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Research Article

Traditional taxonomy underestimates the number of species of *Bokermannohyla* (Amphibia: Anura: Hylidae) diverging in the mountains of southeastern Brazil since the Miocene

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Despite the huge difference in land coverage between mountains and lowlands, most species are indeed found in mountains and foothills. The causes of this pattern have challenged biogeographers and evolutionary biologists. The Espinhaço and Mantiqueira Ranges are large mountain ranges from eastern Brazil that are global biodiversity hotspots located between the Brazilian Atlantic Forest (AF) and the Cerrado. However, Espinhaço and Mantiqueira species diversity may still be underestimated, either due taxonomic complexity or morphological cryptic species complexes. Two hylid frogs, *Bokermannohyla nanuzae* and *Bokermannohyla feioi*, are endemic, respectively, distributed in these two mountain ranges. These species were recently synonymized based on traditional taxonomy. We used data from the mitochondrial DNA (mtDNA) gene *16S* and two nuclear genes to undertake phylogenetic and network, distance-based, and multispecies coalescent analyses on *B. nanuzae*, *B. feioi*, and an extensive outgroup dataset. We tested the monophyly of *B. nanuzae*, as well as the presence of candidate new species. Based on *16S* phylogenetic analysis, We recovered *B. nanuzae* as paraphyletic, with *B. sagarana* nested within it. We recovered two main groups, with the geographic distribution generally corresponding to the Cerrado and AF boundaries. Probably due to ancestral polymorphism, both nuclear haplotype genealogies failed to distinguish *B. nanuzae* from the former *B. feioi* and/or from *B. sagarana*. The time-calibrated mtDNA tree revealed that *B. martinsi*, *B. sagarana*, and *B. nanuzae* have diverged during the Late Miocene, subsequently splitting into the remaining species/lineages during the Plio-Pleistocene. Taken together, our distance-based barcode and nuclear Bayesian analyses identified the former *B. feioi*, referred to as the AF group, as a distinct evolutionary lineage from *B. nanuzae* (Cerrado group). We provide the first insights into how different evolutionary lineages speciated in the highlands of southeastern Brazil and revalidated *B. feioi* for the AF group.

Key words: Atlantic Forest, barcode, campo rupestre, cryptic species, Espinhaço range, Mantiqueira range, molecular taxonomy, species revalidation

Introduction

Mountains are considered an important source of origin and maintenance of Earth's biodiversity. Compared to lowlands, mountains harbor 87% of the global species

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of amphibians, birds, and mammals, despite having a much lower land area (25% vs. 75%; Rahbek et al., 2019). Recently, Humboldt's enigma, proposed by Rahbek et al. (2019), recalls that large-scale geographic patterns remain a problem to explain why most species are found in mountainous regions and their foothills considering that the two primary variables that are proposed to underpin high levels of allopatric speciation in mountains are complex topography and climate oscillation. In the first scenario, height and distance can increase population isolation, which is usually linked to long-term evolutionary processes such as isolation by distance (IBD) and isolation by environment (IBE) (Huang et al., 2016; Tóth et al., 2019). In the second case, speciation can be linked to the impact of historical climate changes on habitat isolation/reconnection (e.g., last glacial maximum, LGM), and the presence of narrow thermal tolerances in tropical highlands can also split populations (Hoorn et al., 2018; Perrigo et al., 2020).

Eastern Brazil harbours the second most extensive South American network of mountain ranges, surpassed only by the Andes. The Mantiqueira Range and the Espinhaço Range (hereafter Mantiqueira and Espinhaço) are two nearly connected main orogenic belts of southeastern Brazil. Together, these highlands are distributed along a broad latitudinal range (10°S – 24°S), extending approximately 1600 km from north to south, reaching up to 2890 m a.s.l. (Almeida-Abreu and Renger, 2002; Gontijo-Pascutti et al., 2012). The Mantiqueira is divided into two sections, the southern and northern Mantiqueira (Silva et al., 2018). The southern Mantiqueira extends from eastern São Paulo State along the southern border of Minas Gerais State, whereas the northern section is in the border of the states of Minas Gerais and Espírito Santo (Silva et al., 2018).The Espinhaço is also divided into two main sections (from north to south): the Septentrional Espinhaço in Bahia State and the Meridional Espinhaço, mainly in Minas Gerais State, with the Quadrilátero Ferrífero (hereafter Quadrilátero) region representing its southernmost mountain formation (Leite et al., 2008; Magalhães et al., 2021). The Mantiqueira is fully embedded within the Atlantic Forest domain (AF), while the Meridional Espinhaço is in a transitional zone between the AF to the east and the Cerrado domain to the west, both global biodiversity hotspots (Mittermeier et al., 2005; Figure 1). The Septentrional Espinhaço, despite being influenced by the AF and Cerrado, lies within the Caatinga domain.

The stronger influence of the AF on the Mantiqueira makes it a mostly forested mountain range with small patches of cool-humid, grass-dominated formations usually associated with igneous and metamorphic rocks, such as granite and gneiss, found atop the highest summits known as *campos de altitude* (Safford, 1999). On the other hand, the typical Espinhaço vegetation is the *campo rupestre* (Miola et al., 2021). The *campo rupestre* is a montane, grassy-shrubby, fire-prone vegetation mosaic with rocky outcrops of quartzite or ironstone along with grasslands with patches of transitional vegetation such as cerrado and gallery forests (Silveira et al., 2016).

These tropical highlands shelter an astonishing species richness and endemism of plants (Giulietti et al., 1987; Safford, 1999; Carrijo et al., 2018). Likewise, the Mantiqueira and the Espinhaço are remarkably rich in anuran amphibians (frogs and toads) harbouring 88 and 41 endemic species, respectively (Silva et al., 2018; Pinheiro et al., 2021), with substantial levels of speciation having occurred from the Miocene onwards (Nascimento et al., 2018, Thomé et al., 2020; Carvalho et al., 2021). Describing such a notable diversity is not an easy task, especially when using only traditional taxonomic approaches. This is because there are many species with highly conserved morphology, for which there are no obvious morphological diagnostic characters (cryptic species *sensu stricto*; Chenuil et al., 2019). The increasing availability of multilocus molecular datasets and the application of analytical approaches have allowed cryptic species that were previously assigned to a single species to be identified. For instance, the hylid frog *Aplastodiscus heterophonicus* Pinheiro, Pezzuti, Berneck, Lyra, Lima & Leite, 2021, was described from the southeastern slopes of the Meridional Espinhaço and is morphologically indistinguishable from *A. cavicola,* which occurs in the Quadrilátero and the Mantiqueira (Pinheiro et al., 2021). However, molecular and acoustic data indicate the two species are not even sister taxa (Pinheiro et al., 2021). Similarly, the leptodactylid frog *Leptodactylus avivoca* Carvalho, Seger, Magalhães, Lourenço & Haddad, 2021, which occurs in the northern portion of the Meridional Espinhaço, is morphologically indistinguishable from *Leptodactylus cunicularius* Sazima & Bokermann, 1978, a species distributed in the Mantiqueira and southern Espinhaço (Carvalho et al., 2021). These species are highly genetically divergent and were also not recovered as sister species.

Nonetheless, there are several species endemic to these two mountain ranges (see Silva et al., 2018 for examples) for which genetic data have never been used to investigate their taxonomy or evolutionary history. Among these species is the hylid frog *Bokermannohyla nanuzae* (Bokermann & Sazima, 1973), a permanent stream dweller described from Serra do Cipó, at the

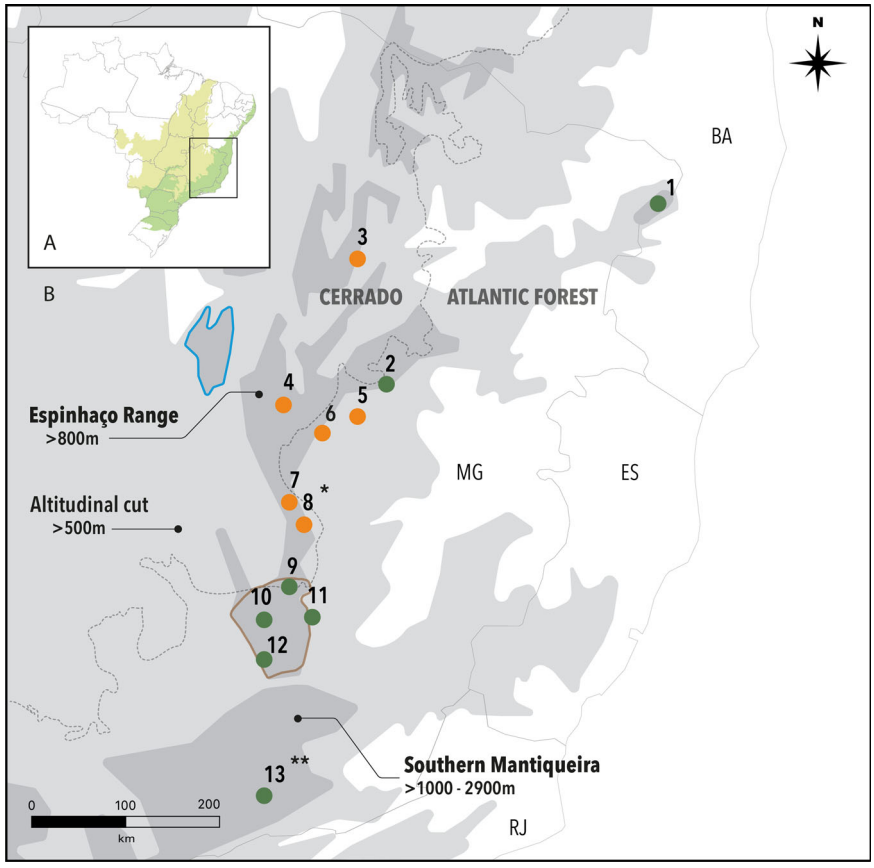

Fig. 1. Geographic distribution of Cerrado (pale yellow) and Atlantic Forest (green) domains in Brazil (A) and occurrence localities of *Bokermannohyla nanuzae* (Locality Numbers, LN 1–13) in Meridional Espinhaço (North, Central, and Quadrilátero), Southern Mantiqueira, and close related mountains in Minas Gerais State, southern Brazil (B). Distinct colours in localities represent mitochondrial DNA lineages found in this study (see Table 1). Blue line for Serra do Cabral (*B. sagarana* range), brown line for Quadrilátero (*B. martinsi* range), orange circles for North and Central Espinhaço group, and green circles for Alto do Cariri, Serra Negra, Quadrilátero and Mantiqueira group. The dotted gray line represents the limits between domains shown in (A), mountain ranges and altitudinal cut (> 500 m a.s.l.) are represented in grayscale. Brazilian states: BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro. **B. nanuzae* and ***B. feioi* type localities.

southern portion of the Meridional Espinhaço (Eterovick and Sazima, 2004). The species was considered endemic to the Quadrilátero and the Meridional Espinhaço, occurring from south to north, from the municipality of Ouro Preto (Lourenço et al., 2006) to the municipality of São Gonçalo do Rio Preto, at the central portion of the Meridional Espinhaço, in the state of Minas Gerais (Leite et al., 2006). However, Walker et al. (2015), based on traditional taxonomic methods (i.e., external morphology, morphometry, and acoustic data), synonymized *Bokermannohyla feioi* (Napoli & Caramaschi, 2004), described and known only from Serra do Ibitipoca, at the southern Mantiqueira, with *B. nanuzae*, and extended the geographic range of this species beyond the Espinhaço to the Mantiqueira. Recently, Pezzuti et al. (2021), based on larval morphology, doubted this synonymy, and raised the possibility that *B. feioi* could be a valid species, distributed both in the Quadrilátero and the southern Mantiqueira (Fig. 1).

Considering the current taxonomic rearrangement of *B. nanuzae* (Walker et al., 2015), we conducted a series of phylogenetic and molecular taxonomic assessments including an extensive outgroup sampling. We explored the mitochondrial *16S* rDNA phylogenetic relationships, distance-based analyses, two nuclear gene networks, and coalescent Bayesian methods to test the monophyly of *B. nanuzae*, assess potential cryptic species hidden under its name and/or any genetic substructure compatible with candidate species, and estimate diversification times. Finally, we explored the climatic differences of each major mitochondrial species group in multivariate space.

Materials and methods

Sampling and data collection

We obtained samples (muscle and liver) from 87 specimens of *B. nanuzae* from 13 localities along its entire distribution: Meridional Espinhaço (which we divided in North, Central, and Quadrilátero) and Southern Mantiqueira (Fig. 1). We included some of the specimens reported by Walker et al. (2015) and samples from the type localities of *B. nanuzae* and *B. feioi* (Table 1). Outgroup selection was based on two extensive phylogenetic analysis (Duellman et al., 2016; Faivovich et al., 2005): *Boana lundii* (Burmeister, 1856) ($n = 1$), *Bokermannohyla oxente* Lugli & Haddad, 2006 ($n = 1$); *Bokermannohyla alvarengai* (Bokermann, 1956) ($n = 2$), *Bokermannohyla hylax* (Heyer, 1985) ($n = 1$), *Bokermannohyla circumdata* (Cope, 1871) ($n = 2$), *Bokermannohyla ibitipoca* (Caramaschi & Feio, 1990) ($n = 2$); *Bokermannohyla* sp. ($n = 1$), *Bokermannohyla astartea* (Bokermann, 1967) (n = 1); *Bokermannohyla saxicola* (Bokermann, 1964) ($n = 2$), *Bokermannohyla martinsi* (Bokermann, 1964) ($n = 2$), and *Bokermannohyla saganara* Leite, Pezzuti & Drummond, 2011 ($n = 1$). Most of the samples were obtained from the Amphibian Collections of the Museu de Ciências Naturais da Pontifícia Universidade Católica de Minas Gerais (MCNAM) and Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais (UFMG), and we additionally used sequences available in GenBank (see Supplemental Table S1).

Molecular marker selection and laboratory procedures

We chose the mitochondrial *16S* rDNA because it has been considered an informative DNA barcoding marker for vertebrates (e.g., Vences et al., 2005). In amphibians, it has been used in phylogenetic studies of large-scale primary species hypotheses (Vacher et al., 2020), delimitation, and identification of species with highly conserved morphology (e.g., Hasan et al., 2012; Townsend et al., 2015).

We extracted genomic DNA using the DNeasy Blood & Tissue kit (Qiagen), following the protocol described by the manufacturer for vertebrate samples. We amplified the *16S* rDNA by Polymerase Chain Reaction (PCR) and sequenced with primers 16Sar-L and 16Sbr-H as described in Palumbi et al., (1991). For PCRs, we used the following conditions, using approximately 10–50 ng genomic DNA: 2.5 µL of 10× reaction buffer (100 mM Tris-HCl, pH 8.3, and 500 mM KCl), 2.4 µL of 50 mM $MgCl_2$, 2 µL of the dNTP mix (2 mM each), 0.5 µL of each primer at 10 µM, 0.5 U of Taq polymerase (Thermo Fisher) and Milli-Q water qsp 25 µL. The PCR was performed on a Veriti 96-Well Thermal Cycler (Thermo Fisher), with the following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of amplification for 45 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, with a final extension step at 72 °C for 7 min.

We also selected two nuclear DNA fragments to explore species limits and presence of substructure in *B. nanuzae*, one located in a conserved coding DNA region (Beta-crystallin gene, *CRY-B*) and another in a non-coding region (DiSulphide Isomerase A6 precursor intron 6, *DI-A6-int6*). The nuclear markers were amplified and sequenced as follows: (i) *CRY-B* with primers Cryb1Ls and Cryb2Ls (Dolman & Phillips, 2004), and (ii) *DI-A6-int6* with primers MVZ37 and MVZ38 (Bell et al., 2012). PCRs conditions were similar to those used for *16S*, but with an annealing temperature of 48 °C.

Bidirectional sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher), following the protocol recommended by the manufacturer. Sequencing runs were performed on an ABI 3730 DNA Analyzer (Thermo Fisher). We assembled and analysed the final sequences from the sequencing readings using the Geneious Prime® (Biomatters Ltd.). All the newly generated sequences were deposited in GenBank (see Supplemental Table S1).

Genetic variation, recombination, and neutrality tests

All DNA sequences were aligned with MAFFT v.7.450 (Katoh et al., 2002; Katoh and Standley, 2013), using the automatic function to select the algorithm according to the data size. The alignment is available on Figshare (Brunes, 2022). We assessed nuclear sequence variation through PHASE software v.2.1 (Stephens et al., 2001) implemented in DnaSP v.5 (Librado and Rozas, 2009). We performed preliminary analysis for each gene with multiple independent runs, different seed numbers, and 100 interactions. We checked the consistence across runs, selected the most likely phase, and reduced multiple-base indels to a single evolutionary step. Then, we evaluated the presence of recombination events using the Difference of Sums of Squares (DSS) test implemented in TOPALI v.2.5 (Milne et al., 2004) with window size of 70 bp, steps 10 bp-long and 100 bootstrap repetitions. In the case of significative results, we performed an "auto-partition alignment" based on the DSS results.

Mitochondrial and nuclear polymorphism levels were calculated in DnaSP v.6 (Librado and Rozas, 2009)

Table 1. Localities of *Bokermannohyla nanuzae* in Meridional Espinhaço (North, Central, and Quadrilátero), Southern Mantiqueira, and close related mountains in Minas Gerais State, with geographic information and sample sizes (N). LN, Locality number (see Fig. 1).

LN	Mountain region	Locality	Municipality	Latitude	Longitude	N
1	Alto do Cariri	Alto Cariri	Santa Maria do Salto	−16.4224	−40.0577	2
2	Central Espinhaço	Serra Negra	Itamarandiba	−18.0109	−42.7418	5
3	North Espinhaço	Serra de Botumirim	Botumirim	−16.8662	−43.0090	7
4	Central Espinhaço	Diamantina	Diamantina	−18.1760	−43.7142	10
5	Central Espinhaço	Serra do Ambrósio	Rio Vermelho	−18.2931	−43.0082	9
6	Central Espinhaço	Pico do Itambé	Santo Antônio do Itambé	−18.4297	−43.3384	10
7	Central Espinhaço	Conceição do Mato Dentro	Conceição do Mato Dentro	−19.0592	−43.6557	1
8	Central Espinhaço	Serra do Cipó[a]	Santana do Riacho	−19.2596	−43.5310	10
9	Quadrilátero	Serra da Piedade	Caeté	−19.8363	−43.6802	9
10	Quadrilátero	Serra do Gandarela	Rio Acima	−20.1279	−43.8906	3
11	Quadrilátero	Serra do Caraça	Catas Altas	−20.0992	−43.4867	6
12	Quadrilátero	Congonhas	Congonhas	−20.4723	−43.8928	5
13	Southern Mantiqueira	Serra do Ibitipoca[b]	Lima Duarte	−21.7119	−43.9029	10

[a]*B. nanuzae.*
[b]*B. feioi* type localities.

including segregation sites (S), number of haplotypes (h), haplotype diversity (Hd), and population mutation (theta). Genetic distances (*p*-distance; mean, min, and max) among species and main groups were calculated in MEGA7 (Kumar et al., 2016) considering the uncorrected pairwise and the Jukes-Cantor model (i.e., all substitution rates are equal; JC model). Small gaps and missing data were treated using the pairwise deletion criteria. Departures from the model of neutral equilibrium were tested in the major mitochondrial groups with Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) in DnaSP v.6 (Rozas et al., 2017). The significance of values was determined using 10,000 coalescent simulations.

Phylogenetic analyses, haplotype genealogies, and pairwise distances

Phylogenetic relationships were inferred by Bayesian Inference (BI) and Maximum likelihood (ML) for mitochondrial data in MrBayes v.3.2.6 (Huelsenbeck and Ronquist, 2001) and RAxML v.8 (Stamatakis, 2014), respectively, via plugin in Geneious using individuals listed in the in Supplemental Table S1. For the BI analysis, we performed two replicate runs with 1,000,000 chain length, sampling every 200 generations with four Markov chain Monte Carlo (MCMC) running simultaneously, and checked if all parameters had effective sample sizes (ESS) values greater than 200. For ML analysis, we ran a rapid bootstrap analysis with 1,000 nonparametric replicates and then used the majority rule to obtain a consensus tree. Both trees were edited using the FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/). The evaluation of the supported clades was based on posterior probability (PP) values $\geq$ 0.95 (Huelsenbeck and Ronquist, 2001) for BI, and bootstrap proportions (BP) $\geq$ 70% (Hillis & Bull, 1993) for ML analyses. Nucleotide substitution models were selected in jModelTest v.0.1.1 (Posada, 2008) under the Bayesian information criterion (BIC) and in the model available to be implemented in both phylogenetic analyses.

Nuclear patterns of haplotype distribution were investigated for each gene separately using a method of haplotype genealogies. Specifically, Haploviewer (beta version; available at http://www.cibiv.at/~greg/haploviewer) was used to convert a maximum parsimony tree produced with DNAPARS function available in PHYLIP v.3.69 package (Felsenstein, 2005) in gene haplotype genealogies. Haplotype distribution was explored by classifying them following the mitochondrial DNA groups.

Dating

We investigated diversification times (tMRCA—time to the most recent common ancestor) through the mtDNA alone and together with the nuclear DNA (nuDNA) through a time-calibrated mtDNA gene tree and a species tree using StarBEAST2 v.2.6.4 (Bouckaert et al., 2014). For both, the mutation rate was calibrated as 0.00277 mutations per site per million years, as performed by Lemmon et al. (2007) for hylids with the same molecular marker (*16S*), due to the absence of an appropriate fossil calibration. We tested the molecular clock expectations for each gene (data not shown) in MEGA7 (Kumar et al., 2016). We then set a strict molecular clock with the yule tree prior for the time-calibrated *16S* gene tree, assuming a constant lineage

birth rate for each branch in the tree. We performed an analysis of 50,000,000 generations, sampling the results every 5,000 generations. For the species tree, we ran the analysis as follows: a strict molecular clock for *16S* and *CRY-B*, a relaxed log-normal clock for *DI-A6-int6*, a constant lineage birth and yule tree prior. Here, we increased the number of generations to 100,000,000 sampling every 10,000 and worked with a subset of 17 individuals representative for each species/clade (see Supplemental Table S1) using both mitochondrial and nuclear genes. In both analyses, we set the nucleotide models following the results of jModelTest, as mentioned before. The convergence of the Markov chain simulations was checked in Tracer v.1.7.2 (Rambaut et al., 2018) assessing the ESS values greater than 200. The final trees were summarized using TreeAnnotator v.2.6.4 (Drummond & Rambaut, 2007), discarding the first 10% as burn-in.

Species delimitation

We explored the molecular species limits among what is currently known as *B. nanuzae* and related species using two recursive methods of pairwise distance-based barcode clustering and two tree distinct multilocus approaches. For the first category, we opted to focus only on distance-based instead of tree-based DNA barcodes methods (e.g., GMYC and PTP) because their results are considered more taxonomically conservative (Vacher et al., 2020), given that tree-based DNA barcodes methods may overestimate the number of candidate species. Nevertheless, we performed a preliminary analysis with GMYC (Fujisawa & Barraclough, 2013) using the package 'splits' v. 1.0-20 (Ezard et al., 2009) of the R platform v. 4.1.2 (R Core Team, 2021). The analysis requires an ultrametric tree, and we used a haplotype tree derived from BEAST 2.5 (Bouckaert et al., 2019) for this purpose. The results were not significant (p-value = 0.5778662), so we do not show or discuss the data.

We applied our data (90 *16S* sequences) in the Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012) and the Assemble Species by Automatic Partitioning (ASAP, Puillandre et al., 2021), both under the JC nucleotide substitution model. Although both methods belong to the same category, the ASAP is an improved version of ABGD, because it includes a scoring system (based on a combination between probability and slope) and does not require a prior specification of the intraspecific distance (P) between pairs (Pmin and Pmax). This made both methods directly comparable to examine the impact of *a priori*-defined intraspecific genetic distances on the results. We applied the default settings for the ASAP analysis (recursive split pval = 0.01, showing the 10 best scores) and reported only the partition with the best ASAP score (lowest value/the first partition) and the partitions with significant p-values.

For the ABGD, we first ran the analysis using all default parameters including both P-values (Pmin: 0.001 and Pmax: 0.1) with 10 steps, minimum relative gap width of 1.5 and 20 bins for distance distribution (data not shown). Considering that we are not undertaking an initial species hypothesis study, we then took advantage of the requirement of minimum P prior (Pmin) to set a value using the current taxonomy, avoiding oversplit patterns. We used the split between *B. sagarana* ($n = 1$) and *B. martinsi* ($n = 2$), two morphologically distinct species, as a positive control, and the potential split within *B. martinsi* as a negative. We then set Pmin value based on the lowest prior maximal distance results of the first run (see results section; PMD = 0.003) and analysed the first and the last estimated partitions. Finally, we also used the barcode gap (i.e., the limit between intraspecific and interspecific distances) to evaluate the results of the two methods.

Regarding the multilocus approaches, we applied two distinct methods: (i) a combined distance-based method and (ii) a Bayesian method that deals with incomplete lineage sorting. For the first, we used the complete individual dataset to generate individual genetic distances (p-uncorrected) matrix for each nuDNA gene (*CRY-B* and *DI-A6-int6*) in MEGA7 (Kumar et al., 2016). Then, we used POFAD v.1.03 (Joly & Bruneau 2006) to combine the data and visualized the standardized multilocus network in SplitsTree v.4.12.3 (Huson & Bryant 2006). Bayesian species delimitation analysis was performed in the software BPP v.2.2 (Yang & Rannala, 2010), following a recent tutorial (Flouri et al., 2020) and using the resulting species tree from the StarBEAST analysis as the guide tree. In this case, we used only the two nuclear fragments and the same subset of individuals' representative for each species/clade, for which we lacked nuclear data (see Supplemental Table S1). For priors of ancestral population size (theta) and root age (tau), we choose a combination of large theta (1, 10) and shallow divergencies (2, 2000) that has been suggested for models with a small number of species (Leaché & Fujita, 2010; Yang & Rannala, 2010). We repeated the analyses three times with different seed numbers to ensure that the results were consistent.

Climatic differences

We intended to perform direct climate comparisons to investigate if there are temperature and precipitation differences between localities of *B. nanuzae* gathered in

the mitochondrial lineages. We used this multivariate approach due to our lack of occurrence points required to conduct rigorous ecological niche models to explore the past distribution and test for niche divergence (Wisz et al., 2008). We started the analyses with 19 bioclimatic variables plus elevation data (1 × 1 km grid cell; Fick & Hijmans, 2017), and filtered them using a Factorial Analysis (FCA), via R environment using the function 'fa.parallel' from the package 'psych' v.2.1.9. Then, we used Principal Component Analysis (PCA) in PAST4 software (Hammer et al., 2001) to freely have access to the climatic profile of each group in the multivariate space.

Results

Genetic variation, recombination and neutrality tests

We successfully sequenced the *16S* gene (446 bp) of 87 *Bokermannohyla nanuzae* individuals, representing 13 localities and 18 haplotypes (*Hd* = 0.90). The overall nucleotide diversity was 0.010–0.032 (Table 2). Polymorphisms within the two major groups (see below) were the same at the haplotype level, and similar regarding haplotype and nucleotide diversity. None of the neutrality tests were significant.

The DSS recombination test did not detect any recombination event in *CRY-B* in a fragment of 232 bp in 83 individuals. For *DI-A6-int6*, we obtained a fragment of 470 bp long for 75 individuals. The recombination test revealed one significant recombination hotspot only in one of the two nuclear genes analysed here (*DI-A6-int6*, 288 bp, Score = 9.92). In this case, the alignment was divided into two nucleotide fragments ranging from 1 to 293 and 294 to 470. Both fragments and the entire sequence were subjected to preliminary phylogenetic examination (data not shown). We chose to keep only the first 293 bp, considering that the purpose of this study is to analyse genetic relationships and we aim to eliminate any false representation induced by random mutations, therefore, without evolutionary signal. All polymorphisms within the two nuclear fragments were higher in *DI-A6-int6* than in *CRY-B* (Table 2).

Phylogenetic analyses, haplotype genealogies, and pairwise distances

Both the BI and the ML analyses based on *16S* (GTR + G, gamma shape = 0.09) produced the same topology (ML tree not shown) and did not recover *B. nanuzae sensu* Walker et al. (2015) as monophyletic (Fig. 2). Instead, our analyses recovered this taxon divided into two maximally supported clades: one containing individuals from the type locality of *B. nanuzae* (Serra do Cipó, Espinhaço Meridional), and the other comprising specimens from the type-locality of the former *B. feioi* (Parque Estadual do Ibitipoca [Ibitipoca State Park], Mantiqueira), with *B. sagarana* as the sister clade of *B. nanuzae sensu stricto* (PP = 0.95, BP = 95). The distribution of the two clades roughly coincides with the limits of the Cerrado and the AF, regarding individuals from: (i) North/Central Espinhaço Meridional (LN 3-8), which we called the Cerrado group despite the fact that Serra do Ambrósio (LN 5) and Pico do Itambé (LN 6) are located at the western edge of the AF (PP = 0.99, BP = 99), and (ii) Quadrilátero and Southern Mantiqueira (LN = 9–13) plus two northeastern localities (PP = 1.00, BP = 100), one from Central Espinhaço (Serra Negra, LN 2), and the other outside the Espinhaço meridional range (Alto do Cariri, LN 1), which we called as the AF group (PP = 1.00, BP = 100). Both analyses recovered *B. martinsi* as the sister clade of the AF group plus *B. sagarana* and the Cerrado group (PP = 0.9, BP = 89; Fig. 2), followed by *B. saxicola* (PP = 0.99, BP = 89), and the other outgroups.

Both nuclear haplotype genealogies failed to distinguish *B. sagarana* from the group of *B. nanuzae* haplotypes (Fig. 3A and B). The *CRY-B* demonstrated a trend

Table 2. Polymorphisms and neutrality tests for *Bokermannohyla nanuzae*. Cerrado group includes clades from North & Central Meridional Espinhaço and Atlantic Forest group includes clades from Alto Cariri, Serra Negra, Quadrilátero, and Southern Mantiqueira.

	Polymorphisms					Neutrality tests	
Fragment (size)	**N**	**SS**	**h**	**HD**	**Π [95% CI]**	**Tajima's D**	**Fu's Fs**
16S (446 bp)							
All	87	44	18	0.90	0.031 [0.010–0.032]	–	–
Cerrado group	47	19	9	0.85	0.009 [0.005 – 0.015]	−0.1640 ns	1.4825 ns
Atlantic Forest group	40	16	9	0.77	0.007 [0.004 – 0.013]	−0.0241 ns	0.7466 ns
CRY-B (231 bp)	166	14	16	0.89	0.009 [0.004 – 0.019]	–	–
DI-A6-int6 (293 bp)	150	44	55	0.93	0.024 [0.014 – 0.048]	–	–

Note: N = number of individuals or nuclear phases; SS = number of segregating sites; h = number of haplotypes; HD = haplotypic diversity; Π = nucleotide diversity corrected by fragment size.

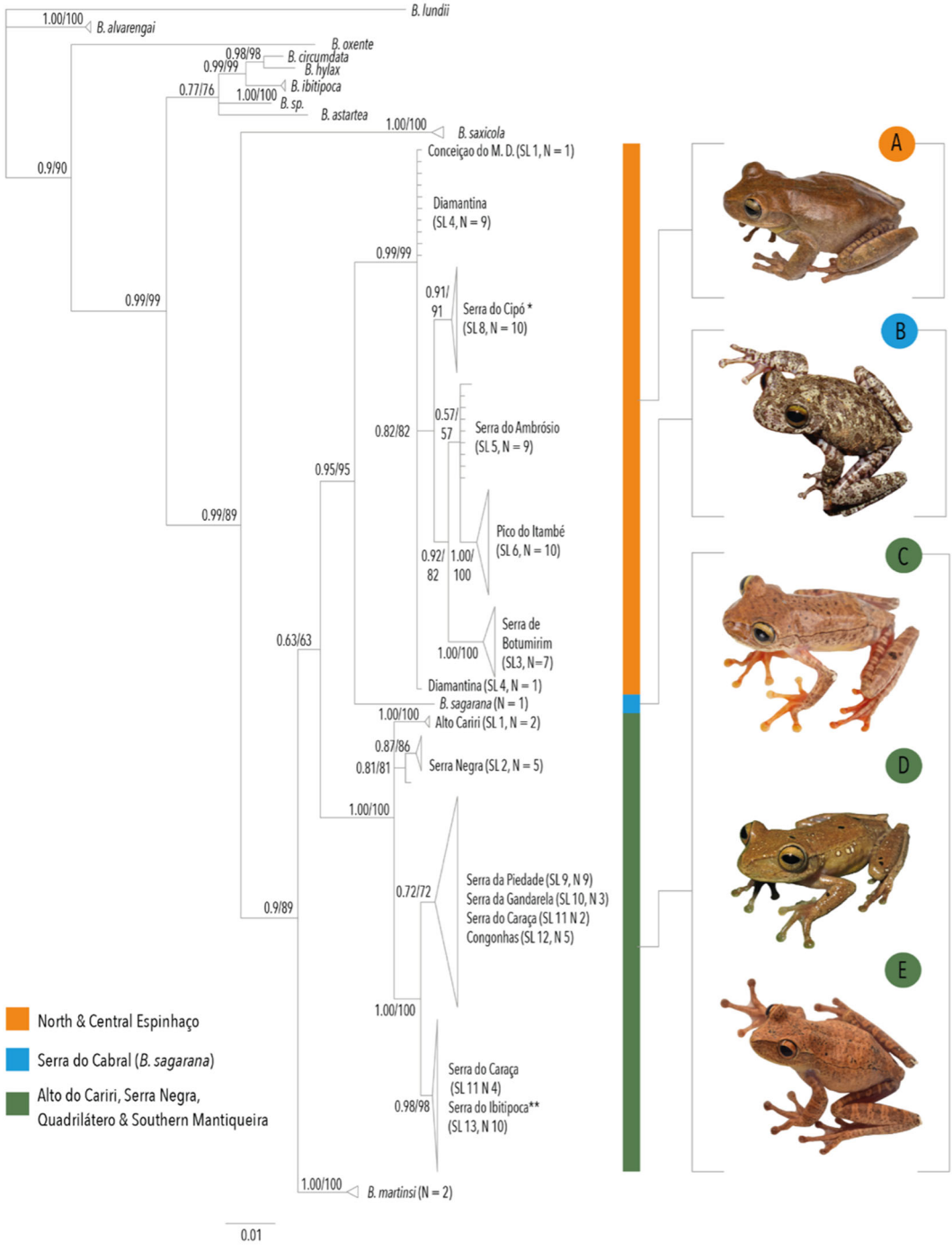

Fig. 2. The 50% majority rule consensus haplotype tree from Bayesian inference analysis of the mitochondrial gene fragment (*16S*) of *Bokermannohyla nanuzae* and outgroups derived from MrBayes. Bayesian posterior probabilities and bootstrap values for ML are represented in black near the nodes. Colours represent the haplotypes distribution along the Meridional Espinhaço (North, Central, and Quadrilátero), Southern Mantiqueira, and close related mountains. Tip labels contain voucher numbers, species, and locality number (LN, see Table 1) A, *B. nanuzae* (Serra do Cipó); B, *B. sagarana* (Serra do Cabral); C, *B. nanuzae* (Alto do Cariri), D, *B. nanuzae* (Serra Negra), and E, former *B. feioi* (Serra do Ibitipoca).

towards the separation of Cerrado (North and Central Espinhaço Meridional) and AF groups (Alto Cariri, Serra Negra, Quadrilátero and Southern Mantiqueira). In detail, haplotype sharing was present in *CRY-B* between *B. sagarana*, Cerrado and AF groups, and in *DI-A6-int6* only between Cerrado and AF groups. In *CRY-B*, all

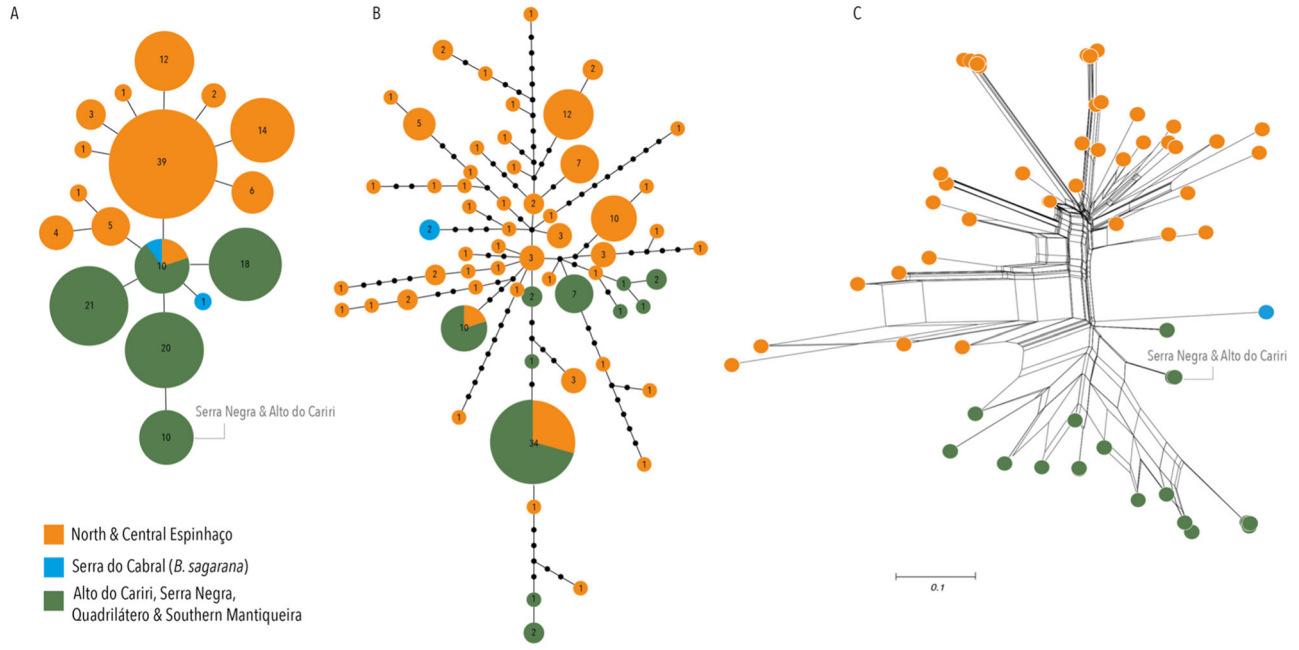

Fig. 3. Nuclear haplotype distribution of *Bokermannohyla nanuzae* and *B. sagarana*: A) *CRY-B*, B) *DI-A6-int6* genealogies, and C) multilocus distance network. Colours represent the major mitochondrial groups (see Fig. 2). North and Central Meridional Espinhaço represent the Cerrado group; and Alto Cariri, Serra Negra, Quadrilátero, and Southern Mantiqueira represent the Atlantic Forest group. Numbers inside circles represent haplotype frequency and mutations are edges.

haplotypes from the Alto Cariri and Serra Negra, the two northeastern localities (LN 1 and 2) were also placed with the haplotypes from Quadrilátero and Southern Mantiqueira, in a single and external haplotype.

Uncorrected pairwise (*p*-uncorrected) between valid-related species were 4.53% (*B. sagarana* and *B. martinsi*). Between *B. nanuzae* (Cerrado & AF) and *B. martinsi* *p*-distances ranged from 3.7% to 5.6%. Distances between *B. nanuzae* and *B. sagarana* were as follows: i) Cerrado group *p*-distances 3.9–5.6% and ii) AF group 5.1–6%. Therefore, *B. sagarana* was more distant to the AF group (maximum of 6%) than the Cerrado group (maximum 5.6%) of *B. nanuzae*. Within *B. nanuzae* groups, the minimum uncorrected *p*-distance between Cerrado and AF groups was 4.4% and the maximum was 6.3%. All mean distances corrected with the JC model were 0.1–0.2% greater than uncorrected distances (Table 3).

Dating

Diversification times based on the tMRCA' calculated in the time-calibrated mtDNA gene tree suggest that the group consisted of *B. martinsi*, *B. sagarana*, and *B. nanuzae* began diversifying during the Late Miocene (Fig. 4A). The initial diversification of *B. sagarana* and the *B. nanuzae* Cerrado group occurred between the Late Miocene and the Pliocene (4.3–8.66 Ma). The Cerrado and AF groups diverged concurrently between the Pliocene and the beginning of the Pleistocene (2–4.4 Ma), and the individuals from Quadrilátero and Southern Mantiqueira (1.11–2.78), Serra Negra (0.3–2,09 Ma), and Alto Cariri (0–0.73 Ma), diverged throughout the Pleistocene.

The following nucleotide substitution models were added in the species tree analyses in StartBEAST: GTR with estimated frequencies for *CRY-B* and HKY with equal frequencies plus an invariant site proportion of 0.0778 for *DI-A6-int6*. The species tree failed to recover well-supported relationships among *B. martinsi*, *B. sagarana*, and *B. nanuzae* (Cerrado and AF groups), with low posterior probability values (PP = 0.40 and 0.55). On the densiTree visualization, conflicts between gene trees are emphasized (Fig. 4B). Diversification times were calculated only for basal splits within well-supported clades: initial diversification of *B. martinsi*, *B. sagarana*, and *B. nanuzae* began within 16.4–7.73 Ma, during the Middle-Late Miocene.

Species delimitation

The two distance-based tools for investigating candidate species showed partially similar results (Fig. 5A). The best partition selected using the lower ASAP-score found four candidate species in our *16S* dataset: *B.*

Table 3. *16S* mean genetic distances between *B. sagarana*, *B. martinsi*, *B. nanuzae*, and its main groups: *p*-uncorrected (lower left) and Jukes-Cantor model (upper right).

Species/groups	*B. martinsi*	*B. sagarana*	*B. nanuzae* (Cerrado)	*B. nanuzae* (AF)
B. martinsi	–	4.67	4.56	5
B. sagarana	4.53	–	4.9	6.05
B. nanuzae (Cerrado)	4.42 (3.7–5.3)	4.75 (3.9–5.6)	–	5.62
B. nanuzae (AF)	4.83 (4.2–5.6)	5.81 (5.1–6.0)	5.41(4.4–6.3)	–

Note: p-uncorrected min and max values in parenthesis. Values are shown in percentage.

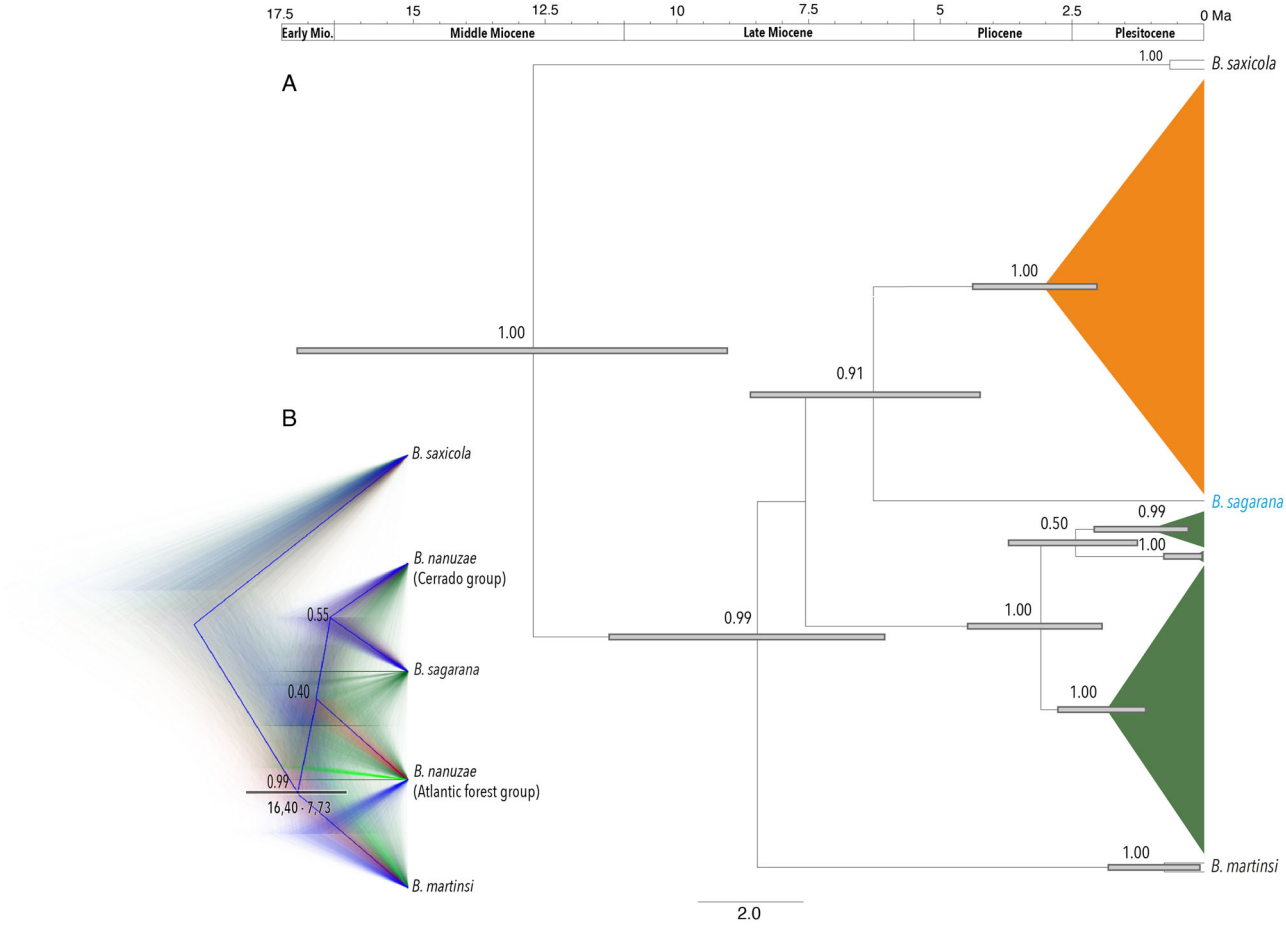

Fig. 4. (A) Maximum clade time-calibrated mitochondrial tree (*16S*) of *Bokermannohyla nanuzae*, close related taxa, and outgroup derived from BEAST 2.5. Cerrado group in orange and Atlantic Forest group in green (see Fig. 2 for more information). (B) DensiTree representation of the species tree of *B. nanuzae* and close related taxa. Consensus trees are in solid blue as the most popular trees, the next most popular tree is in red, the third most popular in green and the rest is light green. Node numbers represent the posterior probabilities (above). Gray bars represent the 95% HPD of the mean times to the most recent common ancestor (tMRCA).

sagarana, *B. martinsi*, *B. nanuzae* from Central and North Meridional Espinhaço (the Cerrado group), and *B. nanuzae* from Quadrilátero, Southern Mantiqueira, Alto Cariri, and Serra Negra (the AF group) (Score: 2.5, Threshold. dist. of 0.025, *p*-value $>$ 0.1, see Supplemental Table S2). The significant partitions (*p*-value $<$ 0.1) found 10 and 18 candidate species. Therefore, both added some candidate species within *B. nanuzae* and considered the samples from Alto Cariri as a candidate species. The ABGD analyses found that the barcode gap distance was of 0.031 and suggested the presence of four partitions, the first three with the same number of candidate species ($n = 7$, Fig. 5 does not show the second and third partitions), varying in the prior maximal distance (PMD) from 0.003 to 0.006, and the last with 4 groups (PMD = 0.01, Fig. 4), the same suggested by the best partition found by ASAP.

The multilocus network clearly separated the nuclear haplotypes from the two major *B. nanuzae* groups, with *B. sagarana* being more similar to the AF group than to the Cerrado group (Fig. 3C). Furthermore, haplotypes from Alto Cariri and Serra Negra were found within the AF group. BPP method using only the nuclear fragments showed high posterior probability for the presence of three species: *B. sagarana*, *B. nanuzae* Cerrado group, and *B. nanuzae* Atlantic Forest group (Fig. 5B).

Climatic differences

The Parallel analysis suggested that the number of climatic factors was three (data not shown): BIO10 (Mean Temperature of Warmest Quarter), BIO15 (Precipitation Seasonality), and BIO4 (Temperature Seasonality). The three first components of the PCA explained a total of 49.92%, 30.47%, and 19.61%, respectively, of the bioclimatic variation of the 13 localities of occurrences of *B. nanuzae*. In general, the distribution of the localities in the first two axes presented two groups corresponding with the mitochondrial lineages with a diagonal pattern (Fig. 6). In one hand, North and its subsequent Central Espinhaço locality represented the upper multivariate limit of the PC1. On the other hand, the locality from Alto Cariri highlighted the upper limit of the PC2. The PCA also showed the presence of an environmental overlapped area, geographically corresponding to major Central Espinhaço and the Quadrilátero. Finally, three localities latitudinally distant, located on the side of the Atlantic Forest, and representing the Serra Negra, Quadrilátero, and Southern Mantiqueira (see Fig. 1; LN 2, 11 and 13, respectively), occupied very close multivariate spaces in a distinct area.

Taxonomic implications

Our results, based on phylogenetic molecular analyses, have shown that there are at least two species hidden under the name *B. nanuzae*. Despite there is no current phenotypic character differentiating any population within the species (Walker et al. 2015; but see Pezzuti et al. 2021), we recovered it as paraphyletic, with *B. sagarana*, a phenotypically diagnosable species (see discussion), nested within it in both our BI analyses and in our species tree analysis. The most conservative results of our species delimitation analyses also split *B. nanuzae* in two species, coincident with the two main clades of our tree: a Western clade, distributed in Northern/Central Meridional Espinhaço (Cerrado group), and a mostly Eastern clade, distributed in the Quadrilátero, Southern Mantiqueira, Serra Negra, and Alto do Cariri (AF group). The phylogenetic, molecular, and climatic data presented herein support these two clades as distinct evolutionary lineages that should be treated as different species. The Cerrado group includes the type locality of *B. nanuzae* (Serra do Cipó, municipality of Jaboticatubas—currently municipality of Santana do Riacho, state of Minas Gerais, Brazil) and it is the clade that the name *Bokermannohyla nanuzae* (Bokermann & Sazima, 1973) should apply. There is also an available name for the AF group, which is currently under the synonymy of *B. nanuzae*, where it was placed by Walker et al. (2015). We therefore revalidate *Bokermannohyla feioi* (Napoli & Caramaschi, 2004; type locality Ibitipoca State Park, district of Conceição do Ibitipoca, municipality of Lima Duarte, state of Minas Gerais, Brazil) and apply this name to the populations from the Quadrilátero, Southern Mantiqueira, Serra Negra, and Alto do Cariri.

Bokermannohyla feioi (Napoli & Caramaschi, 2004)
Hyla feioi Napoli & Caramaschi, 2004
Bokermannohyla feioi Faivovich, Haddad, Garcia, Frost, Campbell & Wheeler, 2005
Boana feioi Wiens, Fetzner, Parkinson & Reeder, 2005

Diagnosis. There are no phenotypic characters that unambiguously differentiate *B. feioi* from *B. nanuzae* (Walker et al., 2015; but see the Phenotypic variation between species section below), such that these are two cryptic species. Nevertheless, there are some molecular diagnostic characters. *Bokermannohyla feioi* differs from *B. nanuzae* at multiple sites ($n = 18$) for a 446 bp fragment of the mitochondrial DNA gene *16S*.

Phenotypic variation between species. Although *B. feioi* and *B. nanuzae* are phenotypically indistinguishable (Walker et al., 2015), there are some characters that, despite overlapping (i.e., not usefull for a sharp diagnosis), help to diferentiate these species. Walker et al. (2015) found that ca. 76% of the specimens of *B. feioi* (referred by them as *B.* cf. *nanuzae* and *B. feioi*) had a complete dorsolateral line, whereas in 67% of the *B. nanuzae* individuals the line is incomplete or absent. Walker et al. (2015) also reported that all specimens of *B. feioi* ($n = 19$) analysed had transverse bars on dorsum, while 83% of the *B. nanuzae* ($n = 50$) individuals lacked these bars, and *B.* cf. *nanuzae* ($n = 134$) individuals showed 50% of variation for each state.

The vocalization of *B. feioi* and *B. nanuzae* share great similarity, although some call parameters varied between these species (Walker et al., 2015). However, due to overlap of temporal and spectral parameters

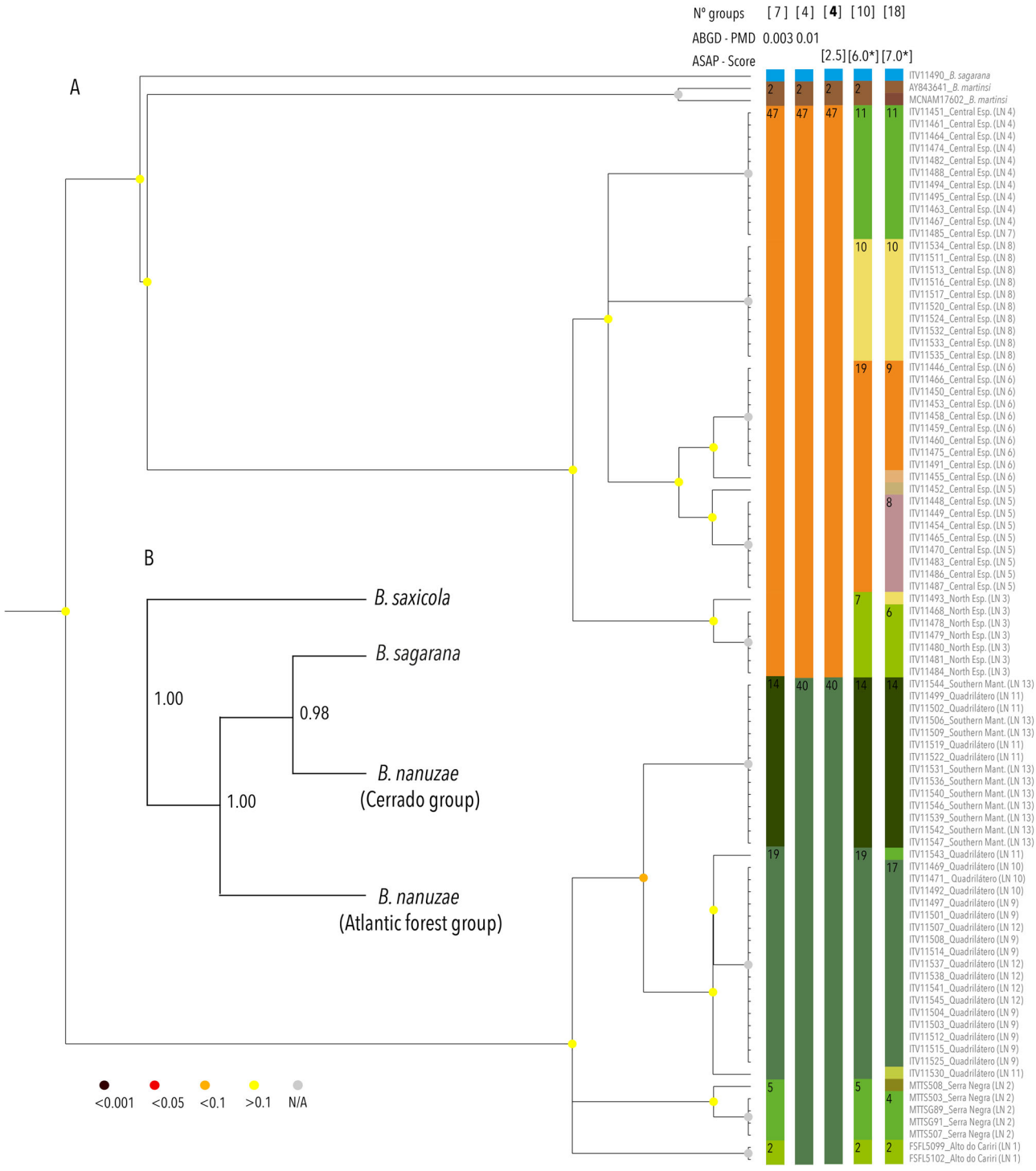

Fig. 5. (A) Dendrogram and bars representation of the groups of individuals belonging to each candidate species proposed by ABDG and ASAP based on *16S* distances corrected with JC model. Above bars, group number in bold represent the partition in the vicinity of the barcode gap. PMD, prior maximal distance of ABGD analysis. Within bars, numbers indicate the number of specimens. The colour scale of the bars is the same as the pattern shown in Fig. 2 for partitions with four groups and the number of colours increases in partitions with more groups, sometimes corresponding to locations (see individual information in grey). Colours in the nodes correspond to their likelihood of merging with known distances within each group (from dark to light), where an unlikely group is indicated by a dark colour. Uncalculated nodes (N/A) in grey. *p-value < 0.01. Cerrado group: Central and North Meridional Espinhaço and Atlantic Forest group: Alto do Cariri, Serra Negra, Quadrilátero, and Southern Mantiqueira; and (B) Nuclear Baeysian species delimitation of a guided tree (BPP). Values near nodes represent speciation probability.

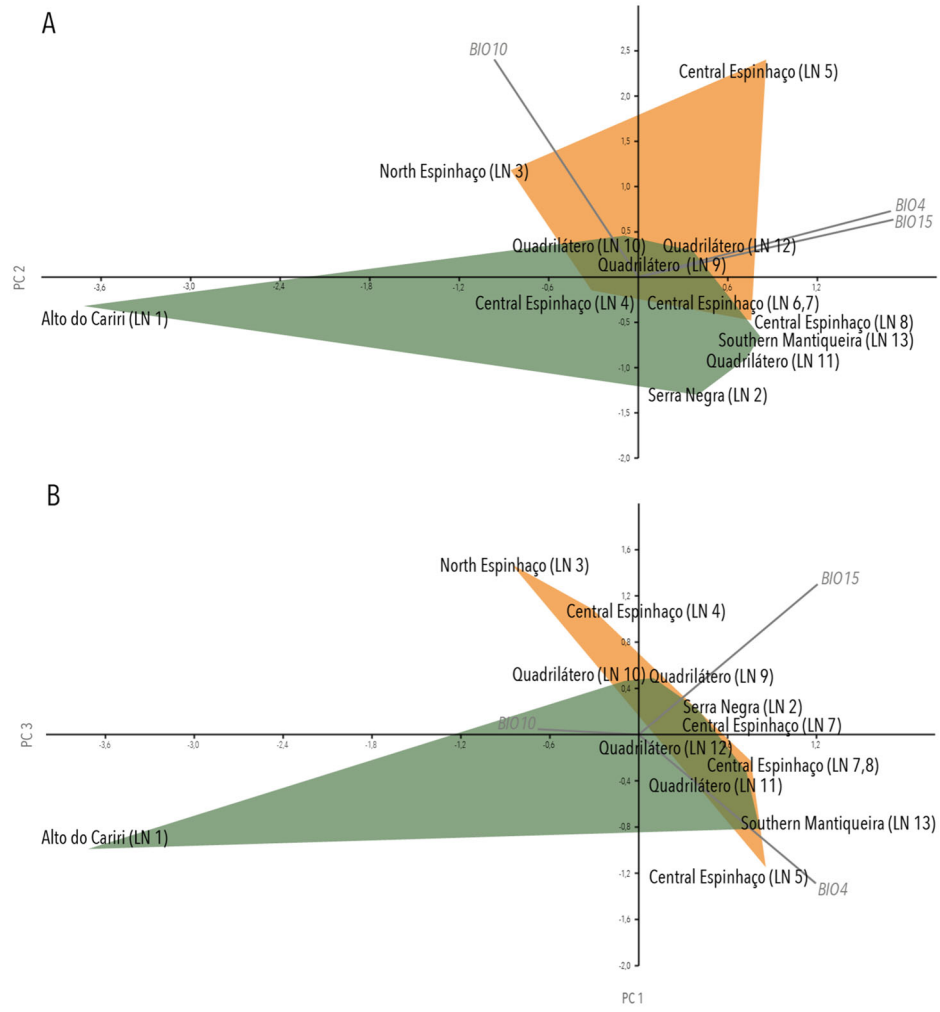

Fig. 6. Climatic differences for *Bokermannohyla nanuzae* based on 13 localities evidenced by Principal Component analysis: (a) PC1 and PC2 and (b) PC1 and PC3. Grey lines indicate the three bioclimatic variables selected from FCA analysis. Coloured polygons represent the convex hulls of the two major mitochondrial groups: Cerrado in orange (Central and North Meridional Espinhaço) and Atlantic Forest in green (Serra Negra, Alto do Cariri, Quadrilátero, and Southern Mantiqueira). BIO4 (Temperature Seasonality), BIO10 (Mean Temperature of Warmest Quarter), and BIO15 (Precipitation Seasonality).

(Carvalho et al., 2012; Walker et al., 2015) and variation caused by factors such as social context (Lima et al., 2014), until a thorough acoustic study is undertaken, vocalization does not seem to be helpful in distinguishing these species.

Regarding tadpoles, the most common labial tooth row formula (LTRF) (ca. 81% of the individuals) in *B. feioi* is 2(2)/4(1), whereas 80% of the tadpoles of *B. nanuzae* have LTRF 2(2)/5(1) (Walker et al., 2015). Pezzuti et al. (2021) found LTRF 2(2)/4(1) in ca. 85% of the analysed specimens of *B. feioi* (therein referred as *B.* aff. *nanuzae*) from the Quadrilátero. The most frequent (ca. 72% of the individuals) tail colour pattern in tadpoles of *B. feioi* is marbled with irregular dark blotches (Walker et al., 2015). Indeed, most specimens of *B. feioi* tadpoles from the Quadrilátero showed a marbled tail (Pezzuti et al., 2021), whereas 60% of the tadpoles of *B. nanuzae* are homogeneously black/brown (Walker et al., 2015). Also, in life, tadpoles of *B. feioi* have two small longitudinal golden stripes in the snout region, one on each side of the body, between eyes and snout tip (Napoli & Caramaschi, 2004; Pezzuti et al., 2021), whereas this character seems to be absent in *B. nanuzae* (Bokermann & Sazima, 1973; Walker et al., 2015).

Distribution. *Bokermannohyla feioi* occurs in mountainous regions within the interior Atlantic Forest of Minas Gerais State, Brazil. The species is known from Serra do Ibitipoca, in the municipality of Lima Duarte (type locality), in the Southern Mantiqueira; from several localities in the Quadrilátero Ferrrífero region, in the

southernmost Meridional Espinhaço; from Serra Negra, municipality of Itamarandiba, in the eastern slopes of the Meridional Espinhaço; and from Alto Cariri, in the municipality of Santa Maria do Salto, in the border of Minas Gerais and Bahia States. Altitudinal range of the species is ~840–1,400 m a.s.l.

Discussion

Species delimitation

Mitochondrial genetic distances based on the *16S* fragment were significantly high among *B. martinsi*, *B. sagarana*, *B. nanuzae*, and the resurrected *B. feioi*. Some authors have proposed a threshold based on pairwise genetic distance (barcode gap) to help distinguish candidate species, especially the cryptic ones (Fouquet et al., 2007; Garnica et al., 2016). In lowlands, the delimitation of Amazonian frogs for example, was shown to have a high validation rate using a threshold of 3% (Vacher et al., 2020). Although comparing levels of diversification across low- and highlands is not straightforward, we observed mean interspecific *p*-distance values much greater here.

Additional threshold strategies are commonly applied to refine analysis of barcode discovery and here we attempted to overcome the oversplitting of candidate species by using the split between *B. sagarana* and *B. martinsi* as a positive control and the split within *B. martinsi* as a negative control in analyses of barcode gap discovery. Considering that the significant partition with the highest number of candidate species (ASAP, $n = 18$, p-value < 0.01) showed that each of the two individuals of *B. martinsi* could represent a candidate species, we discard this result. In two partitions, all individuals of *B. feioi* from Serra Negra and Alto do Cariri were assigned as two potential candidate species (ABGD, $n = 7$ and ASAP, $n = 10$, p-value < 0.01), but showed low divergence when compared with individuals from the other AF localities Quadrilátero and Southern Mantiqueira (1.5–2.0%), that probably represents a historical substructure outcome. Together, these results lead us to not consider them as candidate species, deserving further genomic exploration.

Within *B. nanuzae*, high levels of populational substructure were also attributed to candidate species (ASAP, $n = 10$, p-value < 0.01). Therefore, the best partition found by ASAP (lower score) recovered four candidate species: *B. martinsi*, *B. sagarana*, *B. nanuzae*, and *B. feioi*, a result that was also recovered by the ABGD analysis. Although species endemic to low- and highlands are susceptible to differing evolutionary influences in time and space, ABGD's barcode gap distance of 3.1% is broadly comparable to the threshold discovered by Vacher et al. (2020).

Regarding the analyses of nuDNA fragments, most of *B. nanuzae* and *B. feioi* haplotypes were separated in the haplotype genealogies, and *B. sagarana* haplotypes were located at the centre of both genealogies (see discussion below). Bayesian species delimitation approach validated the speciation splits of the *B. sagarana*, *B. nanuzae*, and *B. feioi*. Although *B. sagarana* is a phenotypically diagnosable species restricted to Serra do Cabral (see below), the specimens of *B. nanuzae* from the Cerrado and AF groups appear to be morphologically cryptic (Walker et al., 2015). Cryptic diversity in the Espinhaço has been described in rock frogs (genus *Thoropa*; Sabbag et al., 2018), monkey frogs (genus *Pithecopus*; Ramos et al., 2019), and thin-toed frogs (genus *Leptodactylus*; Carvalho et al., 2021), demonstrating that divergent lineages with conserved morphology are abundant in this region.

Taxonomy and evolutionary history of *B. nanuzae* and *B. feioi*

We investigated the evolutionary history of *B. nanuzae* and closely related species for the first time and our phylogenetic findings showed that the species as it was previously known (Walker et al., 2015) does not constitute a monophyletic group. In fact, our BI and ML analyses recovered *B. sagarana*, a phenotypically diagnosable species, nested within *B. nanuzae sensu stricto*, implying the resurrection of the species name *B. feioi* herein. Although *B. nanuzae* and *B. feioi* seem to be phenotypically cryptic (Walker et al., 2015), *B. sagarana* differs from both species by having a dorsal colour pattern composed of irregular dark-grey blotches resembling a lichenous rock (dorsal colour uniform, spotted, or with transversal bars in *B. nanuzae* and *B. feioi*) and a double nuptial pad covered with small spicules (absent in *B. nanuzae* and *B. feioi*; Leite et al., 2011).

Monophyly is one of the most frequently used criteria for identifying species that are diverging in the evolutionary *continuum,* including the presence of barriers to gene flow (de Queiroz, 1998, 2007). Thus, in light of our molecular results, there are only two plausible scenarios. In the first one, we accept *B. nanuzae sensu* Walker et al. (2015), keeping *B. feioi* and adding *B. sagarana* under its synonymy. However, the phenotypic characters differentiating *B. sagarana* to the other two species are markedly conspicuous, making it possible to separate these species both in life and in preservation. Therefore, we regard this as the less accurate scenario, hiding diversity and masking the evolutionary

knowledge accumulated about this lineage. The second scenario is the one we considered as more accurate, with *B. nanuzae* being composed of the populations from North/Central Meridional Espinhaço (LN 3–8) and applying the resurrected name *B. feioi* to the populations from Quadrilátero (LN 9–12), Southern Mantiqueira (LN 13), Serra Negra (LN2), and Alto do Cariri (LN1). Therefore, we choose a scenario with two phenotypically cryptic species, aiming to reflect the evolutionary diversity of the clade in its taxonomy, maintaining the phenotypically conspicuous *B. sagarana* as a valid species and considering the marked molecular diversity and the historical biogeography of the clade. Moreover, keeping the three species as valid taxa will stimulate studies aiming to unveil phenotypic diversity and evolution in this clade, because morphological and acoustic studies will be now developed under this new evolutionary scheme. Additionally, focusing conservation efforts on a single species name rather than the three evolutionary independent lineages might more likely threaten a loss of genetic diversity, particularly for mountain endemic species.

The nuDNA data did not recover the mitochondrial clades, but corroborated the close relationship of *B. nanuzae, B. feioi* and *B. sagarana* by exhibiting ancestral polymorphism in the coding *CRY-B* gene between these three lineages, suggesting a pattern of incomplete lineage sorting (ILS). Likewise, the haplotype genealogy of the non-coding *DI-A6-int6* gene showed that *B. sagarana* haplotypes are also nested within haplotypes from North and Central Meridional Espinhaço. Patterns of ILS are common in Neotropical anuran amphibians with high levels of structure in mtDNA and these conflicts are often attributed to the faster rate of evolution of mtDNA than nuDNA (Sabbag et al., 2018, 2022; Thomé et al., 2012; Santos et al., 2020). For instance, ancestral polymorphism between morphological and cryptic species uncovered by mtDNA was found in two of the five studied nuclear markers in phyllomedusid treefrogs of the *Pithecopus rohdei* (Mertens, 1926) complex (from the AF), and *Pithecopus megacephalus* (Miranda-Ribeiro, 1926), from the *campo rupestre* of Espinhaço (Ramos et al., 2019). Introgressive hybridization in divergent lineages may also be a cause of mitonuclear discordance (e.g., Magalhães et al., 2021). However, we found no evidence of gene flow between species/groups in either the mitochondrial or nuclear loci.

Interestingly, the geographic ranges of *B. nanuzae* and *B. feioi* showed a striking pattern, with both species roughly distributed along a north-south latitudinal gradient, without the presence of any obvious geographic barrier. The boundaries between the geographic ranges of both species are somewhat coincident with the ecotonal area between the Cerrado and AF domains (Miola et al., 2021), suggesting that the distribution of species and domains might be related. Considering this north-south latitudinal gradient, the two species also seem to have diverged from each other without the influence of geographic distance, as would be expected in an IBD scenario (Slatkin, 1987, 1993).

Pleistocene climatic fluctuations are one of the likely explanations of relatively high speciation rates in Neotropical mountains (Rull, 2006). Here we found high levels of nucleotide diversity within both species in the *16S* gene (maximum 1.5% and 1.3% to *B. nanuzae* and *B. feioi*, respectively) and no evidence of secondary contact and recent expansion that could be attributed to the population contraction and expansion expected to have occurred during the Pleistocene climatic cycles. However, for *B. feioi*, the external position of the unique nuclear haplotype of Serra Negra and Alto Cariri (*CRY-B*), suggests that these marginal areas could be the result of recent expansion events. This result was also detected in a population of *B. saxicola* from Serra Negra (Nascimento et al., 2018). Population expansion in another *Bokermannohyla* species, *B. alvarengai*, was inferred to have occurred throughout the last glacial maximum (Oliveira et al., 2021).

It is also important to consider the Espinhaço and Mantiqueira old geology and landscape mosaic when discussing speciation. During the Pleistocene oscillations, a scenario of more stable climatic conditions in the highlands favoured speciation due to long-term fragmentation and limited hybridization (Hopper, 2009; Hopper et al., 2016), rather than a pattern of cycles of fragmentation and expansion. In particular, *B. nanuzae* exhibited high levels of substructure between localities (populations) associated with isolation on sky islands and no signs of interspecific gene flow. Mechanisms such as heterozygosity retention and significant post-mating barriers would avoid the deleterious effects of inbreeding, as predicted by the "Old, Climatically Buffered, Infertile Landscapes" (OCBIL) theory (Hopper, 2018). Furthermore, *B. nanuzae* and *B. feioi* speciation could be the result of niche differentiation along environmental gradients acting silently as a barrier and decreasing the gene flow (e.g., Cerrado and Caatinga clades in Werneck et al. (2012)).

The climatic differences between sampled localities of *B. nanuzae* and *B. feioi* in multivariate space revealed that each group has its own distinct climate envelope with an overlapped area. Some localities in the AF and Cerrado groups appear to be associated with particular temperature seasonality. The climatic differences of localities within the AF group appear to be shaped by

rainfall, while high temperatures appear to be more related with the Cerrado group. Climate data supported the separation trend in both groups/species, not only between distant but also between neighbouring localities, as revealed by the mitochondrial phylogeny. For instance, Serra Negra (LN2) and Serra do Ambrósio (LN5), represented by *B. feioi* and *B. nanuzae*, respectively, are two geographically adjacent localities included in the AF limits but clearly separated by PC2, indicating that they may represent different climatic niches. These findings support a niche differentiation between *B. nanuzae* and *B. feioi*. At the same time, some intermediate localities (e.g., LN 2 and 5) are compatible with areas where hybridization might be possible.

The combination of molecular and climatic data provided the first indications of the likely processes that resulted in the speciation of two divergent evolutionary lineages throughout the Plio-Pleistocene, *B. nanuzae* and *B. feioi*. We here lack data to assess the role of *B. sagarana* in the diversification of these species and estimates of its diversification time should be interpreted with caution, considering the possibility of population subdivision and therefore longer coalescence times being detected with larger sampling (e.g., Edwards & Beerli 2000). Also, temporal diversification based on organelle loci can inflate the coalescent time, as recently showed for *B. saxicola* (Oswald et al., 2022). Future research could focus on sampling efforts and the use of faster-evolving nuclear markers such as microsatellites or SNPs to test hypothesis of species diversification combined with niche modelling analysis and prediction of potential hybridization areas and the presence of gene flow between species. Finally, thermal physiology experiments will greatly add to our climatic niche comparison, because examining the range of thermal preferences and tolerances of species will make the potential eco-physiological differences between *B. nanuzae* and *B. feioi* to adapt to distinct biomes more accurate.

Supplemental material

Supplemental material for this article can be accessed here: https://doi.org/10.1080/14772000.2022.2156001.

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