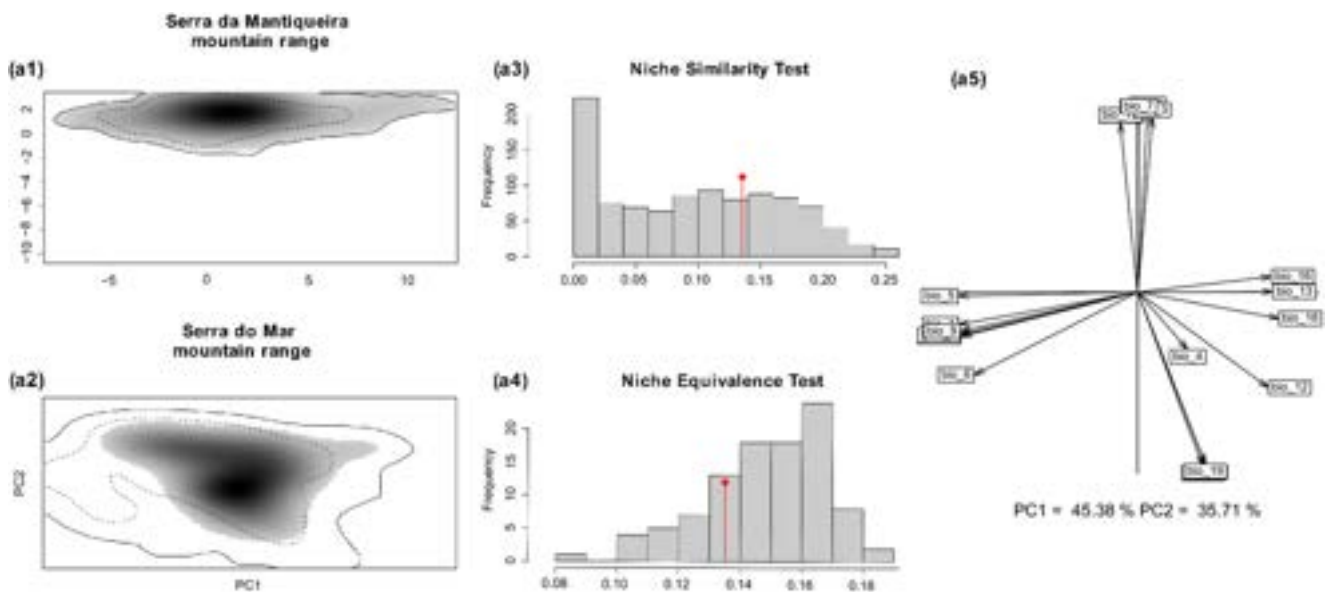


Fig. 7 Niche comparison among clade of *Boana polytaenia*. Panels (a1) and (a2) depict the environmental space occupied by *B. polytaenia* Serra da Mantiqueira clade and *B. polytaenia* Serra do Mar clade, respectively. Areas with the highest density of occurrences are represented by darker cells, while the lines depict the available background. Panel (a3) represents the bidirectional niche similarity test, for both

directions conducted (*B. polytaenia* Serra da Mantiqueira clade → *B. polytaenia* Serra do Mar clade, $p=0.32$; *B. polytaenia* Serra do Mar clade → *B. polytaenia* Serra da Mantiqueira clade, $p=0.30$). Panel (a4) presents the niche equivalence test ($p=0.78$). The red line shows the observed overlap. In the PCA-env plot (a5), the contribution of each variable to the environmental space is indicated

when comparing the climatic niches of the two clades, we observed a limited overlap and no significant similarity or equivalence between them. This may suggest that each clade occupies a distinct ecological niche, potentially shaped by historical climate fluctuations.

The phylogeographic separation between the two clades of *B. polytaenia* corresponds geographically to the southeastern mountain ranges of the Atlantic Forest (AF), as indicated in the recently presented phylogeny (Faivovich et al., 2021). This phylogeographic break refers to the first moment of diversification between these clades, which occurred between the Plio-Pleistocene boundaries (ca. 3.16 Mya), as recovered by the dated species tree. The Pliocene was marked by significant environmental changes. These included intense orogenic activity, culminating in the latest Andean uplift and the closure of the Isthmus of Panama, and climatic changes resulting in drier and cooler global climate, setting the stage for the glaciations of the Pleistocene (Haffer, 1969; Mckay et al., 2012; Rull, 2020). Thousands of years after this initial split, already in Pleistocene, a second moment of diversification occurred, which divided each clade of *B. polytaenia* into two lineages each (ca. 2.44 Mya for Serra do Mar clade and ca. 1.79 Mya for Serra da Mantiqueira clade). During the Pleistocene period, climate oscillations intensified (Head & Gibbard, 2005), and several Neotropical taxa, such as birds, mammals, anurans, snakes, ants, and plants, also underwent diversification processes attributed to climate change along the Serra do Mar

and Serra da Mantiqueira (Grazziotin et al., 2006; Mata et al., 2009; Cardoso et al., 2015; Bolívar-Leguizamón et al., 2020).

The phylogeographical break between the clades correspond geographically to the Continental Rift of Southern Brazil (CRSB), located between Serra da Mantiqueira and Serra do Mar, also known as the Rio Paraíba do Sul valley. During the Quaternary period, this region underwent phases of tectonic reactivation caused by ancient structural faults, and sea level changes generated by cycles of climate change, which promoted modification along the CRSB and caused lineages isolation (Siddal et al., 2010; Hansen et al., 2013; Souza, 2015). In this region, Pirani et al. (2022) have also demonstrated that the Paraíba do Sul River may have played a role in the diversification process of the treefrog *Dendropsophus elegans* (Wied-Neuwied, 1824). Therefore, the past climate along CRSB appears to have acted as a barrier that likely promoted genetic divergence between clades, with the topographic complexity of the region potentially reinforcing this process.

The presence of unique mtDNA haplotypes by lineage and structured clades in each mountain range suggest distinct genetic groupings maintained by the region. However, climate appears to be the main factor driving lineage divergence within *B. polytaenia*. The Serra da Mantiqueira clade shows a stronger association with colder climate environments, according to the ENM results, and predominantly inhabits higher altitude areas (Cruz & Feio, 2007;

Zornosa-Torres et al., 2020). In contrast, the distribution of the Serra do Mar clade demonstrates an opposite response to past climatic fluctuations compared to the Serra da Mantiqueira clade. During colder periods, such as LGM, the Serra da Mantiqueira clade expands its climatically suitable areas, while the Serra do Mar clade experiences retraction. Conversely, during warmer periods, the Serra do Mar clade expanded its distribution range while the other clade retracted. These contrasting patterns suggest distinct environmental preferences between each clade, as reflected in the difference between the contributions of the selected climatic variable to each clade niche, with the suitable areas of Serra da Mantiqueira clade strongly determined by temperature-related variables, whereas the Serra do Mar clade suitable areas with variables related to water availability. This is supported by the lack of similarity and equivalence between the niches of the two clades, implying they respond differently to environmental changes. Therefore, Pleistocene climate oscillations likely played a crucial role in shaping the diversification within *B. polytaenia*. Montane habitats, such as southern AF mountain ranges, are expected to be more susceptible to vicarious processes due to climate change (Hewitt, 2004), thereby promoting lineage divergence.

During glacial periods, such as the LGM, tropical forests were contracted and replaced by cold-adapted environments (Behling & Negrelle, 2001). However, each bioclimatic region of the Atlantic Forest demonstrates different responses to climate change. While the northern AF maintained stable areas that functioned as climate refuges, the southern portion experienced several forest contractions and changes in climatic conditions that did not favor the species of this region of the biome (Carnaval & Moritz, 2008; Carnaval et al., 2009). As observed through the ENM performed separately for each clade of *B. polytaenia*, the climate models do not predict the entire geographic distribution of the species, with each model predicting only the specific range of its respective clade. Moreover, the climate niche overlap indicates low similarity between the clades. The niche equivalency and similarity tests were non-significant in both directions, reinforcing that each clade occupies distinct climate spaces. The divergent response to the paleoclimatic dynamics can be interpreted as a reflection of distinct ecological adaptations of each clade to the specific climate regime of each mountainous region. Such ecological separation is consistent with the niche partitioning, which suggests that phylogenetically closely related lineages may differentiate their niches over time, mainly as a response to environmental pressures (e.g. competition), although the retention of ancestral niche traits might also be expected (Wiens & Graham, 2005). The results support the idea of distinct ecological adaptations between the clades.

High altitude taxa in the AF demonstrates different responses to lower temperatures when compared to lowland taxa (Carnaval et al., 2014). During the Pleistocene, higher elevations and latitudes protected taxa from the effects of climate change, providing suitable conditions for highland species to expand their range during periods of colder climates, such LGM (Amaro et al., 2012; Rodrigues et al., 2009). Given the evidence that cold periods during the Pleistocene possibly played a role in the demographic history of *B. polytaenia*, we expected the Serra da Mantiqueira clade to have benefited from these climatic shifts, expanding its climatically suitable areas primarily during colder periods and the demographic analyses to reveal expansions of their lineages during the LGM, corroborating the ENM results. To avoid violating the assumptions of the BSP model, which requires that samples to come from a single panmictic population with sufficient genetic variation (Drummond et al., 2005; Heled & Drummond, 2008), we restricted the analysis to the two lineages (Mantiqueira-East and Mar-South) with the largest sample sizes and the highest levels of genetic diversity. The demographic patterns recovered for these lineages are consistent with independent ecological and biogeographic expectations, supporting a robust framework for inferring broad demographic trends over time. Nonetheless, although the demographic analysis examined only one lineage of each *B. polytaenia* clade and did not reveal significant changes in the last 600 Kya, subtle oscillations in both lineages can be observed in the last 100 Kya (Graph A and B in Fig. 4), suggesting possible subtle historical demographic changes. These small variations may suggest that climate changes since the LIG period have exerted a modest yet persistent influence on demography of *B. polytaenia*. The increase in genetic variation and, consequently, the increase in coalescence time, influenced by the high level of geographic structuring and division between clades in the mountain ranges, masked possible major oscillations in the effective population size of lineages between glacial periods (Jesus et al., 2006). Overall, the combined genetic and ecological evidence suggests that historical climatic shifts shaped the diversification of *B. polytaenia* across southeastern Brazil.

Despite the strong geographic structure and high genetic diversity recovered through mtDNA, we found clear evidence of incongruity between mtDNA and nuDNA through the sharing of nuclear haplotypes between different lineages. These markers incongruities may indicate differences in mutation-accumulation and recombination rates between mitochondrial and nuclear loci, which occur at a faster rate in the mitochondrial genome, differences in effective population size, incomplete nuclear lineage classification, demographic asymmetry, and other processes (Prugnolle & de Meeus, 2002; Lynch, 2007; Thomé et al., 2010; Toews &

Brelsford, 2012). Our results support a scenario of incomplete lineage sorting, which can be interpreted as evidence for the retention of ancestral polymorphism for nuclear loci (Peters et al., 2007; Lopes et al., 2021). The incomplete lineage sorting occurs when ancestral polymorphism does not become definitively fixed in new populations, allowing diverged lineages to share genetic variations for a long period of time. This process is more common in periods of rapid diversification, when larger effective population size delay fixation of variations and results in the retention of ancestral polymorphisms (Maddison, 1997). The sharing of nuDNA haplotypes between lineages suggests the retention of ancestral polymorphism of nuclear loci, underscoring the complexity of lineage divergence and the challenges in accurately phylogenetic relationship using different genetic markers.

Considering the recovered demographic patterns, the strong genetic structuring of the clades, the lack of niche similarity between clades, and the non-reciprocal geographic predictions for the species distribution recovered by ENM, our findings suggest the existence of distinct evolutionary clades within *B. polytaenia*. The results obtained through the ENM may reflect the broad ecological tolerance of *B. polytaenia* species, which is known to be active and reproduce all year round in different populations and environments (Bertolucci & Rodrigues, 2002; Heyer et al., 1990; Rodrigues, 2015; Pinheiro et al., 2012). This continuous activity throughout the year suggests a possible adaptation to variable seasonal and thermal conditions, potentially contributing to the persistence of the species lineages in the face of climatic fluctuations, as well as to the historical demographic and distributional patterns identified in our study. Furthermore, as the taxonomic framework proposed by Faivovich et al. (2021), which includes populations previously treated under the synonym of *B. beckeri*, *B. latistriata*, and *B. bandeirantes*, it is plausible that these clades represent separate evolutionary units sharing a common ancestor. Despite this clear differentiation, the sharing of nuclear haplotypes between lineages suggests the retention of ancestral polymorphism at nuclear loci, underscoring the complexity of lineage divergence and the challenges in accurately resolving phylogenetic relationships of a taxonomic species complex. However, future studies may investigate possible genetic exchanges between the clades within each mountain range or even investigate the speciation process in one of the clades. The use of more variable nuclear markers, such as Single Nucleotide Polymorphisms (SNPs) (Warwick et al., 2021), offers greater possibilities to assess the phylogeographic structure of species, evaluate the connectivity of lineages, and construct more detailed models of gene flow. Additionally, these markers can help determine

whether the distribution of genetic diversity in this species is associated with conservation units.

Plio-Pleistocene climate change has played an important role in the diversification of *B. polytaenia*. However, topographic complexity may have played a secondary role in this process, as the *B. polytaenia* clades (Serra da Mantiqueira clade and Serra do mar clade) appear to be well-structured among each mountain range of southern AF. It is likely that the species climate preferences are strongly associated with the higher altitude regions, mainly for *B. polytaenia* Serra da Mantiqueira clade. Periods of colder climate likely promote the expansion of suitable areas for the Serra da Mantiqueira clade and consequently influencing the distribution of genetic diversity. Conversely, for Serra do Mar clade, these same periods reduced the suitable habitats. The lowlands, such as the Vale do Rio Paraíba do Sul, appear to have different climatic conditions than the mountain tops, likely acting as a barrier to dispersal and contributing to the observed genetic structuring of the clades. Consequently, the high genetic diversity within each mountain range reflects both limited gene flow across lowland barriers and differential response to past climate changes. The biogeographic pattern recovered in our study is strongly observed in other anurans species, where lineages show a similar phylogenetic structure, with species occurring in the Serra da Mantiqueira: *Aplastodiscus arildae*, *Holoaden luederwaldti*, *H. bradei*, *Ischnocnema holti*, and *Physalaemus feioi*; and their respective sister taxa in Serra do Mar: *Aplastodiscus* sp., *H. suarezi*, *H. pholeter*, *I. lactea*, and *P. olfersii* (Berneck et al., 2016; Cassini et al., 2010; Canedo & Haddad, 2012; Martins & Zaher, 2013; Pombal et al., 2008; Silva-Soares et al., 2018b). This pattern may suggest similar phylogenetic histories throughout many anuran taxa in both Serra do Mar e Serra da Mantiqueira. Overall, the climate has been a key factor driving the spatial distribution and genetic variation of *B. polytaenia*. Furthermore, the strong genetic structure, niche differences and non-overlapping distribution models, suggests the potential existence of distinct evolutionary units within *B. polytaenia*, which warrants further investigation.

Conclusion

Boana polytaenia exhibit phylogeographic separation, through two clades associated with the Serra da Mantiqueira and Serra do Mar. The initial divergence for *B. polytaenia* is dated during the Plio-Pleistocene transition period (ca. 3.16 Mya). Pleistocene climatic oscillations played an important role in the diversification of the two *B. polytaenia* clades, with periods of lower temperatures favoring the expansion of suitable areas for *B. polytaenia* Serra da Mantiqueira

clade, while for *B. polytaenia* Serra do Mar clade these areas experienced retraction. The divergence observed between the clades is supported by strong genetic structure within each mountain range. Furthermore, the absence of climatic niche overlap, the distinct influence of past climate change on each clad, and the taxonomic complexity involving *B. polytaenia* highlight the need for a comprehensive phylogenetic revision of the *B. polytaenia* clade.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11692-025-09653-y>.

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Author Contributions I.K.R.N. performed the analyses, wrote the main text, prepared all the figures and tables and formatted the manuscript. F.C. and D.J.S. revised the text and helped in performing the analyses.

Data Availability The DNA sequences used are available on GenBank, generated from the work of Faivovich et al. (2021), and the accession numbers are in Table S1 of the Supplementary information. The georeferenced points used in the ENM in this study are in Table S6 in the Supplementary Information and are available in online biodiversity databases.

Declarations

Competing Interests The authors declare no competing interests.

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