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Research Article

Historical connections between Atlantic Forest and Amazonia drove genetic and ecological diversity in *Lithobates palmipes* (Anura, Ranidae)

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The Atlantic and Amazon rainforests have a shared but unclear past, with intermittent connections resulting from historical climate change. We investigate these connections by studying the phylogeography and climatic niche of the disjunct distributed frog *Lithobates palmipes*. We sequenced two fragments of mitochondrial DNA from Atlantic Forest (AtF) and Amazonia (AmF) individuals and evaluated how genetic diversity is distributed in space and whether past demographic changes occurred. Also, we evaluated the existence of past suitable connections between biomes for *L. palmipes* through ecological niche models (ENM) and tested for niche divergence. The AtF group is nested within the AmF group and closely related to individuals from eastern Amazonia, a pattern recovered in many species that used northeast connection routes. We found evidence of recurrent use of connections in different directions and time during the Pleistocene, resulting in genetic structure between biomes, with no signal of demographic change and evidence of niche divergence across both genetic groups. ENMs indicated suitable areas connecting forests throughout northeastern Brazil during the Pleistocene. Mitochondrial lineages do not match biomes exactly. One lineage is composed of AtF populations and eastern Amazonia individuals. The other is composed of western Amazonia individuals, suggesting an effect of past climatic heterogeneity within the Amazonia forest. This is the first evidence that this route drove genetic and ecological diversity for amphibians recently, a group with habits and ecological requirements different from other vertebrates that have been shown to use this putative corridor.

Key words: Demography, lineage delimitation, *Lithoboate*s, Neotropics, Niche divergence, Pleistocene, rainforests

Introduction

The causes of the impressive biological diversity in the Atlantic and Amazon forests are still a matter of debate among researchers, who have evoked mainly historical elements of the landscape as drivers of the processes responsible for current patterns (Rull & Carnaval,

2020). These two tropical rainforests are separated by a diagonal of open formations (DOF) formed by the semi-arid Caatinga, the Cerrado savannas, and the Chaco (Collevatti et al., 2013; Werneck, 2011). Contrary to present-day scenario, where these rainforests present unique climatic characteristics and biotas (Carnaval et al., 2014; Longo et al., 2018), they underwent periods of intense biological exchanges. Globally, higher temperatures are believed to have increased water availability through glacial melting and promoted forest

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expansions (Por, 1992). Glacial periods, in contrast, reduced global temperatures and favoured open, savanna-like biomes, while reducing rainforest areas and sea level (Por, 1992).

Such intermittent connections among humid tropical forests in the Neotropics have played critical roles in shaping current patterns of biological diversity and distribution (Costa, 2003; Thomé et al., 2016). From different routes that have possibly linked the Atlantic and Amazon forests, the southern one is known to be the oldest (Pre-Pleistocene) and most important for biotic diversification (Ledo & Colli, 2017). This connection coupled western Amazonia to southern Atlantic Forest and its interruption caused the vicariant diversification of sister genera and species (Batalha-Filho et al., 2013). The other two routes, which connected eastern Amazonia to northern Atlantic Forest along the coastal region and through Cerrado and Caatinga biomes, occurred during interglacial periods of the Pleistocene (Wang et al., 2004). Those connections probably were stablished and interrupted many times, since at least 50 glacial ages every 40–100 thousand years occurred during the Pleistocene (Baker et al., 2020; Lisiecki & Raymo, 2005). The repeated interruption of gene flow during Pleistocene climatic cycles after the establishment of the DOF in these northern routes culminated in cycles of differentiation of intraspecific lineages (Prates et al., 2016a). Present-day gallery forests in the Cerrado and some *Brejos de Altitude* (upper-elevation moister enclaves) within the Caatinga would therefore be remnants of a wider forest more similar to eastern Amazonia Forest with respect to vegetation and precipitation pattern (Carnaval et al., 2009).

The premise that connections were governed by climate cycles as well as the spatiotemporal dynamics of these connections still need further investigation. It is possible that biomes remained connected during the Last Maximum Glacial (LGM) (Sobral-Souza et al., 2015) or even never became isolated during other periods of aridity. Climatic changes would have influenced floristic composition in the connecting routes by favouring plants more or less tolerant to water and thermal stress, instead of promoting a total shift from forest to open biomes in each cycle (Ledo & Colli, 2017). In a scenario like this, ecological and physiological attributes of different taxa have also influenced their effective use of these connections (Zamudio et al., 2016). Hence, a more complete understanding of the effects of such connections on Neotropical diversification can be achieved through phylogeographic studies of species with different life histories (Prates et al., 2016b).

Amphibians form a diverse group regarding ecophysiological restrictions (Bovo et al., 2018), with species presenting different responses to climate change (Blaustein et al., 2010; Miller et al., 2018; Alan Pounds et al., 2006). They are also recognized for their site fidelity, frequently presenting clear effects of geological changes on their genetic structure, making some authors questions the existence of widespread amphibians in the Neotropics (Arteaga et al., 2016; Beddek et al., 2018; Zeh et al., 2003). Some phylogeographic studies demonstrated the importance of past climatic change and the southern route in the diversification of forest amphibians in South America (Fouquet et al., 2012a; Fouquet et al., 2012b). However, information on the effects of connections on northeastern Atlantic Forest amphibian's diversity and ecology are scarce.

The family Ranidae, although extremely diverse, is represented by only three described species in South America (Frost, 2020): *Lithobates vaillanti* (Brocchi, 1877), *L. bwana* (Hillis & de Sá, 1988) and *L. palmipes* (Spix, 1824) (Hillis & Wilcox, 2005). These species originated approximately 28 million years ago, during the formation of the Panama Isthmus and the uplift of the Andes (F. E. A. Coelho, F. Camurugi and A. A. Garda, personal communication). *Lithobates palmipes*, one of these four species, is mainly associated with lowland tropical rainforests and widely distributed in Amazonia. This species also occurs in northeastern Atlantic Forest, and this disjunct distribution suggests the importance of connections between biomes in its evolutionary history, also raising the possibility that it comprises a species complex (Santa-Cruz et al., 2016). As the species probably originated in Amazonia, its phylogeography can clarify the connection's role on the species/lineage's diversification. Although both Amazonia and Atlantic forests are tropical rainforests, they have highly heterogeneous and distinct geomorphological histories and climatic conditions (Laraque et al., 2007; Zamborlini Saiter et al., 2016). Therefore, connections could also have led to climatic niche evolution within *L. palmipes*.

Using mitochondrial genetic markers from individuals from widespread localities covering most of the known distribution of the species, we grouped individuals *a priori* according to biomes, and evaluated the effect of the interruption of the connection by (1) testing if groups of *L. palmipes* from each biome (hereafter AmF group and AtF group) comprised reciprocally monophyletic clades that might indicate the existence of a species complex; (2) assessing the effects of such connections on the genetic diversity and demography of groups in each biome; (3) assessing which regions in the past may have been suitable areas for *L. palmipes* connecting the biomes and (4) testing if AtF and AmF groups occupy similar climatic niches through niche overlap, equivalency and similarity analyses, as these biomes nowadays have unique climatic identities. Such as other species with
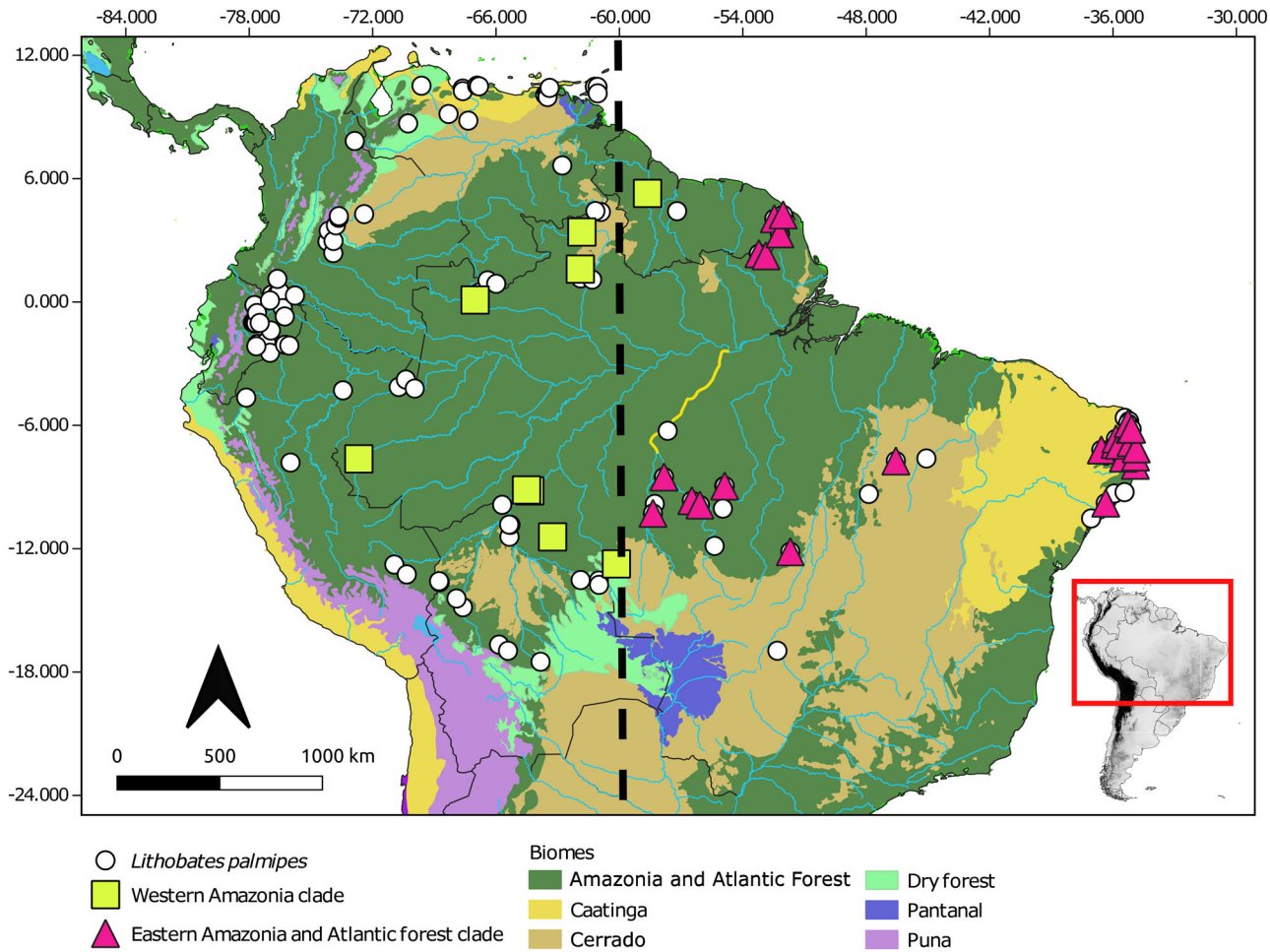
Fig. 1. *Lithobates palmipes* known distribution in Amazonia and Atlantic Forest, with few occurrences in rainforest fragments within Cerrado and Caatinga biomes. Triangles and squares represent localities which we have mtDNA sequences and main clades recovered; the dotted line shows the approximate limit dividing these clades in Amazonia and the river highlighted in yellow represents Tapajós River (see Results and Discussion sections). Biomes adapted from Olson et al. (2001).

similar distribution, we expect that *L. palmipes* comprises a single species with population structure coherent with its disjunct distribution. We expect that the species has dispersed recently, during the Pleistocene, through the northern connection, given its restricted distribution to northeastern Atlantic Forest. Also, we expect that this past dispersal event from Amazonia towards the Atlantic Forest resulted in a recent population expansion in the AtF group, with populations on each biome occupying different climatic niches.

Material and methods

Data acquisition

We gathered liver and muscle samples of 64 *Lithobates palmipes* specimens from 27 localities covering the distribution in the Atlantic and Brazilian Amazonia (Fig. 1)

through donations from Zoological Collections (Supplemental Table S1). We submitted tissues to a protocol of total DNA extraction (adapted from Bruford et al., 1992) and obtained fragments of two mitochondrial genetic markers (mtDNA), *cytochrome oxidase I* (*COI*) and *16S*, through PCR amplification using specific primers and conditions (Supplemental Table S2). Purification and sequencing of PCR products were carried out at Macrogen Inc. We also used sequences available in GenBank ($N = 6$) and obtained through donation ($N = 5$) (see Supplemental Table S1). We checked for stop codons, edited, and aligned sequences using MUSCLE algorithm (Edgar, 2004) in the software Geneious 9.0.5.

Dated gene tree reconstruction

To reconstruct a time-calibrated Bayesian gene tree for *Lithobates palmipes* we used BEAST 1.8.4 (Drummond

et al., 2012), including only individuals for which we had both *COI* and *16S* genes available. We used a concatenated alignment, rooting the tree using *L. vaillanti* as an outgroup. Although *L. vaillanti* is not the closest relative to *L. palmipes*, it is the only species with both *16S* and *COI* available in GenBank. We used PartitionFinder2 (Lanfear et al., 2017) to find substitution models for the following partitions: *16S* and the *COI* first position of each codon (TRNEF + I), *COI* second position of each codon (HKY), *COI* third position of each codon (HKY + G). We then used PAUP* v. 4.0a169 (Swofford, 2002) to test for a strict molecular clock using likelihood ratio tests under AICc and BIC criterion with HKY + I + G as substitution model. Because the results support a clock-like model (null hypothesis cannot be rejected), we set a strict molecular clock with a mutation rate of 0.0069 sites per million years (Macey et al., 1998), which is compatible with that estimated for *16S* for different Ranid species (Chen et al., 2013; Jongsma et al., 2018). We set the mutation rate with a uniform distribution, setting bounds to 0.01 and 0.003 to accommodate for heterogeneity in mutation rates among partitions described above. Highest posterior density (HPD) of divergence times in each node were estimated using the BEAST 1.8.4 (Drummond et al., 2012). We used coalescent constant size as tree prior and ran three independent MCMC chains to check for convergence, each with 50,000,000 generations, sampling every 5,000. We combined log files and trees using LogCombiner 1.8.4 (Drummond et al., 2012) and assessed chain convergence and stationarity (ESS > 200) using Tracer 1.7.1 (Rambaut et al., 2014). Consensus tree was estimated using TreeAnnotator 1.8.4 (Drummond et al., 2012), discarding 20% of the trees as burn-in.

To account for individuals that we only have *16S* and estimate in which clade they are grouped, we also built a gene tree only for this fragment. We generated the tree following the same procedures described previously, only changing the substitution model (TN93 + G) estimated using PartitionFinder2, and using a strict clock with no mutation rate, since here we are interested in topology only. Because there are more species with *16S* available in Genbank, here we added *L. bwana*, *L. juliani* (Hillis & de Sá, 1988) and *L. warszewitschii* (Schmidt, 1857) as outgroups.

Lineage delimitation

We assessed putative gene lineages evolving independently comparing congruent groups recovered by different species delimitation analyses. We performed Poisson Tree Process in its maximum likelihood and Bayesian implementation (PTP and bPTP, Zhang et al., 2013) using its website platform (species.h-its.org). Both PTP and bPTP differentiate cladogenetic events within and among lineages (Zhang et al., 2013). We performed 500,000 MCMC generations using as input the Bayesian tree obtained with Beast. The remaining parameters were set as default. Bayesian implementation of the general mixed Yule-coalescent model analysis (bGMYC, Reid & Carstens, 2012) is an analysis commonly used to delimit species by accounting for uncertainty in tree topology. Before the analysis, we confirmed that our data violate premises of GMYC model ($P < 0.05$) by using 'P2C2M.GMYC' R package (Fonseca et al., 2021), therefore we did not perform bGMYC analysis.

We also performed two distance-based analyses to estimate the number of clusters and compared to PTP, bPTP results. The first, Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012), groups sequences based on genetic distances, and creates clusters in which the distance between two random samples, each from different clusters, will always be larger than the distance of sequences within clusters (Puillandre et al., 2012). We ran ABGD in its website platform (bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using K2P distance, minimum and maximum prior distances of 0.001 and 0.01, respectively, 15 recursive runs, and relative gap width (X) of 1.0. The second distance-based analysis was the Bayesian Analysis of Population Structure (BAPS 6; Corander et al., 2008). First, we performed a mixture model with a range of 1–10 k, then we used the result to run an admixture model (Corander & Marttinen, 2006) with 100 iterations, 200 individuals of reference for each subpopulation and 20 iterations for each individual.

Genetic diversity in space and demography

To understand the effects of a recent disconnection among biomes (see Results section) in *Lithobates palmipes*, we grouped individuals *a priori* by biome (AtF and AmF group). First, we performed an analysis of molecular variance (AMOVA) for *COI* and *16S* separately, to assess if genetic diversity is higher within or between biomes using 50,000 permutations in Arlequin 3.5 (Excoffier & Lischer, 2010). We estimated summary statistics, such as number of segregating sites (S), number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (π) for each marker and group in DnaSP 6 (Rozas et al., 2017). We also estimated genetic distance between groups in MEGA7 (Kumar et al., 2016). To visually assess genetic diversity in geographic space, we generated haplotype networks for each marker using Popart 1.7 (Leigh & Bryant, 2015), and using

concatenated alignment we used spatial interpolation of genetic distances in Alleles in Space software (Miller, 2005). The latter uses genetic distances between individuals coupled with geographic coordinates to generate a 3D graph, in which X and Y axis represent geographic space, and Z genetic distances between individuals. Peaks along Z represent areas with discontinuity between populations, suggesting reduced or no gene flow.

To evaluate past demographic changes in population size, we performed Tajima's D and Fu's F neutrality test in Arlequin 3.5 with 50,000 permutations for each group and genetic marker. Significant negative values can be interpreted as an indication of population expansion, while positive values support population contraction. AMOVA, summary statistics, and neutrality tests were also performed for groups recovered in the lineage delimitation analysis (see Results section).

We also performed a continuous spatiotemporal Bayesian approach in BEAST 1.10.4 using the same alignment with 60 individuals that we sequenced *COI* and *16S*. A lognormal relaxed random walk (RRW) model was used as a time-heterogeneous approach that allows for variation in diffusion rates across branches of the phylogeny and simultaneously estimates ancestral traits and topology in a Bayesian framework (Lemey et al., 2010). We enforced the Jitter option with 'multivariateTraitLikelihood' to add random noise to samples with identical coordinates. We used the skyride model as demographic prior for RRW analyses to estimate effective population sizes through time (Minin et al., 2008). We implemented the same partitioned substitution model and mtDNA mutation rate prior set as described earlier in the dated genealogical tree section and a coalescent model as tree prior. We performed three independent runs of 10^8 generations, each sampled at every 1,000 steps. We visually assessed convergence of the parameters (ESS) of MCMC runs using Tracer 1.7, combined runs and trees after removing a 25% burn-in using Log Combiner 1.10.4, and annotated the MCC tree using TreeAnnotator 1.10.4. A visual representation of the spatiotemporal diffusion of lineages was exported from spreaD3 0.9.6 (Bielejec et al., 2016).

Ecological niche modelling (ENM)

To build ecological niche modelling (ENM), we gathered *L. palmipes* occurrence points from VertNet (Constable et al., 2010) and from classic papers on taxonomy and distribution of *Lithobates* species (Hillis & de Sá, 1988; Hillis & Wilcox, 2005). We also used occurrence points from localities from where we have tissues. We used a total of 136 occurrence points (23

from Atlantic Forest and 113 from Amazonia). To characterize climatic niches (also known as Grinnellian niche), we used present day bioclimatic variables (Booth et al., 2014) available in 'WorldClim1' (www.worldclim.org, Hijmans et al., 2005) in 2.5 arc minute resolution. To avoid over parametrization and due to collinearity, we removed variables with high covariance values ($r > 0.8$), using at the end 7 out of the 19 climatic variables available: Isothermality (bio03), temperature seasonality (bio04), minimum temperature of coldest month (bio06), temperature annual range (bio07), annual precipitation (bio12), precipitation of driest month (bio14) and precipitation seasonality (bio15).

We first built an ecological niche model (ENM) based on occurrence points from AtF and AmF groups together using MAXENT 3.3.3k (Phillips et al., 2006) in R package 'ENMeval' 0.3.0 (Muscarella et al., 2014). We build the model using 10,000 random points across South America as background, using all occurrence points, defining the training and the testing data using the method 'Checkboard1' and using six different combinations of feature class (FC): L, LQ, H, LQH, LQHP and LQHPT (L = linear, Q = quadratic, H = hinge, P = product and T = threshold). To avoid overfitting models, we applied regularization multipliers (RM) ranging from 0.5 to 4, with increments of 0.5. We selected the best model based on Akaike's information criterion corrected for small sample sizes (AICc). To assess model performance, we used four indexes. First, we calculated the average values of the area under the curve (AUC) and AUCdiff (AUC of the training data – AUC of the testing data) that is positively correlated to model overfitting (Muscarella et al., 2014). Because AUC has been criticized as a good estimator of model performance (Jiménez-Valverde, 2012; Lobo et al., 2008), we also calculated the True Skill Statics (TSS), that ranges from −1 to +1, with 0 being a model no different from random classification of suitable areas (Allouche et al., 2006). In addition, we evaluated model performance using the Boyce Index, appropriate for models built using presence-background (Boyce et al., 2002). It also ranges from −1 to +1, with positive values indicating that the model is consistent with species distribution, values close to 0 suggesting that the model is not different from random, and negative values suggesting that the model found low suitability in areas where the species occurs (Hirzel et al., 2006; Sillero et al., 2021).

To assess the existence of suitable areas that could have been used as dispersion routes between biomes, we projected the model built to three different times in the past, using 'WorldClim1' climatic data for Holocene (6

thousand years ago, under global climate model MIROC-ESM and CCSM4), Last Glacial Maximum (LGM, 21 thousand years ago, under global climate model MIROC-ESM and CCSM4) and Last Inter Glacial (LIG, 130 thousand years ago, Otto-Bliesner et al., 2006). Even though niche conservatism is one of the premises of projecting ENMs for past conditions, we believe that projecting group's ENMs can be informative about possible routes used by the species to reach Atlantic Forest (Ledo & Colli, 2017).

Niche conservatism/divergence

As the species has a wide and disjunct distribution range in different biomes, we tested if the AmF and AtF population groups differ in their climatic niches or if the niche is conserved, even in such different regions. For that, we performed niche overlap, similarity and equivalency tests in R package 'Ecospat' (Di Cola et al., 2017) as proposed by Broennimann et al. (2012).

First, we extracted the bioclimatic information of each occurrence and background point using the same variables of the ENM. We used as background the ecoregions in which each group occurs, following the ecoregion classification proposed by Olson et al. (2001). We summarized the environmental space available for both groups performing a principal component analysis (PCA) and combining the background of each group (Broennimann et al., 2012). Second, we estimated PCA scores for occurrence and background points and performed a Kernel density function for AmF, AtF, and background. Using occurrence densities, we estimated overlap between groups using the Schoerner's *D* index, that ranges from 0 (no overlap) to 1 (total overlap) (Schoener, 1968). We considered the following classes of overlap suggested by Rödder & Engler (2011): $0 - 0.2 =$ no or very limited overlap, $0.2 - 0.4 =$ low, $0.4 - 0.6 =$ moderate, $0.6 - 0.8 =$ high, $0.8 - 1.0 =$ very high.

Using 'Ecospat' in R environment (Di Cola et al., 2017), we tested for niche divergence using 'alternative = lower' in niche equivalence test with 100 permutations to test if dissimilarity between groups differs from what is expected by chance $(P < 0.05)$ (Broennimann et al., 2012). To test if dissimilarity between groups is due to differences in background, we performed a similarity test with 1,000 permutations, also using 'alternative = lower' (Broennimann et al., 2012). If similarity test is significant $(P < 0.05)$, then backgrounds are more dissimilar than expected by chance, and any difference found in equivalence test is due to the dissimilarity between backgrounds, otherwise $(P > 0.05)$ dissimilarity between backgrounds is no greater than expected by chance, and niches have diverged.
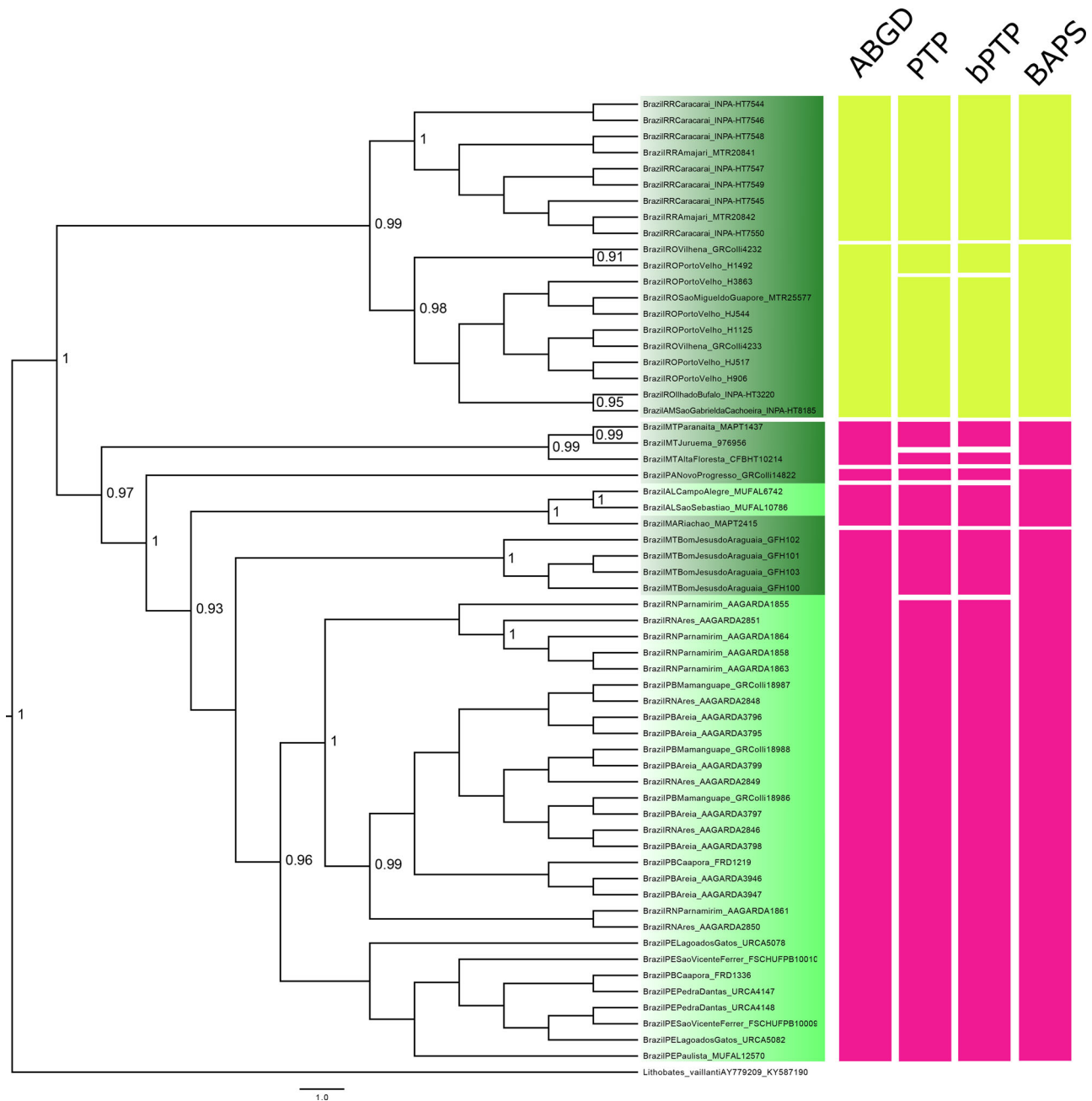
Results

Gene trees and lineages delimitation

We successfully obtained sequences from 82 individuals (*16S*: 72, 370 bp, Genbank accession numbers: OM832098–OM832167; *COI*: 65, 627 bp, Genbank accession numbers: OM832590–OM832648) from localities covering the whole distribution of *L. palmipes* in the Atlantic Forest and a great part of its range in Amazonia. The concatenated alignment used to build the dated tree was composed of 60 individuals, for which *COI* and *16S* sequences were available. We recovered a tree with high posterior probability (PP) separating the outgroup and *L. palmipes*. Within *L. palmipes*, we found two clades with high support (PP: 1; Fig. 2): the first composed exclusively of individuals from western Amazonia, and the second composed of individuals from eastern Amazonia and Atlantic Forest. The same pattern was recovered in the 16S gene tree, with individuals from French Guiana in the same clade of eastern Amazonia and Atlantic Forest, and individuals from Guyana grouped with western Amazonia (Supplemental Fig. S1).

All lineage delimitations analyses, either based on genetic distance or trees, separated the same two clades with high node support recovered in the tree (Fig. 2). Based on our dated tree, these main lineages diverged recently, during Early Pleistocene at 1.167 million years ago (HPD 0.466–2.358; Fig. 3). Also, all lineage delimitation analyses recovered at least two lineages within western Amazonia. Despite these congruences, delimitation analyses were discordant about putative lineages within the eastern Amazonia + Atlantic Forest clade. While ABGD and BAPS grouped individuals from this groups in just a few lineages, bPTP and PTP split them in more lineages, some of them composed of one individual (Fig. 2).

Summary statistics and demography

For both *COI* and *16S*, AmF group is more diverse than the AtF group in all parameters estimated (Table 1). The AMOVA result shows that variance is significantly distributed between AmF and AtF groups, instead of within groups $(P < 0.05,$ Table 2). However, we found a moderate value of Fst $(COI = 0.602, \quad P < 0.05;$ $16S = 0.590, \quad P < 0.05)$ and low genetic distances $(COI = 0.015; 16S = 0.008)$ between groups, reinforcing the lack of reciprocal monophyly between lineages, with

Fig. 2. Mitochondrial genealogy (COI and 16S) of *Lithobates palmipes* samples and lineage delimitation results using PTP, bPTP, ABGD and BAPS. Note the conflicting results among different lineage delimitation analyses. Despite incongruences, all analyses grouped individuals from eastern Amazonia within the same lineage, or as sister lineage of Atlantic Forest individuals (pink colored), separated from individuals from western Amazonia (green colored). Dark green tips represent individuals from the AmF group, and light green individuals from the AtF group. Colorless tip is the outgroup. Numbers on each node represent posterior probabilities (PP), and nodes with no information have a PP lower than 0.9. Scale bar of 1.0 represents the number of substitutions per site.

certain genetic structure in each biome regarding mitochondrial DNA. Genetic variance is better explained by lineages recovered in lineage delimitation (Table 2), probably due to incomplete lineage sorting. This becomes even more evident through haplotype networks, with some AmF group haplotypes also occurring in the Atlantic Forest (Fig. 4). Also, when grouping according to lineages, Eastern AmF + AtF lineage presented higher values than Western AmF for all summary statistics (Table 1), and we recovered higher Fst values ($COI = 0.769$, $P < 0.05$; $16S = 0.681$, $P < 0.05$), and genetic distance ($COI = 0.018$; $16S = 0.01$). Interpolation

Table 1. Summary statistics for each gene of *Lithobates palmipes* and grouping per biome (AtF and AmF) and per lineage recovered on lineage delimitation analysis (Western AmF and Eastern AmF + AtF).

Locus	Lineage	Length	N	S	H	Hd	π	Tajima's D	*P*-value	Fu's F	*P*-value
16S	All	370	72	6	9	0.845	0.00685	1.991	0.9	−0.103	0.5
	AtF		33	2	3	0.573	0.00179	0.535	0.7	0.577	0.5
	AmF		40	6	7	0.801	0.00535	0.874	0.8	−0.207	0.4
	Eastern AmF + AtF		49	4	6	0.752	0.00354	0.706	0.7	−0.574	0.3
	Western AmF		23	3	3	0.593	0.00314	1.095	0.8	1.744	0.8
COI	All	627	65	25	18	0.879	0.01012	0.322	0.6	−1.169	0.3
	AtF		34	8	6	0.677	0.00267	−0.563	0.3	−0.127	0.5
	AmF		31	20	12	0.847	0.00918	0.119	0.6	−0.516	0.4
	Eastern AmF + AtF		44	16	12	0.801	0.00437	−0.936	0.1	−2.702	0.1
	Western AmF		21	8	6	0.695	0.00360	−0.177	0.4	−0.039	0.5

Length in base pairs; N – number of samples; S – polymorphic sites; H – number of haplotypes; Hd – haplotype diversity; π – nucleotide diversity; *P*-values of the Tajima's D and Fu's F test; AtF – Atlantic forest; AmF – Amazon Forest.

Table 2. AMOVA results showing variance between and within *Lithobates palmipes* groups using COI and 16S separately, grouping according to biomes (Atlantic forest and Amazon forest).

| Gene	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value	ΦFC
COI	Among biomes	1	85.59	2.58	60.22	<0.05	0.60
	Within biomes	63	107.66	1.70	39.78		
	Total	64	193.26	4.29			
	Among lineages	1	116.55	4.05	76.92	<0.05	0.76
	Withing lineages	63	76.70	1.21	23.08		
	Total	64	193.26	5.27			
16S	Among biomes	1	34.51	0.95	59.02	<0.05	0.59
	Within biomes	70	46.7	0.66	40.98		
	Total	71	80.79	1.61			
	Among lineages	1	39.80	1.25	68.15	<0.05	0.68
	Within lineages	70	40.98	0.58	31.85		
	Total	71	80.79	1.83			

of genetic distances in the geographic space shows a high peak of genetic discontinuity in space in the Cerrado and Caatinga regions, with some discontinuity within Amazonia (Supplemental Fig. S2).

According to the RRW diffusion model (Fig. 5), the dispersion of *L. palmipes* most likely began in the southeastern Amazonia around $500 \sim 450$ ka, with individuals spreading concomitantly to northwestern Amazonia and to the Atlantic Forest around 290 ka. Interestingly, the populations that reached eastern Amazonia at Maranhão state originated from the Atlantic Forest population rather than eastern Amazonia ones. Moreover, the southernmost populations of the Atlantic Forest (Alagoas state) colonized this region more recently from the split between Maranhão population (starting around 150 ka) rather than those populations that spread northwards reaching Rio Grande do Norte State since the Atlantic Forest population was established around 290 ka. Another interesting geographic pattern highlighted by the analysis is that populations from southwestern Amazonia (Rondônia state) are not genetically more related to populations from southern Amazonia (Mato Grosso or Pará states), although the geographic distance between then

(~300 km away) is relatively short compared to other samples. In summary, this analysis indicates that at least two independent colonization events have occurred in the Atlantic Forest and that *L. palmipes* populations have crossed the present day Caatinga more than once.

ENM for current and past conditions

The ENM built with current climatic conditions for the entire distribution of *L. palmipes* recovered suitable areas coherent with the species' distribution (AUC = 0.80, AUCdiff = 0.01, TSS = 0.51, Boyce index = 0.97, see Supplemental Table S2), and few suitable areas outside the known range, in southern Atlantic Forest and west of the Andes (Fig. 6). In the past, suitable areas for *L. palmipes* were wider than now. Based on our model, even regions now occupied by the Cerrado savannas were suitable for *L. palmipes*, reaching its maximum expansion 21 thousand years ago, during the LGM under MIROC global climate model, and 6 thousand years ago under CCSM4 global climate model. However, during the LIG suitable areas were wider when compared to the projection for other time periods under CCSM4 global climate model. Stable
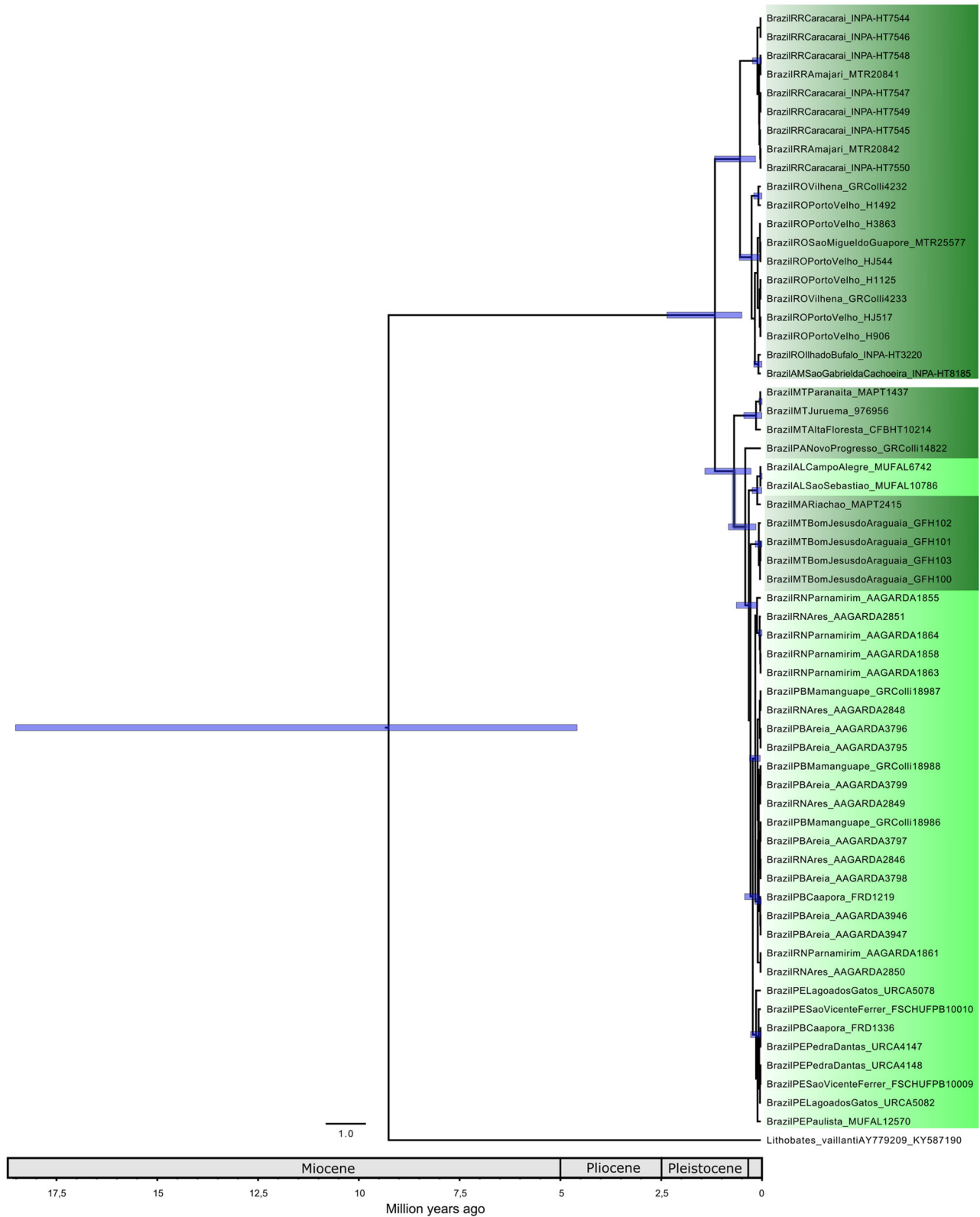
Fig. 3. *Lithobates palmipes* dated gene tree. Main intraspecific clades diverged recently, during the Pleistocene. Colors represent if individuals are from Amazonia (dark green) or Atlantic Forest (light green). White space separating colors separates western Amazonia clade from eastern Amazonia/Atlantic Forest clade. Purple bars on each node represent 95% HPD (highest posterior density) confidence interval in divergence times. Scale bar of 1.0 represents the number of substitutions per site.
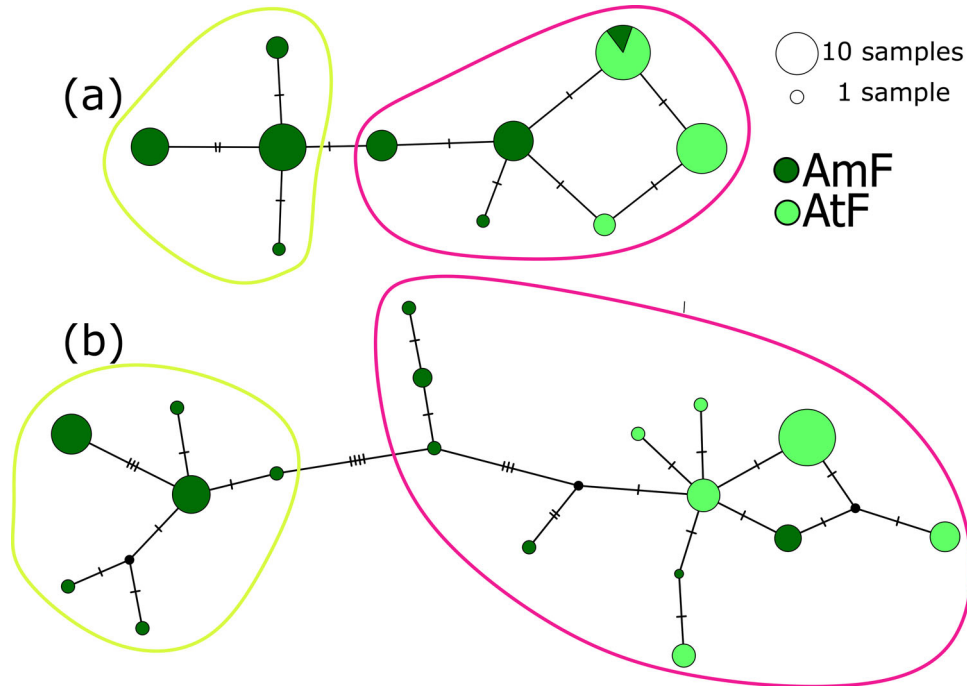
Fig. 4. Haplotype network of *Lithobates palmipes* for (a) 16S and (b) COI molecular markers. AmF – Amazon forest; AtF – Atlantic forest. Green and pink circles represent western Amazonia clade and eastern Amazonia/Atlantic forest clade.

areas for *L. palmipes* were mainly concentrated in western Amazonia, a small portion of eastern Amazonia, central and northeast Atlantic Forest. Interestingly, there is an expansion of suitable areas eastward, and through the northeast coastline, recovering suitable areas almost connecting Amazonia and the Atlantic Forest through the northeast.

Niche evolution between groups

Niche overlap between AmF and AtF is limited ($D = 0.004$, Fig. 7). In addition, we found evidence that climatic niches are more different than expected by chance (equivalence test $P = 0.01$), while backgrounds are not, and such low overlap can be explained by niche divergence (similarity test $P = 0.77$).

Discussion

Quaternary connections between Amazonia and Atlantic Forest caused the disjunct distribution of *Lithobates palmipes*, and here we found that groups in each biome are not reciprocally monophyletic, possibly due to incomplete lineage sorting after more than one recent dispersion. We support the origin of *L. palmipes* in Amazonia, with no demographic change after dispersal to the Atlantic Forest since late Pleistocene. Those dispersal events occurred through a Northeastern connection

between the Amazonia and the Atlantic Forest and drove rapid niche divergence between groups in each biome. Among the possible connections between AtF and AmF biomes proposed in the literature, the one through northeastern Brazil is described as the most recent (Ledo & Colli, 2017; Por, 1992). This connection probably resulted in the allopatric distribution of sister species of birds in these biome (Berv & Prum, 2014), and genetic structure and current disjunct distributions in reptiles and mammals (Costa, 2003; Prates et al., 2016a; Zamudio & Greene, 1997). Here, we found that the Northeastern connection was also important to the evolutive history of an amphibian species.

Forest connections and their effects on Neotropical biota

We found that *L. palmipes* has an actual wide distribution in South America, not comprising a species complex, confirming previous results with smaller sampling (Fouquet et al., 2007). Despite this wide distribution, our results indicate some degree of genetic structure between populations in each biome (Table 2, Fig. 3, and Supplemental Fig. S2). This likely results from the reduction or recent complete halt in gene flow between AmF and AtF groups due to the expansion and establishment of the DOF (Collevatti et al., 2013). We support an Amazonian origin of *L. palmipes*, given the
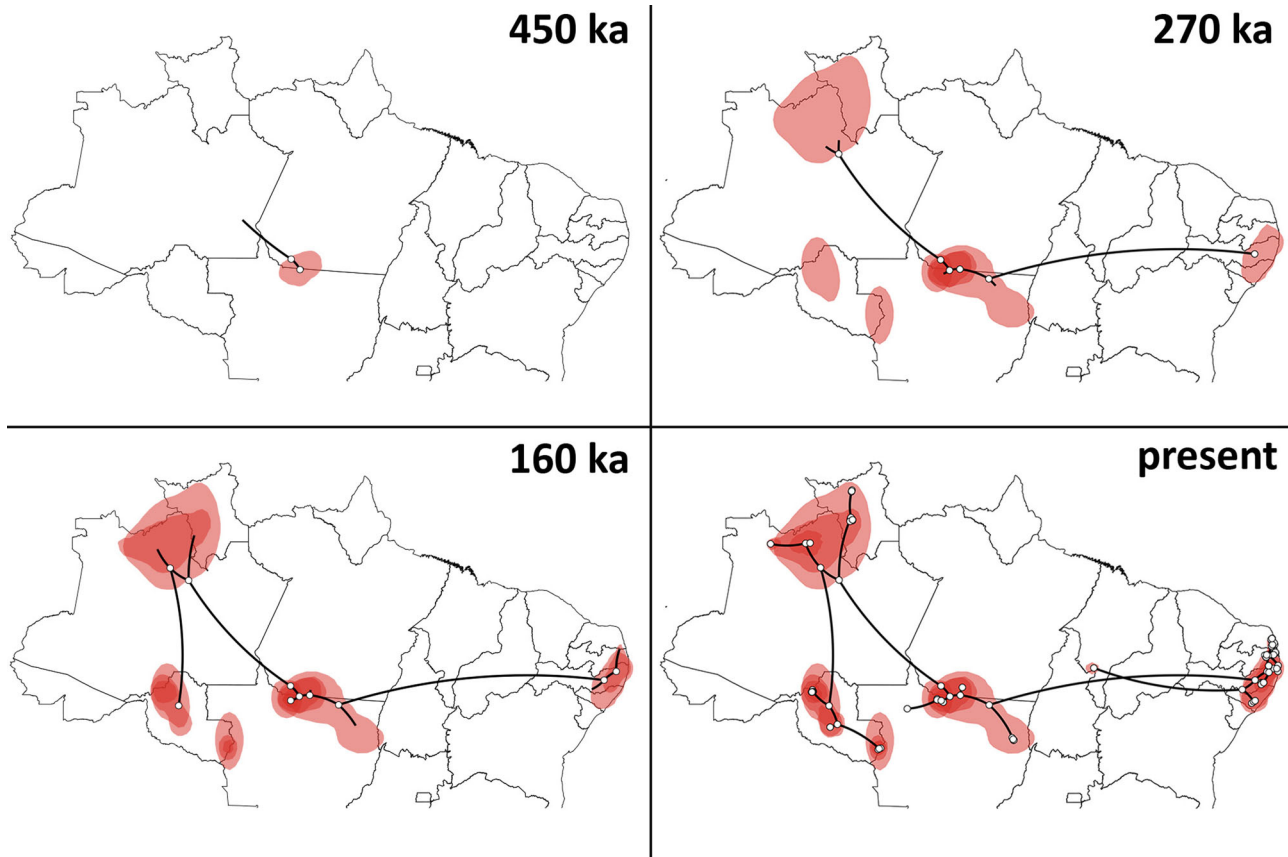
Fig. 5. Continuous spatial diffusion analysis of *Lithobates palmipes* populations (Amazonia and Atlantic Forest) based on a Bayesian phylogeographic analysis in BEAST. We depict four-time intervals in years^{-3} (ka) derived from the mean node ages estimated in the maximum clade credibility tree (Relaxed Random Walk model). The red areas represent the 80%-HPD uncertainty in the location of ancestral branches. The dark to light color gradient represents older and more recent diffusion events, respectively. Note that the species likely originated in Amazonia, dispersed to the Atlantic Forest and then dispersed again returning to Amazonia, likely using a Northeastern connection among forests during the Pleistocene.

higher values in summary statistics for AmF when compared to AtF group, and AtF being nested within AmF. This was expected, since *L. palmipes* originated after a dispersal from west to east of the Andes cordillera (F. E. A. Coelho, F. Camurugi and A. A. Garda, personal communication), and it is in accordance with previous findings that Amazonia Forest was an important source of diversity in South America (Antonelli et al., 2018). In species with Amazonian origin, such as the lizards *Anolis punctatus* (Daudin, 1802) and *A. ortonii* (Cope, 1868), a bottleneck followed by population growth after reaching the Atlantic Forest was interpreted as a signal of a dispersal event between biomes (Prates et al., 2016a). Conversely, the lack of a bottleneck signal in another lizard species, *Polychrus marmoratus* (Linnaeus, 1758), might have been caused by its continuous occurrence within forest connections, followed by isolation (Prates et al., 2016a). Here, our results do not support a bottleneck or population expansion in mtDNA for both AmF and AtF groups. Statistical analysis using more

loci will likely confirm no demographic changes, supporting a scenario in which *L. palmipes* occurred throughout the connection used to reach the Atlantic Forest, as demographic effects are expected to be more pervasive in the matrilineal and haploid mitochondrial markers.

The Northeastern connection was probably the only route used by *L. palmipes* to reach the Atlantic Forest, given its present distribution restricted to the Atlantic Forest northwards of the São Francisco River, its shallow genetic divergence with respect to Amazonia, and the occurrence of shared haplotypes. Interesting, this route was likely used more than once and in different directions. For example, our spatiotemporal results suggest that *Lithobates palmipes* used it to reach the Atlantic Forest, get back to the Amazonia and then disperse to the Atlantic Forest again during different times throughout late Pleistocene, reinforcing the intermittent nature of such forest corridors among biomes, a pattern similar to the one recovered for *Bothrops bilineata*
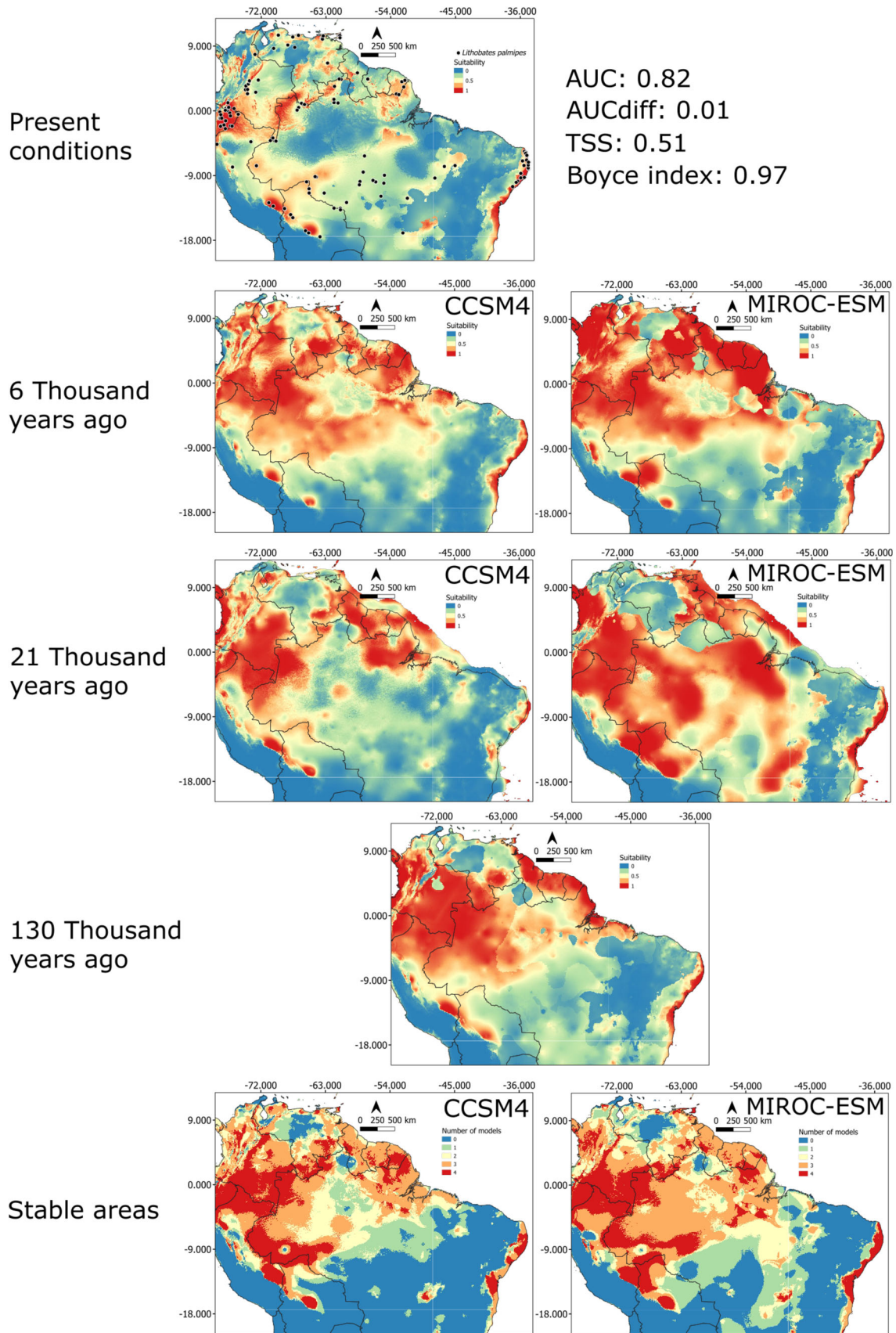
Fig. 6. Ecological niche model for *Lithobates palmipes* for present and past climatic conditions during the Holocene (6 thousand years ago), Pleistocene Last Glacial Maximum (LGM, 21 thousand years ago) under global climate model MIROC-ESM and CCSM4, Pleistocene Last Interglacial (LIG, 130 thousand years ago) based on data published by Otto-Bliesner et al. (2006), and stable areas through these times. Indexes used to assess model performance are Area Under the Curve (AUC), the difference between AUC of the training data and AUC of the testing data (AUCdiff), True Skill Statistics (TSS), and Boyce index.
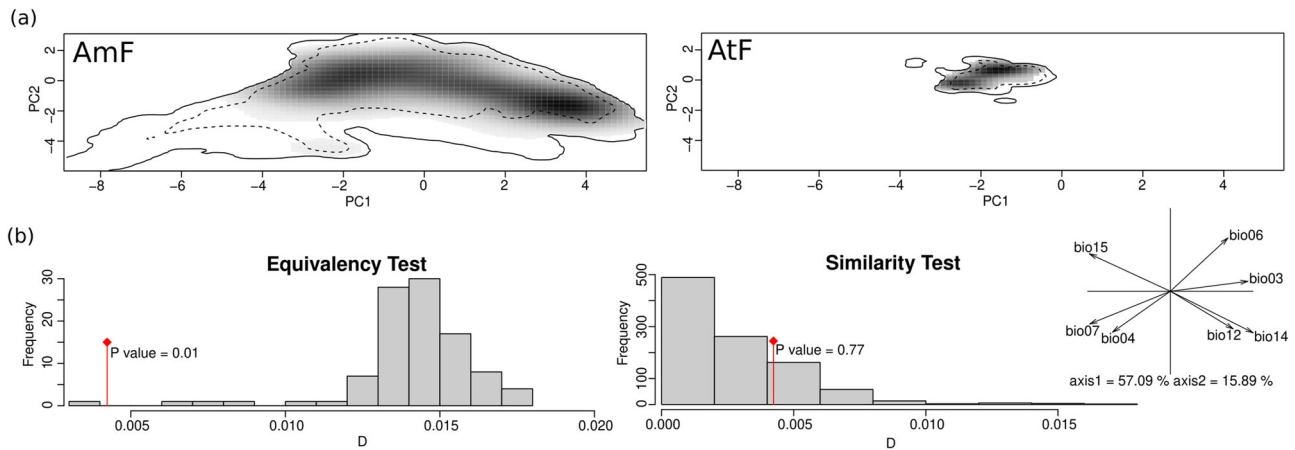
Fig. 7. (a) PCA plot summarizing the environmental space available for both groups (AmF and AtF group). Dashed and solid lines show 100% and 50% of the entire summarized background available, respectively. Density of occurrence in environmental space is represented in shades of grey. The darker the colour, the higher the density of occurrence. (b) Histograms showing observed overlap value (*D* index) against null model generated in similarity and equivalency tests, and correlation circle showing variables contribution in PCA.

(Wied-Neuwied, 1821) (Dal Vechio et al., 2018). Although the exact timing of establishment of those connections remains obscure, it most likely occurred during middle to late Pleistocene, which agrees with our initial predictions.

A possible alternative would be that *L. palmipes* may have used a southern route, expanded northeastward, as reported for *Adelophryne* and *Dendrophryniscus* (Fouquet et al., 2012a; Fouquet et al., 2012b), and then becoming extinct in southern Atlantic Forest. However, this is likely not the case because *L. palmipes* in the Atlantic Forest diverged recently, and individuals are closely related to individuals from eastern Amazonia, a common pattern observed in other species that more likely used a Northeastern connection (Costa, 2003; Fouquet et al., 2012b; Gehara et al., 2014). In addition, we found support for a Northeastern connection using ENMs.

Regardless of some similarities in the past, Amazonia and Atlantic Forest now hold unique environmental characteristics (Carnaval et al., 2014; Longo et al., 2018), and *L. palmipes* occurs in both, despite these climatic differences. During the process of speciation, species can keep occupying niches as similar as possible (i.e., niche conservatism), or they can shift to environments with conditions much different from their ancestor (i.e., niche divergence) (Pyron et al., 2015). Some species of the *Lithobates palmipes* species group diversified after dispersal events followed by niche divergence, and now these species occupy a diversified set of areas with low niche overlap (F. E. A. Coelho, F. Camurugi and A. A. Garda, personal communication). The same holds true for *L. palmipes*, which reached the Atlantic Forest during Pleistocene and now occupies two biomes

with limited niche overlap. Similar to our findings, a remarkably fast intraspecific niche divergence was also found in treefrog *Boana raniceps* (Cope, 1862), in which genetic lineages that diverged during the Pleistocene now present a low value of niche overlap (Camurugi et al., 2021), and this may be common in species with wide and/or disjunct distributions such as *L. palmipes*.

Mitochondrial lineages in a widely distributed forest amphibian

Despite having found a genetic structure that matches to some degree the geographic limits of both biomes sampled, we also found two mitochondrial lineages with high node support within *L. palmipes* that are not correspondent with its disjunct distribution. One of these lineages is composed of individuals from eastern Amazonia and Atlantic Forest. Despite the distance separating Atlantic Forest and eastern Amazonia, we cannot discard completely the possibility that some gene flow among these localities still occurs, given that some individuals have been found in gallery forests within the Cerrado biome (Oliveira et al., 2010; Ramalho et al., 2011; Santos & Vaz-Silva, 2012). However, moderate Fst values and the recent divergence between lineages suggest the lack of reciprocal monophyly probably results from incomplete lineage sorting (Fujita et al., 2012).

The other *L. palmipes* lineage is composed exclusively of individuals from western Amazonia. An effect of river basins is commonly evoked to explain patterns of genetic structure matching river systems in aquatic and semi-aquatic species (Pearse et al., 2006; Hubert

et al., 2007; Lawson, 2013; Zhang et al., 2020). In contrast, we found *L. palmipes* mtDNA lineages following a west-east pattern in Amazonia, likely resulting from historical climate change and Tapajós river acting as a barrier. During the last 250 thousand years, western Amazonia was somewhat climatically stable, like southern Atlantic Forest (Cheng et al., 2013). In contrast, eastern Amazonia experienced alternating dry and wet cycles and was more similar to Northeastern Atlantic Forest (Cheng et al., 2013). This influenced the biotic interchange between these biomes, probably favouring dispersal between eastern Amazonia and northeastern Atlantic Forest, and between western Amazonia and southern Atlantic Forest (Cheng et al., 2013; Ledo & Colli, 2017). The suggested border between Western/Eastern regions in modern days is the Tapajós river, that possess an ecotonal nature regarding differences in altitude, climate, and vegetation type (Moraes et al., 2020). Such differences are responsible for a marked change in community composition (Moraes et al., 2016), and it is a barrier to gene flow (Pirani et al., 2019). This may also be the cause of a biogeographical pattern in some species separated in west-east lineages at approximately 60°W (close to Tapajós river) in insects (Hall & Harvey, 2002), reptiles (Geurgas & Rodrigues, 2010; Prates et al., 2016b), birds (D'Horta et al., 2013; Rocha et al., 2015), and amphibians (Mota et al., 2020), similar to what we found in *L. palmipes*.

Phylogeographic studies based only on mtDNA tell the gene evolutionary history and not necessarily the complete species history. Because mtDNA is haploid and maternally inherited, it reflects only gene genealogy of matrilineal lineages that may differ from nuclear markers (Shaw, 2002; Godinho, Crespo, & Ferrand, 2008). Due to mtDNA's haploid nature, it has only half of the effective population size compared to nuclear DNA, resulting in faster allele fixation (Ballard & Whitlock, 2004). We acknowledge the limitations of using only mtDNA markers in our study. However, because we found no marked genetic structure in mtDNA despite its tendency of faster allele fixation, it would be unlikely that the addition of nuclear markers would change the trend in our results. Further studies using multiple loci may reinforce the existence of isolation by environment between west and east Amazonian lineages in *L. palmipes*.

Conclusions

Our results highlight the recent northeastern connection between Amazonia and Atlantic Forest used as a dispersal route by *Lithobates palmipes*, a semi aquatic forest associated ranid frog. This is the first evidence of a

Northeastern route being used by an amphibian, suggesting that it sustained environmental conditions suitable for a group with particular habits and ecological requirements from that reported previously in the literature (Prates et al., 2016a). Furthermore, historical climatic heterogeneity in Amazonia seems to have a stronger effect in generating genetic structure in *L. palmipes* than hydrography.

Disclosure statement

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

Supplemental material

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