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Original Article

Hydrographic basins dictate the genetic structure of the paradoxical frog *Pseudis bolbodactyla* (Anura: Hylidae) in the rivers of Central Brazil

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ABSTRACT

Rivers are prominent landscape features, acting as key promoters of diversification among freshwater organisms. Albeit generally considered potential barriers to species movement, they may also facilitate gene flow and structure populations of semiaquatic species (Riverine Thruway Hypothesis, RTH). We evaluated the role of rivers on the processes responsible for current genetic variation in the semiaquatic frog *Pseudis bolbodactyla*, testing whether each hydrographic basin harbours distinct genetic lineages. We sequenced three markers on 166 samples from 13 localities along the Paraná (PR), Araguaia–Tocantins (AT), and São Francisco (SF) River basins in Brazil. We recovered three populations geographically matching each hydrographic basin. Our results indicate migration among basins, with the best model selected using approximate Bayesian computation, including migration between AT and SF and ancient gene flow from PR to the AT–SF ancestor. Our findings are likely related to the orogenic events in Central Brazil dating to the Late Miocene (5 Mya), when hydrographic basins and the geomorphological features of the Brazilian Shield were formed. This suggests that *P. bolbodactyla* probably represents a species complex, with each lineage occurring in a distinct hydrographic basin, matching the predictions of the RTH.

Keywords: hydrographic basins; Cerrado biome; diversification; population genetics

INTRODUCTION

Rivers are one of the most prominent landscape features, acting as key promoters of diversification among aquatic (Oliveira *et al.* 2019), semiaquatic (Roberto *et al.* 2020), flying (Hayes and Sewlal 2004), and terrestrial organisms in the Neotropics (Coelho *et al.* 2022). After the first realization that primate ranges in the Amazon are delimited by major rivers (Wallace 1852), many studies have evaluated the Riverine Barrier Hypothesis (RBH) using distributional and genetic data of terrestrial, semiaquatic, and flying organisms in the Neotropics and elsewhere (Kopuchian *et al.* 2020, Coelho *et al.* 2022, Janiak *et al.* 2022). Rivers also represent the boundaries of areas of endemism and species turnover in Amazonia and the Atlantic Forest (Silva *et al.* 2005, Carnaval *et al.* 2014), while isolating parts of the Cerrado and compartmentalizing the landscape into highlands and lowlands (Brasil and Alvarenga 1989). Along with the watercourse features (e.g. size, volume, and flow direction), the ecological and natural history idiosyncrasies of the taxa in question also help define effects of rivers on population structure.

Accordingly, the rise of intraspecific phylogeography saw the RBH as one of the first and most directly testable mechanisms to be examined using mitochondrial DNA (mtDNA) sequences (Gascon *et al.* 1998, 2000, Lougheed *et al.* 1999). Indeed, rivers have been shown to drive the differentiation of aquatic or

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semiaquatic fauna in taxa such as caimans (Muniz *et al.* 2017, Roberto *et al.* 2020), snakes (Brandley *et al.* 2010, Ukuwela *et al.* 2013), fish (Hubert *et al.* 2007, Santos *et al.* 2009, Dubut *et al.* 2012), insects (Price *et al.* 2010, Metcalfe *et al.* 2020), anurans (Fonseca *et al.* 2021), and birds (Choueri *et al.* 2017, Thom *et al.* 2020). Although rivers have been frequently shown to act as hard and soft barriers to gene flow (Pyron and Burbrink 2010), they have also presented no effect on the distribution of genetic diversity in other cases (Fluck *et al.* 2020). The degree of genetic structure on opposing river banks has been shown to vary throughout the course of rivers, with middle and lower portions of the huge Amazonian rivers, for example, impeding migration more across margins than upstream regions (Reis *et al.* 2019).

Besides acting as barriers for species and populations across opposing margins, river basins have been shown to facilitate gene flow in semiaquatic and water-dependent species (Lawson 2013, Fonseca *et al.* 2021). At the landscape level, hydrological drainages were shown to function as corridors of connectivity among populations within basins (Lawson 2013, Choueri *et al.* 2017), with migration across populations preferably occurring downstream within each river basin (Thom *et al.* 2020, Fonseca *et al.* 2021). This alternative role of rivers was recently named the Riverine Thruway Hypothesis (RTH), which posits that rivers should act as facilitators of gene flow in species associated with aquatic environments (Fonseca *et al.* 2021), which include fish (Hubert *et al.* 2007) and even birds (Thom *et al.* 2020).

Still, most evolutionary studies on anurans in the Neotropical region have focused on rivers as vicariant barriers (Kaefer *et al.* 2012, Moraes *et al.* 2016, Maia *et al.* 2017, Godinho and da Silva 2018). Furthermore, few studies have examined aquatic or semiaquatic anurans, even though some of these species are strongly associated with rivers and floodplains (e.g. Pseudae, *Lithobates* spp., *Pipa* spp.: Trueb and Cannatella 1986, Hillis and Wilcox 2005, Garda *et al.* 2010). Thus, such semiaquatic species with strong ecological bounds associated with floodplains are expected to follow river histories and be restricted to specific basins.

The South American paradoxical frogs (Hylidae: Pseudinae) are morphologically adapted to live in river floodplains (Aguiar *et al.* 2007, Garda and Cannatella 2007, Garda *et al.* 2010). Their large larvae (up to 270 mm in total length, Santana *et al.* 2016), shrink up to five times their length during metamorphosis into an ordinary-sized adult frog (32.3–52.9 mm snout–vent length, Garda *et al.* 2010). Such habits and geographical distribution make these frogs ideal organisms to test hypotheses of several evolutionary patterns, including biogeographical hypotheses regarding speciation of semiaquatic fauna. Gallardo (1961), for example, suggested a 'one basin–one species' hypothesis for the group, a pattern usually found in strictly aquatic organisms. Recently, a landscape genetics study with *Pseudis tocantins* Caramaschi and Cruz, 1998, which is distributed throughout the floodplains of long and wide rivers in central Brazil, showed that gene flow occurs primarily within basins (Fonseca *et al.* 2021). Among the seven species of the genus, *Pseudis bolbodactyla* Lutz, 1925 occurs in the floodplains of the middle São Francisco River, in the upper Tocantins River, which is part of the upper Araguaia–Tocantins basin , and along the Paranaíba River, which is part of the upper Paraná basin (Garda *et al.* 2010), all of which are watersheds restricted to the Brazilian Shield.

Herein, we test the RTH (i.e. rivers as facilitators to gene flow) and the 'one basin–one species' hypothesis using *P. bolbodactyla* as an organism model. If both hypotheses are corroborated, we expect to see each basin harbouring a distinct lineage with little to no gene flow among basins. We also explore phylogeographical patterns and the underlying processes that drove the current levels of genetic variation within *P. bolbodactyla*. To achieve these goals, we used phylogeographic and phylogenetic analyses to perform population assignment tests, estimate evolutionary relationships and historical demographies, and reconstruct evolutionary history using a Bayesian-based method and testing competing evolutionary models using approximate Bayesian computation (ABC).

MATERIAL AND METHODS

Sample collection and sequencing

We collected 166 tissue samples of *P. bolbodactyla* from 13 localities (Fig. 1; Supporting Information, Table S1; collecting permits #132/2005—CGFAU/LIC and SISBIO 26157). We used the sister species *Pseudis paradoxa* (KP149190) as the outgroup to *P. bolbodactyla* (Garda and Cannatella 2007). Voucher specimens from our field trips are housed in Coleção Herpetológica da Universidade de Brasília, Brasília, Brazil (CHUNB; Appendix 1). Whole genomic DNA was taken from muscle or liver tissues using the Qiagen DNeasy Blood and Tissue Kit (Qiagen). We followed the standard protocol of polymerase chain reaction (PCR) for DNA amplification for one mitochondrial marker [cytochrome *c* oxidase subunit 1 (*COI*)] and two nuclear markers [recombination activating gene 1 (*RAG-1*) and proopiomelanocortin (*POMC*)]. Information on primers used in this study is presented in Table S2. PCR products were cleaned using Exo-Sap-IT (USB Corp.) and sequenced in both directions on a Beckman-Coulter CEQ-8000 automated sequencer. We performed all sequence alignments in Geneious® v.9.1.8 using the MUSCLE alignment algorithm. We used PHASE v.2.1.1 (Stephens and Donnelly 2003) to determine the most probable pair of alleles for both nuclear loci. We ran PHASE with default parameters for 100 iterations, a thinning interval of 1, and a burn-in of 100. To check for consistency between runs, we repeated each PHASE analysis five times. The most appropriate model of nucleotide substitution for the alignments was determined with jModeltest (Darriba *et al.* 2012) using the Bayesian Information Criterion (BIC).

Population assignment and genetic diversity

We performed the Bayesian clustering method implemented in the R package Geneland v.4.0.4 (Guillot *et al.* 2005) to detect geographical discontinuities in both mitochondrial and nuclear loci. This method considers the spatial coordinates of each individual sampled and distributes them into *K* clusters by minimizing deviations from Hardy–Weinberg equilibrium and gametic phase disequilibrium within the groups (Guillot *et al.* 2005). We used the uncorrelated allele frequency model and evaluated support for 1–5 populations with 100 iterations and a burn-in of 1000. Every 100th observation was sampled to reduce autocorrelation. To estimate *K*, we simulated a fixed value of *K* using the above parameters to determine population membership and generate posterior probability maps.

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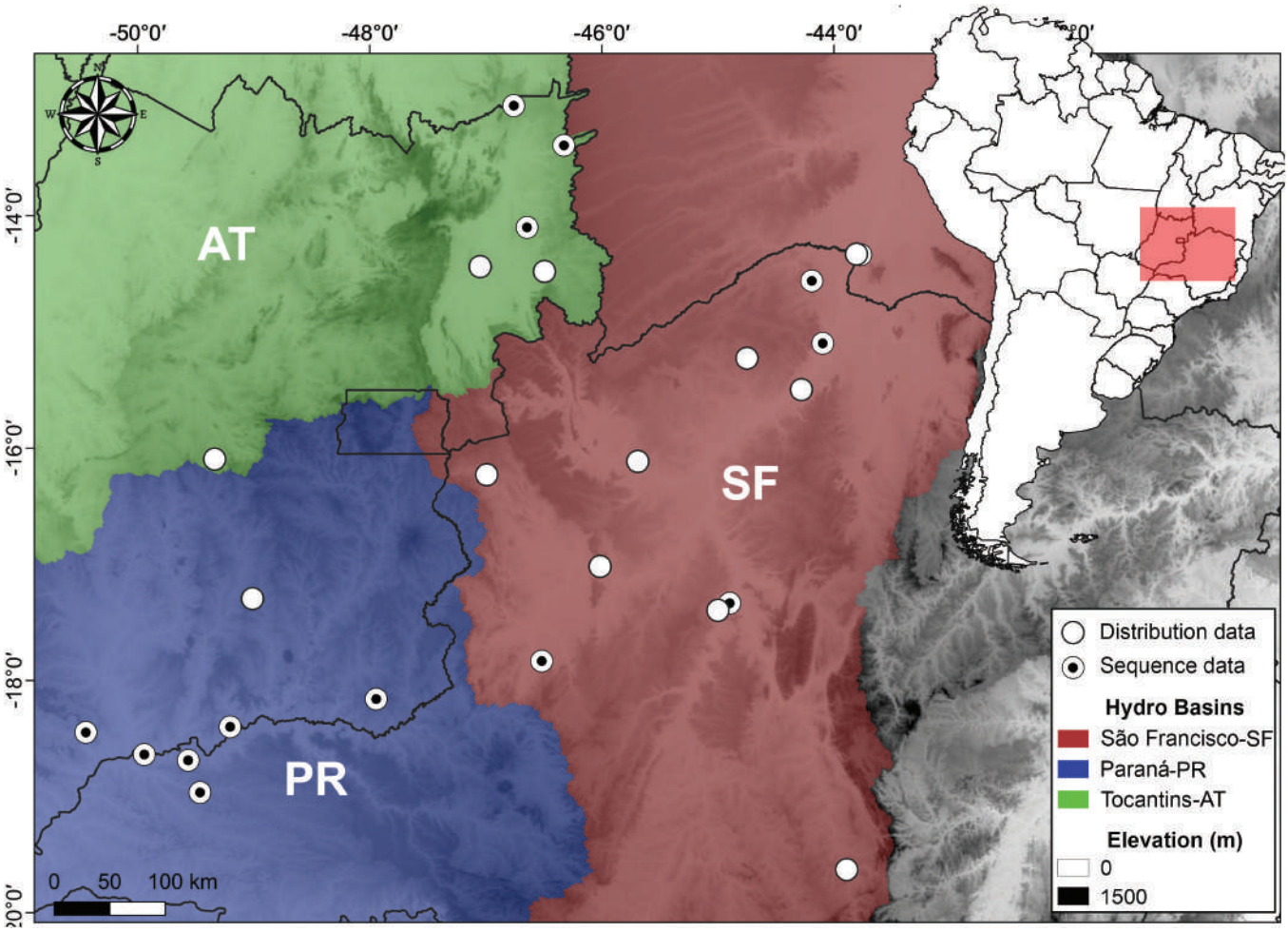

Figure 1. The geographical distribution of *Pseudis bolbodactyla* in central Brazil is shown by white circles and all genetic samples used in this study are illustrated by dotted circles. Black lines are state limits.

Based on the lineages delimited according to the method above, haplotype diversity (Hd) and nucleotide diversity (π) were calculated for each group using DNAsp v.5 (Librado and Rozas 2009). We also performed an analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) partitioning our samples into hierarchical components among lineages to assess how much genetic variation was present within and between lineages. Population substructure was quantified with the fixation index F_{ST} in the program Arlequin (Librado and Rozas 2009) after 10 000 iterations. Finally, we explored the relationships among haplotypes of each locus in POPART (Leigh *et al.* 2015) through the median-joining network method. We identified each lineage using different colours in the haplotype network. We also calculated sequence divergence (uncorrected p-distance) among lineages using MEGA X (Kumar *et al.* 2018) to assess the degree of genetic divergence.

We estimated a Bayesian gene tree for the mtDNA loci (*COI*) using BEAST v.2.6 (Bouckaert *et al.* 2019). We used a Yule speciation prior, implemented a strict clock rate of 0.00957 per Myr (Crawford 2003), and ran the analysis for 50 million generations sampling every 1000 generations. We used Tracer v.1.7.1 (Rambaut *et al.* 2018) to assess effective sample sizes (ESS) of estimated parameters and stationarity, ensuring that all ESS values of all parameters were above 200 (Rambaut *et al.* 2018).

Dated molecular phylogeny

The three lineages found in Geneland (hereafter referred to as AT, SF, and PR; see Results below) were used as terminal taxa for the species tree estimation in *BEAST (Heled and Drummond 2010) using *COI*, *POMC*, and *RAG-1* unliked with no partitions, and an evolutionary rate for *COI* in BEAST v.2.6.3. To infer the timing of lineages divergence we used the mtDNA *COI* mutation rate of 0.957% per lineage per Myr (Crawford 2003). We calibrated the species tree using this mutation rate because there are no fossils available for calibration. We ran the analysis with 200 million generations, sampling every 10 000 generations. Stationarity was determined by visually inspecting trace plots and ensuring that all ESS values were above 200 in Tracer v.1.7.1. The first 10% of sampled genealogies were discarded as burn-in, and the most credible clade was inferred with TreeAnnotator v.2.6.3 (Bouckaert *et al.* 2019). We used DENSITREE (Bouckaert 2010) to produce a tree cloud from sampled trees.

Historical demography and diversification scenarios

We evaluated past changes in the effective population size (*Ne*) of each lineage using the Bayesian Skyline Plot (BSP) method implemented in BEAST v.2.6.3. For the BSP we used an average substitution rate for vertebrate mtDNA of 0.957% per lineage per Myr (Crawford 2003) and the same substitution model for

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COI. Each analysis of BSP was evaluated with a linear substitution model, five groups, 50 million generations, and 10% burn-in. We checked for stationarity by visually inspecting trace plots and ensuring that all values for ESS were above 200 in Tracer v.1.7.1. We took the values from *Ne* over time from Tracer v.1.7.1 and built the BSP curve in Microsoft Excel.

We used ABC to simulate and calculate the posterior probability of competing diversification scenarios. We specified a total of five scenarios with stable population sizes (Fig. 2): (i) no migration among lineages; (ii) gene flow from PR to the ancestor of lineages AT and SF; (iii) gene flow between lineages AT and SF; (iv) gene flow from PR to the ancestor of lineages AT and SF, and gene flow from SF to AT; and (v) gene flow from PR to the ancestor of lineages AT and SF, and gene flow between lineages AT and SF. We constructed these models based on the results of previous analyses and on the biogeographical history of the Paraná Basin. We built diversification scenarios that mirrored our empirical datasets given the number of genetic markers, marker inheritance, number of individuals per marker and lineage, and sequence length. All priors were sampled from a uniform distribution and the value of each prior is available in Supporting Information Table S3. We used the results recovered by *BEAST analysis to set the evolutionary relationships among lineages and both divergence time priors (T_1 and T_2). Then, we used the R package PipeMaster (Gehara *et al.* 2020) for genetic simulations. We performed 100 000 simulations under each demographic scenario and calculated a total of 26 genetic summary statistics from each individual simulation: number of segregating sites (S), nucleotide diversity (π), haplotypic diversity, Tajima's D, Fu and Li's D and F statistics, and fixation index (F_{ST}). Summary statistics were calculated combining all lineages and for each lineage. The only exception was F_{ST}, which was calculated considering only individual lineages. Next, we used the function *postpr* implemented in the R package *abc* (Csilléry *et al.* 2010) to calculate the posterior probability of each diversification scenario given the empirical dataset. To compare our demographic scenarios, we used rejection and mnlogistic methods implemented in the *abc* R package with tolerances of 0.001 and 0.01, respectively. We evaluated the performance of our model by generating a confusion matrix using the *cv4abc* function in the *abc* R package using the mnlogistic method with a tolerance of 0.01. We also used a principal components analysis (PCA) to check if simulated datasets produced summary statistics similar to our observed dataset. Finally, we estimated the posterior probability of divergence times and migration rates under the best model.

RESULTS

Population assignment, genetic diversity and species tree

We successfully amplified gene fragments from 154 samples. The mtDNA gene tree topology recovered three lineages corresponding to the three hydrographic basins: Paraná (PR), Araguaia–Tocantins (AT), and São Francisco (SF) River basins. Overall, these lineages were inferred with strong support [posterior probability (pp) > 0.99; Fig. 3]. However, two individuals from the São Francisco basin (CHUNB42877 and CHUNB42885) were more closely related to individuals from the Araguaia–Tocantins basin. Using all three loci, the Geneland

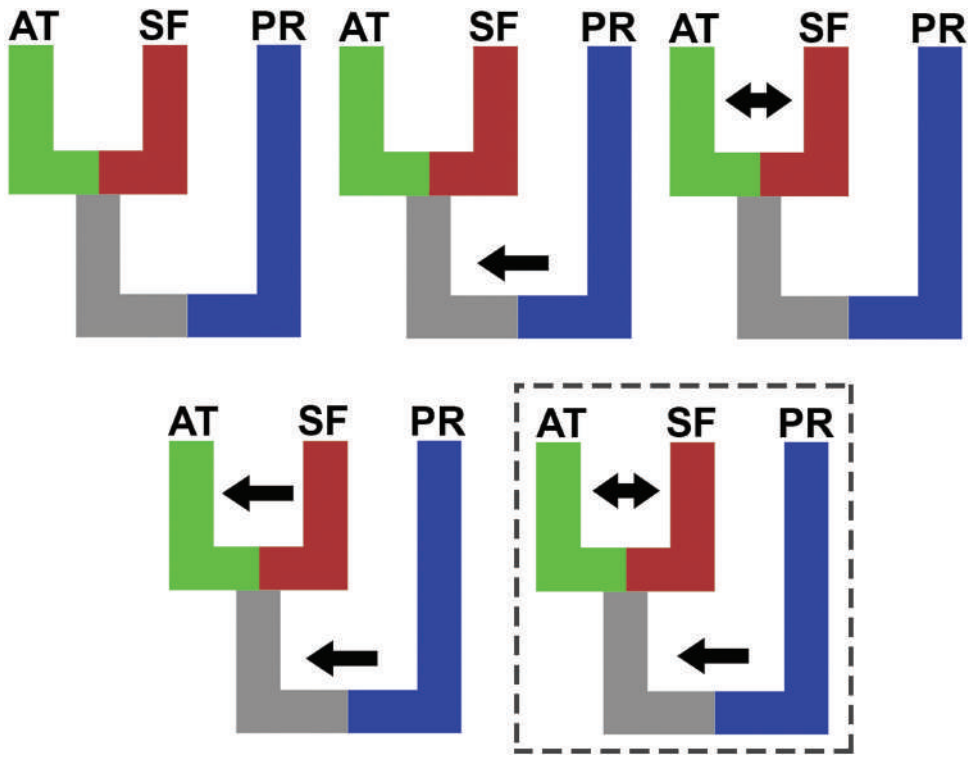

Figure 2. Schematic representation of the six models tested using Approximate Bayesian Computation. A–E represent distinct diversification scenarios of divergence among *Pseudis bolbodactyla* populations (AT, SF, and PR). The direction of the arrows indicates migration direction. The best-supported model is marked by a dashed line box.

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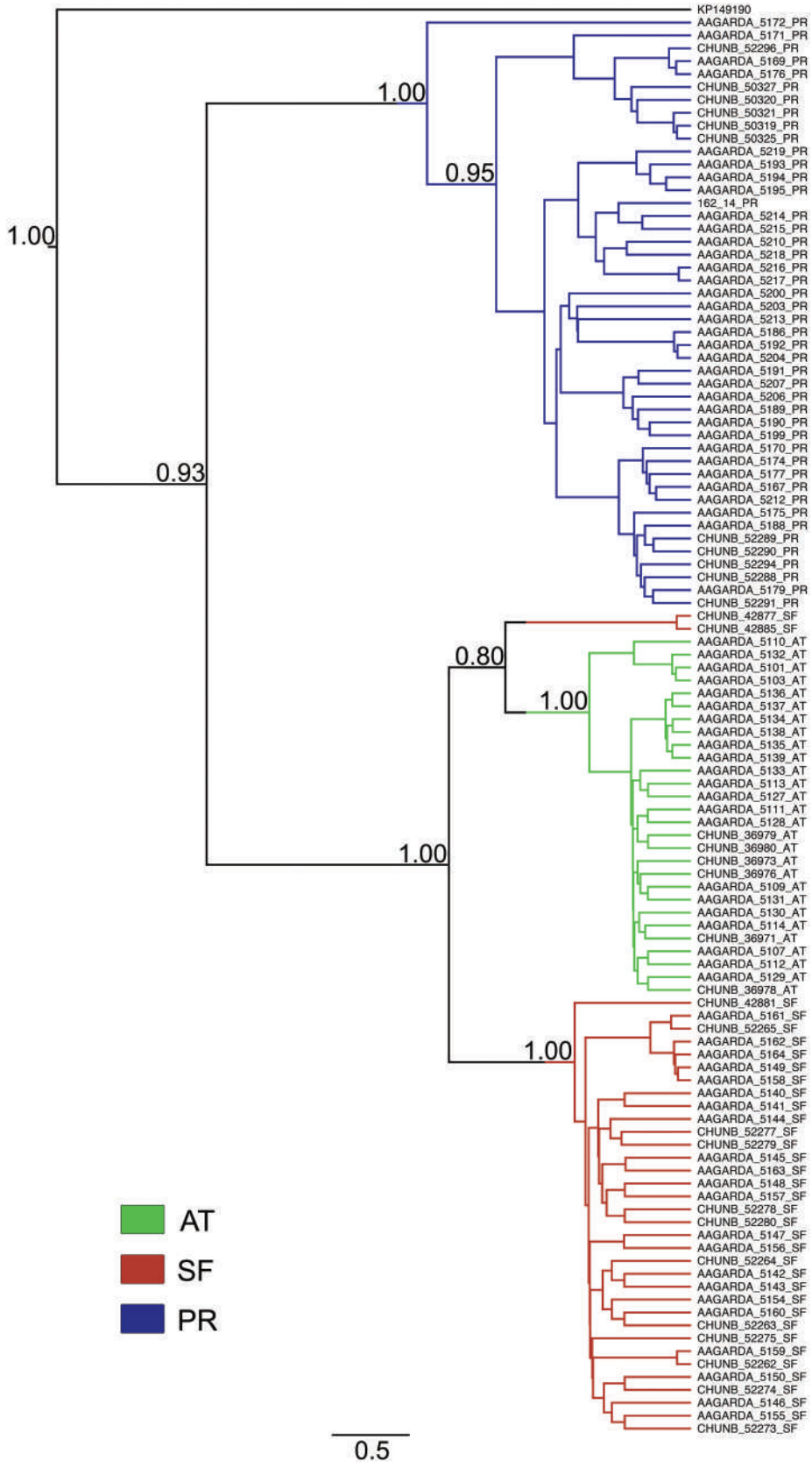

Figure 3. *COI* mitochondrial gene tree inferred from a Bayesian inference analysis in BEAST. All clades are coloured according to the major evolutionary lineages (hydrographic basins). Values on nodes indicate posterior probabilities. Scale bar indicates the rate of base substitutions per site.

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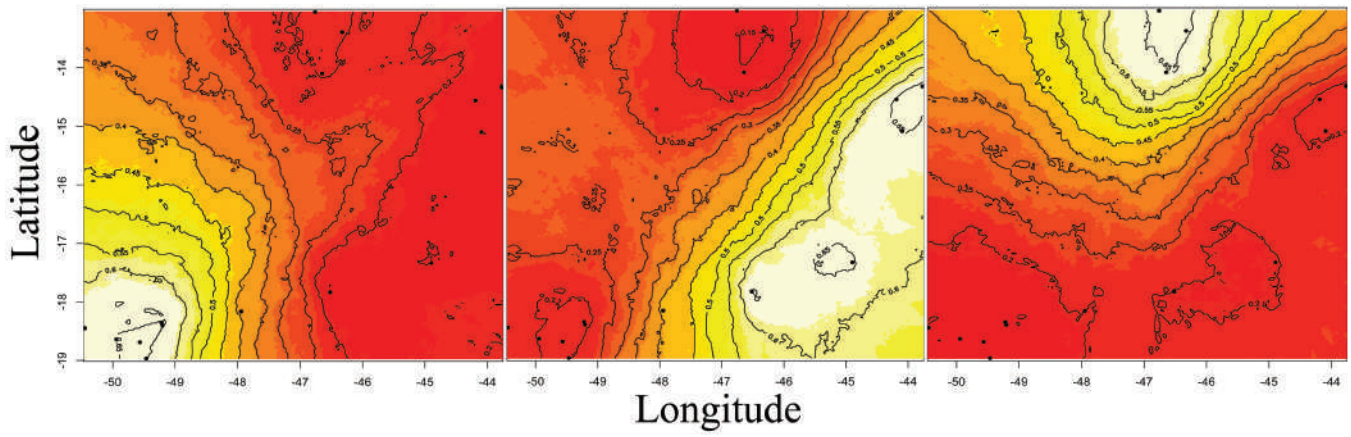

Figure 4. Geneland analysis of *Pseudis bolbodactyla* with posterior probability isoclines, indicating extensions of the genetic populations found (black lines with inclusion probabilities): letf Paraná, center São Francisco, and right Araguaia-Tocantins. Light colour zones in maps indicate the groups of localities with greater probabilities of belonging to the same genetic unit. Black dots indicate locations of the 13 analysed localities.

Table 1. Average uncorrected (p-distance) sequence divergence separating the different *Pseudis bolbodactyla* lineages.

	AT	SF	PR
AT	**0.004**		
SF	0.033	**0.005**	
PR	0.077	0.066	**0.016**

Data in bold type are the intraspecific divergences. AT = Araguaia–Tocantins, SF = São Francisco, PR = Paraná.

analysis detected the same three lineages ($K = 3$; Fig. 4), revealing a clear geographical population structure matching hydrographic basins. Genetic distances among populations ranged from 3.3% to 7.7% (Table 1).

The mtDNA *COI* haplotype network revealed high haplotype diversity, with no shared haplotypes among lineages (Fig. 5). The SF and PR lineages present one dominant haplotype each in the *POMC* haplotype network, while the PR lineage is more diverse. The *RAG-1* haplotype network has one central and most frequent haplotype, which is shared among lineages. According to AMOVA (Table 2), most genetic variation in *COI* occurs among lineages ($F_{ST} = 0.82$; $P < 0.00001$). The nuclear genes presented contrasting results. We found a high index of fixation for *POMC* ($F_{ST} = 0.76033$; $P < 0.00001$) and a low index for *RAG-1* ($F_{ST} = 0.03394$; $P = 0.03089$).

The species tree reconstructed in *BEAST showed high support for AT, SF, and PR lineages ($P = 1$) and estimated the divergence time between the PR lineage and SF–AT around 3.08 Mya [95% highest posterior density (HPD) = 1.56–4.63], during the Plio-Pleistocene (Fig. 6). *BEAST estimated a more recent separation between SF and AT dated to 1.21 Mya (95% HPD = 0.53–1.95), during the Pleistocene.

Historical demography, migration, and diversification scenarios

The BSP for *COI* showed no evidence of demographic fluctuations for the SF and AT lineages, while PR showed a moderate expansion over the past 200,000 years, albeit within the 95% confidence interval (10× expansion around 0.4–0.2 Mya, Fig. 7). The AT and SF lineages have small effective sample sizes (*Ne*) in comparison with PR, which is almost 10 times larger.

The ABC analysis recovered model 5 (Fig. 2; Table 3) as the best diversification scenario, with a probability of 0.86 and 0.99 for rejection and mnlogistic methods, respectively (Table 3). This model predicts a past migration from PR to the ancestor of AT–SF, and current bidirectional gene flow between AT and SF (Fig. 2). A rate of one migrant per generation was inferred for both gene flow from PR to the ancestor of AT–SF (95% HPD: 0.02–1.98) and for the bidirectional migration between AT and SF (95% HPD: 0.09–1.96). ABC analysis had an overall accuracy of 72%, with the best model reaching an accuracy of 92% (Supporting Information, Fig. S1). Our simulated datasets produced summary statistics that were in the range observed in our dataset (Fig. S2).

DISCUSSION

Pseudis bolbodactyla is composed of three deeply diverging lineages (AT, SF, and PR) associated with three unique hydrographic basins. We detected little current gene flow between the AT and SF lineages and high genetic differentiation among hydrographic basins (except for *RAG-1*). This matches the predictions of the RTH and the 'one basin–one species' hypothesis, in which populations or species associated with aquatic environments are genetically structured by hydrographic basins. While rivers facilitate within-basin migration (RTH), populations become isolated in each basin (one basin–one species hypothesis). Both mechanisms therefore simultaneously promote diversification among but intensify gene flow within basins, hence accounting for the pattern observed. This pattern was also found for *P. tocantins* in the Tocantins–Araguaia River system (Fonseca *et al.* 2021). These two rivers run in parallel northward for hundreds of kilometres before merging, and although separated only by moderately high mountains (up to 500 m), gene flow occurs within the basin and primarily downstream (Fonseca *et al.* 2021). While seemingly intuitive, the role of rivers in structuring populations and favouring gene flow is rarely tested explicitly

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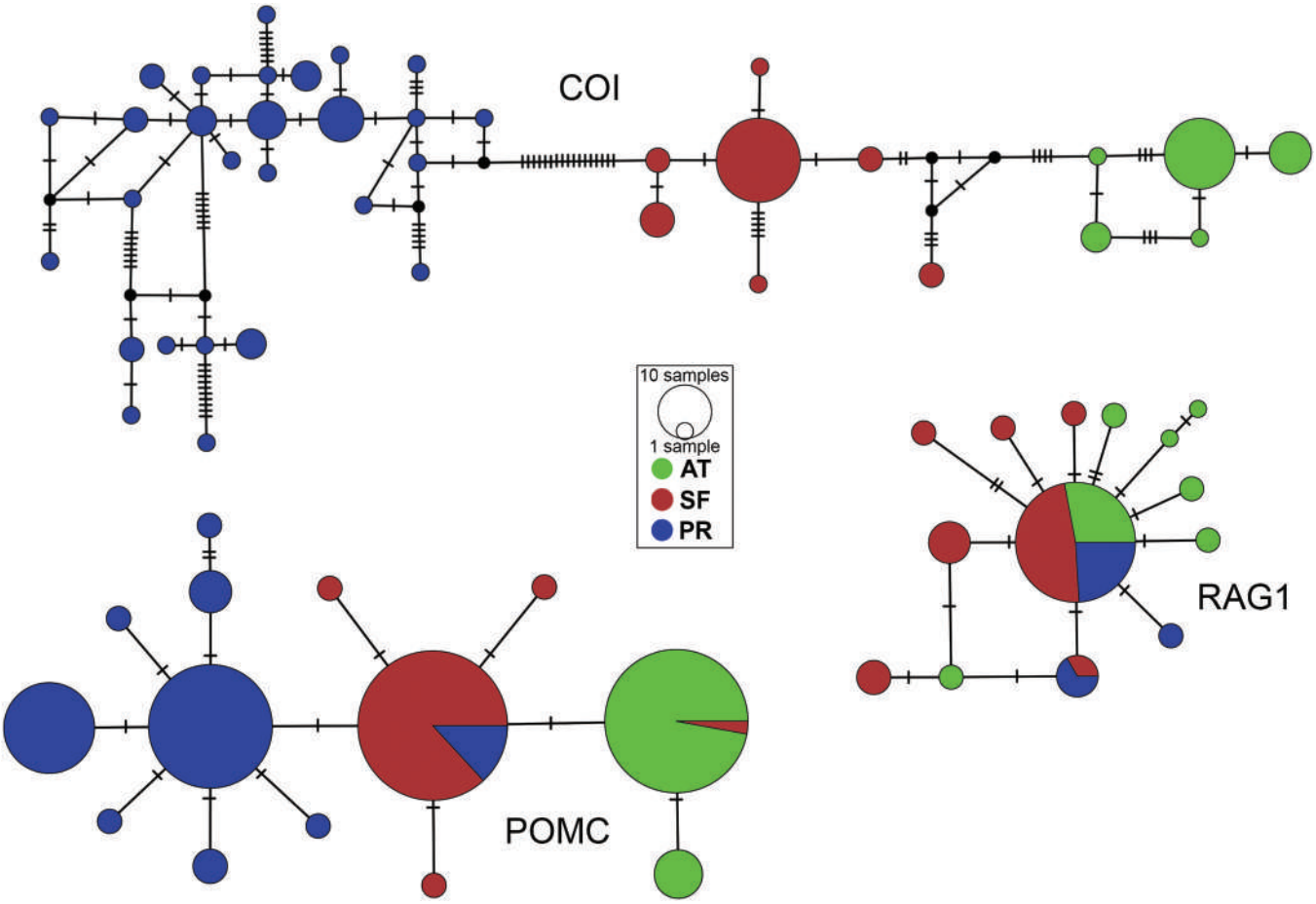

Figure 5. Haplotype networks from neighbour-joining analysis for the three genes (*COI*, *POMC*, and *RAG-1*) of *Pseudis bolbodactyla*. Colours represent populations. Circle sizes are proportional to haplotype frequencies.

Table 2. Percentage of variance explained in the analyses of molecular variance (AMOVAs) between and within populations for *COI*, *POMC*, and *RAG-1* considering three lineages of *Pseudis bolbodactyla*.

Source of variation	*COI*	*POMC*	*RAG-1*
Between populations	82.26%	76.03%	3.39%
Within populations	17.74%	23.97%	96.61%
F_{ST}	0.82256	0.80633	0.03394
P value	<0.00001	<0.00001	0.03089
Interpopulation DF	2	2	2
Intrapopulation DF	109	221	81
Interpopulation SQ	523.82	89.72	2.02
Intrapopulation SQ	168.06	42.71	42.68
Interpopulation VC	7.15	0.61	0.02
Intrapopulation VC	1.54	0.19	0.53

DF = degrees of freedom; SQ = sum of squares; PV = percentage of variation; VC = variance components.

for semiaquatic and terrestrial animals, with the more common finding showing rivers as barriers (Fonseca *et al.* 2021).

The lineages of *P. bolbodactyla* diverged during the Plio-Pleistocene (PR and AT–SF) and Pleistocene (AT and SF), periods marked by orogenic events in central Brazil that led to the present configuration of hydrographic basins (Lundberg *et al.* 1998). Pleistocene climatic fluctuations may have also influenced divergence, but in different ways for each basin. The Araguaia–Tocantins basin attained is current configuration during the end of the Pliocene and throughout the Pleistocene (Brasil and Alvarenga 1989, Del'Arco and Bezerra 1989), allowing the divergence of AT and SF from the shared ancestor with PR under unidirectional migration from PR.

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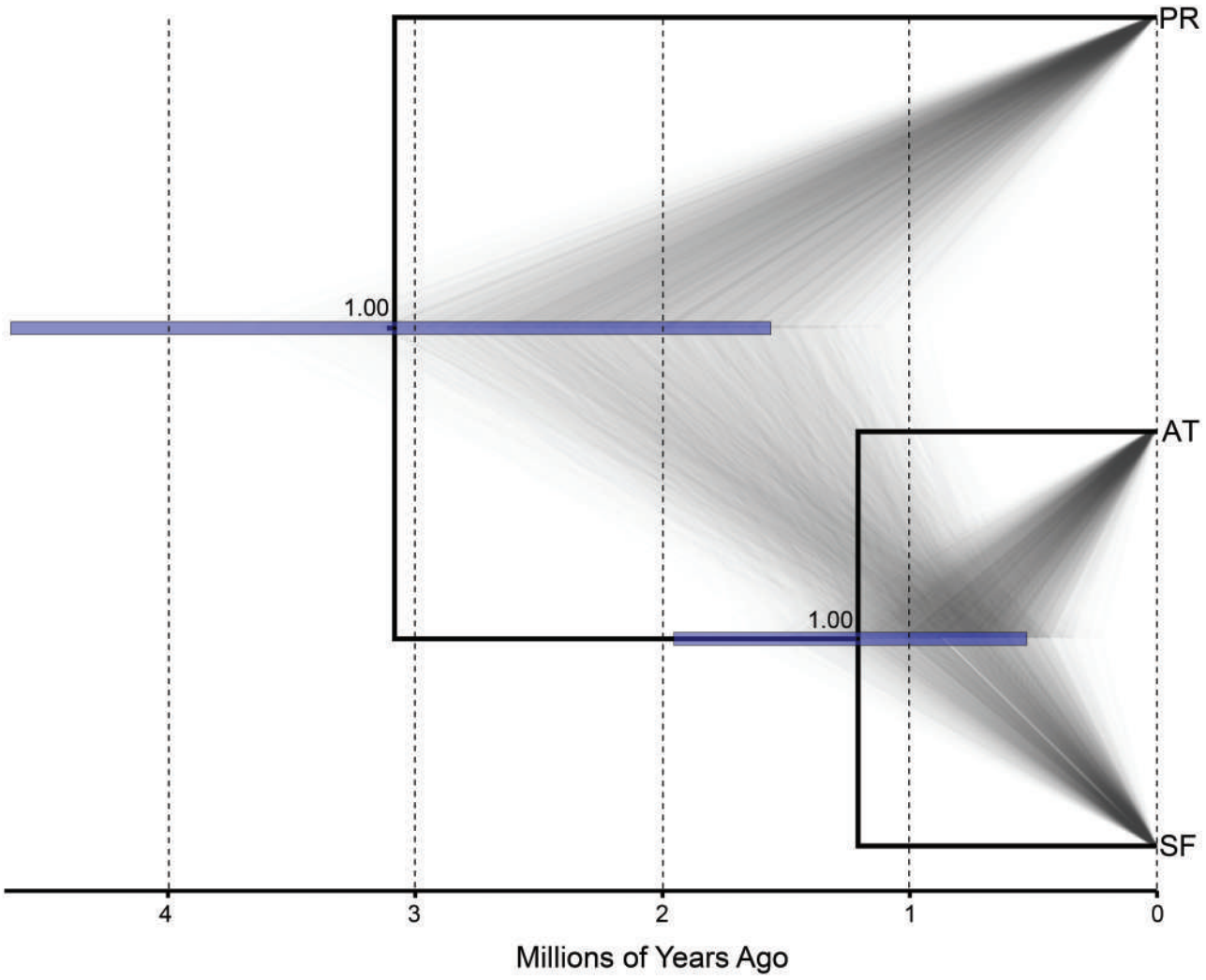

Figure 6. Time-calibrated species tree estimated using *BEAST and populations found in Geneland. Numbers indicate node probabilities. On the background, a tree cloud produced using DENSITREE of sampled trees.

Confinement to and migration among hydrographic basins in central Brazil were probably influenced by the uplift of the central Brazilian plateau and related events of headwater capture (Aquino and Colli 2017). For example, AT has a lower baseline compared to the PR and SF basins, making it more prone to capture headwaters of neighbouring river basins (Saadi 1993, Aquino and Colli 2017). Indeed, the fish fauna in PR is older and more structured, probably having colonized the upper SF and AT basins in headwater capture events (Aquino and Colli 2017). Our PR lineage is also older, and we detected ancient unidirectional migration from PR to the ancestor of AT–SF (model 5; Fig. 2), which suggests that headwater capture events must have played similar roles in the current pattern of genetic diversity and distribution in *P. bolbodactyla*. Our AT and SF lineages diverged more recently with low bidirectional migration until the present day (one migrant per generation; see ABC results) but remained limited to the upper Tocantins River and São Francisco Rivers, respectively. This restricted distribution of AT and SF populations has two possible explanations. First, the middle and lower parts of the São Francisco River run across the Brazilian semiarid Caatinga region, which could limit dispersion into such areas probably due to prolonged and severe droughts reducing the occurrence and duration of suitable water bodies. Second, the Paranã Valley in the upper Tocantins River is isolated from downstream sites by a narrow canyon, which slows the river and forms a large wetland in northeastern Goiás State. There is limited information on the fauna shared by these three hydrographic basins other than fishes (Aquino and Colli 2017).

The Cerrado geomorphological compartmentalization resulting from early Miocene tectonic activities has been implicated in lineage diversification and speciation in this region (Guarnizo *et al.* 2016). This activity led to uplift of the Brazilian Shield, which subsequently started a long process of erosion that shaped the landscape into ancient plateaus, dominated by savanna-like vegetation, and younger valleys with more heterogeneous forest assemblages (Colli 2005). Theoretically, this dynamic history could have resulted in distinct lineages forming in plateaus and valleys (Werneck *et al.* 2011). However, different diversification patterns have been inferred from phylogeographical assessments of the Cerrado biota, with clades changing geographically in two directionality patterns: northwest–southwest (for Cerrado endemics) and southwest–northeast (in groups widespread along

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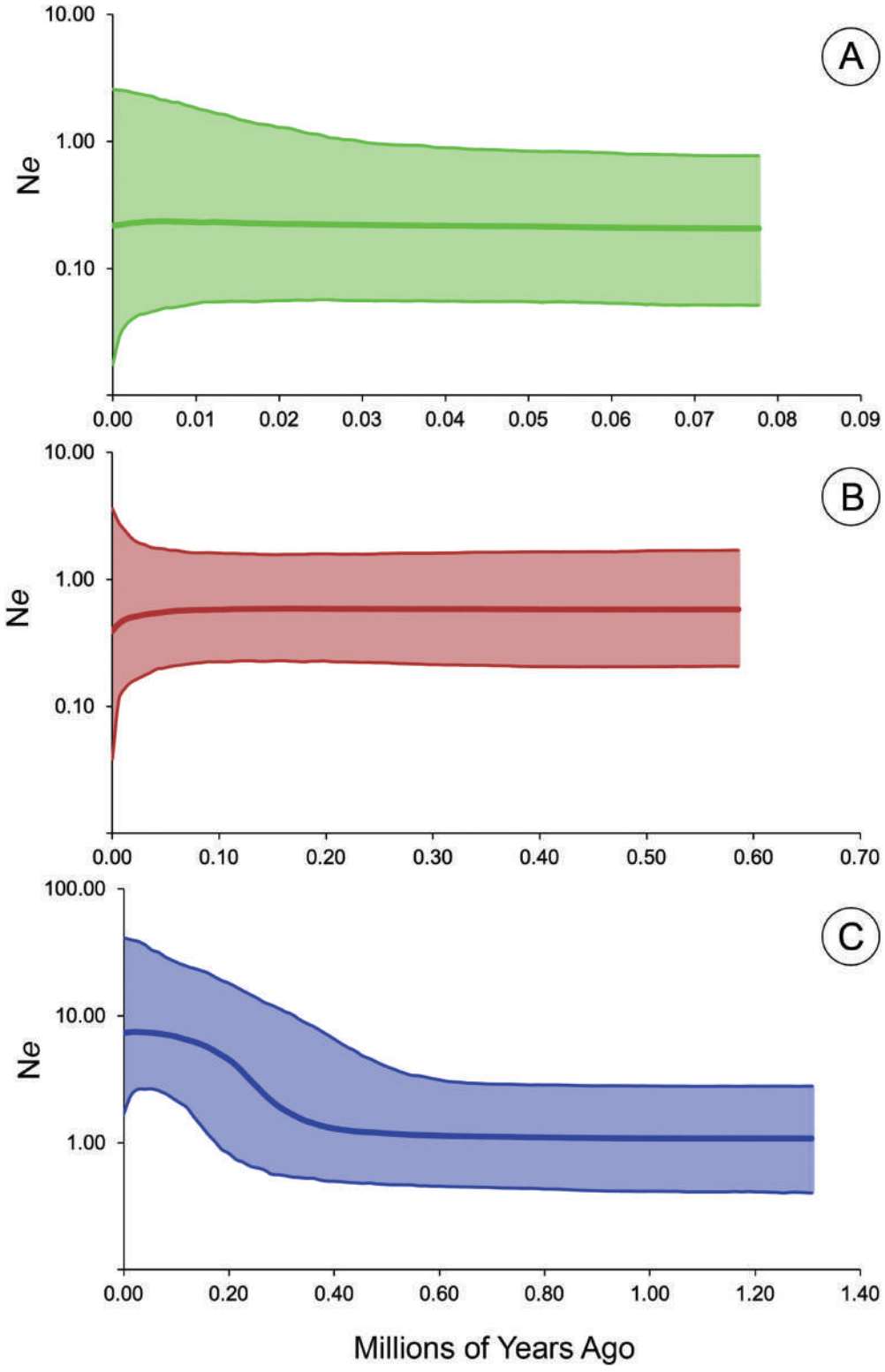

Figure 7. Bayesian skyline plots illustrating effective population sizes (*Ne*) through time of the three *Pseudis bolbodactyla* lineages: A, AT; B, SF; and C, PR. The darker inner line represents median population size, and the coloured area represents 95% highest posterior probability.

the diagonal of open formations, encompassing the Caatinga, Cerrado, and Chaco biomes, Guarnizo *et al.* 2016). Our results show a different pattern for a Cerrado endemic species, with a southeast–northeast directionality shaped by hydrographic basins and demographically influenced by climatic fluctuations during the Pleistocene.

Plants and animals in the diagonal of open formations show idiosyncratic responses to Pleistocene climatic cycles, but most have signs of synchronous population changes (Gehara *et al.* 2017, Bonatelli *et al.* 2022). There is no evidence of demographic fluctuation for the SF and AT populations, while PR shows a moderate expansion over the past 200 000 years. The

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Table 3. Posterior probabilities of model comparisons obtained from five diversification scenarios tested using approximate Bayesian computation (ABC) analysis for *Pseudis bolbodactyla*.

Demographic scenario	ABC method	
	Rejection	Mnlogistic
Model 1	0.02	0
Model 2	0	0
Model 3	0.1	0.01
Model 4	0.02	0
Model 5	**0.86**	**0.99**

The best-fit model is highlighted in bold type.

Paraná basin runs southward, and some areas currently occupied by the species probably experienced colder climates and/or stronger influences of tropical forests during the Pleistocene. As climate changed and forests receded, adequate habitats became available for these tropical, floodplain-dwelling frog species. The timing of this putative expansion for the PR population matches those observed for other open-area species, such as Caatinga dry forest frogs and lizards (Gehara *et al.* 2017).

Such a combined effect of demographic histories in response to climatic fluctuations and the Brazilian plateau uplift is common among many vertebrates in the region (Maciel *et al.* 2010, Prado *et al.* 2012, Oliveira *et al.* 2018). In cane toads [*Rhinella marina* (Linneus 1758) species group], for example, two events of diversification were caused by uplift of the Brazilian Shield during the Miocene, which split them into two main lineages (north and south of the Brazilian Shield, Maciel *et al.* 2010). The Cerrado treefrog *Boana albopunctata* (Spix 1824) shows the same diversification process, with central and southeast Cerrado clades (Prado *et al.* 2012). Within the fossorial but still widely distributed frog *Dermatonotus muelleri* (Boettger, 1885), the Central Brazilian plateau acted as a barrier between two distinct lineages across the open formations of South America (Oliveira *et al.* 2018). Patterns recovered for these species, as well as the timing of events, are similar to what we recovered herein for *P. bolbodactyla*, corroborating Miocene orogenic events as drivers of anuran diversification in Central Brazil. The recent diversification in the Pleistocene for AT and SF probably reflects a concordant geomorphological history between these two drainages, which diverged later than PR drainages (Brasil and Alvarenga 1989, Del'Arco and Bezerra 1989, Turchetto-Zolet *et al.* 2013). The ancient separation of the PR basin (Gray *et al.* 1985, Potter 1997) and its distinct geomorphological history (Gray *et al.* 1985, Campos and Dardenne 1997, Lundberg *et al.* 1998) likely influenced the formation of this lineage.

Taxonomic implications of the one basin–one species hypothesis

Recent taxonomic revisions of paradoxical frogs have compared all species and many populations using morphological, morphometric, larval and bioacoustic characters, and molecular data (Garda *et al.* 2010, Santana *et al.* 2013, 2016). Despite populations of some species being geographically well structured, many cannot be currently distinguished without molecular data. For example, *Lysapsus bolivianus* Gallardo 1961 and *L. limellum* Cope 1862 are virtually identical based on larval, morphological, and bioacoustic characters (Santana *et al.* 2013). Likewise, the sister species *P. paradoxa* (Linnaeus 1858) and *P. platensis* Gallardo 1961 and populations of *P. bolbodactyla* are morphologically and bioacoustically cryptic (Santana *et al.* 2016).

In this study, we found that *P. bolbodactyla* is a complex formed by three distinct lineages, each restricted to a distinct hydrographic basin in central Brazil. Such results corroborate the predictions of the RTH and indicate that its scenarios must be considered as well when studying the landscape genetics of Neotropical species, especially semiaquatic ones. These lineages might correspond to undescribed species, a hypothesis that can be further tested using denser genetic and morphological sampling. As two samples from SF were nested within AT, suggesting gene flow or incomplete lineage sorting, future studies should incorporate genomic data to investigate speciation with gene flow while testing for other factors such as isolation by distance and isolation by resistance (plateaus vs. floodplains). Nonetheless, we suggest that large genetic distances, distinct demographic histories, and the likelihood of allopatric speciation may be similar to those seen in other aquatic and semiaquatic tetrapod fauna such as frogs (*Pipa*, *Lithobates*, *Lysapsus*), cecilians (*Typhlonectes*, *Atretochoana*), lizards (*Dracaena*, *Crocodilurus*, *Potamites*, *Neusticurus*), and snakes (*Helicops*, *Erythrolamprus*).

SUPPLEMENTARY DATA

Supplementary data are available at *Biological Journal of the Linnean Society* online.

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CREDIT STATEMENT

A.A.G. conceived the study and collected samples with D.J.S. A.A.G. and D.J.S. sequenced samples in the laboratories provided by S.L.B. and F.T.B. D.J.S., E.A.M., E.M.F., M.G., E.F.O., and A.A.G. ran the statistical and phylogeographical analyses. D.J.S., E.A.M., and E.F.O. wrote the first version of the manuscript, which was revised by all authors multiple times. S.L.B., F.T.B., and A.A.G. provided funds and logistical support for laboratory and computational analyses.

CONFLICT OF INTEREST

The authors declare no competing interests.

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DATA AVAILABILITY

All sequences are deposited in GenBank (accession numbers can be found in the supplementary data).

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