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LEANDRO J.C.L. MORAES[1,2,*], FERNANDA P. WERNECK[2], ALEXANDRE RÉJAUD[3], MIGUEL T. RODRIGUES[1], IVAN PRATES[4], FRANK GLAW [5], PHILIPPE J.R. KOK[6,7], SANTIAGO R. RON[8], JUAN C. CHAPARRO[9], MARIELA OSORNO-MUÑOZ[10], FRANCISCO DAL VECHIO[1], RENATO S. RECODER[1], SÉRGIO MARQUES-SOUZA[1], ROMMEL R. ROJAS[11], LÉA DEMAY[3], TOMAS HRBEK[12], ANTOINE FOUQUET[3]

[1] *Universidade de São Paulo, Instituto de Biociências, Departamento de Zoologia, São Paulo, SP, Brazil*

[2] *Instituto Nacional de Pesquisas da Amazônia (INPA), Coordenação de Biodiversidade, Av. André Araújo 2936, 69067-375, Manaus, AM, Brazil*

[3] *Laboratoire Evolution et Diversité Biologique, UMR 5174, CNRS, IRD, Université Paul Sabatier, Bâtiment 4R1 31062 cedex 9, 118 Route de Narbonne, 31077 Toulouse, France*

[4] *Department of Ecology and Evolutionary Biology and Museum of Zoology, University of Michigan, Ann Arbor, MI, USA*

[5] *Zoologische Staatssammlung München (ZSM-SNSB), Münchhausenstr. 21, 81247 München, Germany*

[6] *Department of Ecology and Vertebrate Zoology, Faculty of Biology and Environmental Protection, University of Łódź, 12/16 Banacha Str., Łódź 90-237, Poland*

[7] *Department of Life Sciences, The Natural History Museum, London SW7 5BD, United Kingdom*

[8] *Museo de Zoología, Facultad de Ciencias Exactas y Naturales, Pontificia Universidad Católica del Ecuador, Quito, Ecuador*

[9] *Museo de Biodiversidad del Perú, Urbanización Mariscal Gamarra A-61, Zona 2, Cusco, Peru*

[10] *Instituto Amazónico de Investigaciones Científicas SINCHI. Sede enlace. Calle 20 # 5-44, Bogotá, Colombia*

[11] *Facultad de Ciencias Biológicas, Departamento de Ecología y Fauna, Universidad Nacional de la Amazonía Peruana-UNAP, Av. Grau 1072, Iquitos, Peru*

[12] *Universidade Federal do Amazonas, Instituto de Ciências Biológicas, Av. General Rodrigo Octávio Jordão Ramos, 1200, 69080-900, Manaus, AM, Brazil*

* Corresponding author. E-mail: leandro.jclm@gmail.com

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Author contributions: A.F, L.J.C.L.M. and F.P.W. conceived the study. A.F., M.T.R., S.R., J.C.C., F.G., P.J.R.K., M.O.M., R.R.R., T.H., F.D.V., R.S.R. and S.M.S. conducted sampling. A.F., A.R. and L.D. generated DNA sequence data. L.J.C.L.M., A.R. and A.F. analysed the data; L.J.C.L.M. and A.F. led the writing; and all authors reviewed the final draft.

ABSTRACT. Major historical landscape changes have left significant signatures on species diversification. However, how these changes have affected the build-up and maintenance of Amazonia's megadiversity is still debated. Here, we addressed this issue by focusing on the evolutionary history of a pan-Amazonian toad genus that has diversified throughout the Neogene (*Amazophrynella*). Based on a comprehensive spatial and taxonomic sampling (X samples, all nominal species), we delimited Operational Taxonomic Units from mtDNA sequences. We delimited 35 OTUs among which 13 correspond to nominal species, suggesting a vast underestimation of species richness. Next, we inferred time-calibrated phylogenetic relationships among OTUs based on complete mitogenomic data, which confirmed an ancient divergence between two major clades distributed in eastern and western Amazonia respectively. Ancestral area reconstruction analyses suggest that the Andean foothills and the Brazilian Shield region represent the ancient core areas for their diversification. These two clades were likely isolated from each other by lacustrine ecosystems in western Amazonia during the Miocene, and display a pattern of northward and eastward dispersals throughout the Miocene–Pliocene. Given the ecological association of *Amazophrynella* with non-flooded forests, our results reinforces the perception that ancient Amazonian landscape changes had a major impact on the diversification of terrestrial vertebrates.

ADDITIONAL KEYWORDS: Amphibia – biogeography – mitogenomics – Neotropics – Pebas system – *terra firme*

INTRODUCTION

Amazonia has experienced dramatic geomorphologic and climatic changes leading to major landscape changes over the Cenozoic (Hoorn *et al.*, 2010; Albert *et al.*, 2018). Those changes have been invoked to explain this region's high species richness and current biological distributional patterns (Antonelli & Sanmartín, 2011; Leite & Rogers, 2013; Bicudo *et al.*, 2019). However, despite major improvements in our comprehension of these landscape changes (Hoorn *et al.*, 2010; Antonelli *et al.*, 2018), the timing of these changes and how they have affected the diversification of organisms remain unclear. For example, most studies investigating diversification processes within Amazonia focused on birds, which mostly diversified over a relatively recent timeframe (< 5 Ma) within this region (Silva *et al.*, 2019). Other studies have focused on current patterns of community composition in vertebrate groups but did not investigate historical processes (Oliveira *et al.*, 2017; Godinho & da Silva, 2018; Vacher *et al.*, 2020). One of the reasons for the scarcity of biogeographic studies in other animal groups comes from challenges of obtaining a comprehensive spatial sampling due to the difficulty to access many Amazonian regions (Vacher *et al.*, 2020). In fact, the few biogeographic studies investigating the diversification of small terrestrial Amazonian vertebrates such as amphibians and squamates, which supposedly disperse less efficiently than birds and large mammals, generally unraveled much older events on comparable spatial scales, which may suggest a role of ancient historical landscape changes in their diversification (Fouquet *et al.*, 2012a, b, 2014; Kok *et al.*, 2017, 2018; Marques-Souza *et al.*, 2020; Moraes *et al.*, 2020; Réjaud *et al.*, 2020).

During the Neogene (ca. 23–2.5 million years ago–Myr), Amazonia experienced intense geomorphological dynamism related to Andean orogeny in the western portion of the South American plate (Albert *et al.*, 2018; Bicudo *et al.*, 2019). The uplift of this

mountain range notably led to the closing of an estuary at the western end of a hydrographic system running towards the Pacific Ocean some 23 Myr (Hoorn *et al.*, 2010; Bicudo *et al.*, 2019). Consequently, western Amazonia has probably been covered by an enormous lacustrine ecosystem that drained into the Caribbean sea to the north (the current Orinoco drainage) until ca. 9 Myr (the "Pebas System"; Wesselingh & Salo, 2006; Hoorn *et al.*, 2010, 2017). Recent evidence completed the picture by proposing a watershed in western Amazonia segregating a deep aquatic system along the Andes from a central fluviotidal basin covered by extensive seasonally flooded habitats to the east (Bicudo *et al.*, 2019). About 9 Myr, the orogeny of the northern Andes and continuous sedimentation of these aquatic systems, mainly with the young Andean sediments, have ultimately led to a shift of the flow of this proto-Amazon River system toward the Atlantic Ocean (Hoorn *et al.*, 2010). A biologically diverse mega-wetland (the "Acre System"; Latrubesse *et al.*, 2010) has apparently persisted in southwestern Amazonia for ca. 3 million years after the establishment of this eastward flow. Subsequent late Miocene erosion favored the progressive development of non-flooded (*terra firme*) forests and frequent hydrological changes in this region, ultimately leading to the modern configuration of the Amazon River and its tributaries (Albert *et al.*, 2018). It is noteworthy that the timeframe and the amplitude of these changes are still discussed (Hoorn *et al.*, 2010, 2017; Latrubesse *et al.*, 2010) and their consequences on biotic diversification remain elusive.

Anuran amphibians often display distinct biogeographic patterns as compared to other vertebrate taxa because they have finely tuned environmental preferences and functional characteristics often associated with limited dispersal ability (Moraes *et al.*, 2016; Wollenberg-Valero *et al.*, 2019). These characteristics make their populations particularly sensitive to the aforementioned historical events, which ultimately lead to

striking spatial and temporal signatures in their distributional patterns and phylogenetic relationships (Fouquet *et al.*, 2012a, 2014). The tiny toads of the pan-Amazonian genus *Amazophrynella* Fouquet *et al.*, 2012a,b perfectly falls into this description since they all are similarly small-bodied, mostly associated with the leaf-litter of *terra firme* forests, and breed in small temporary ponds (Fouquet *et al.*, 2012a; Rojas *et al.*, 2018). The entire genus appears to display highly conserved ecology, morphology and habitat use (Rojas *et al.*, 2018). Its external morphology is so conserved that until the 1990s, only one described species was supposed to occupy the entire Amazonia. However, the taxonomic knowledge has increased rapidly in recent years, with the description of eleven new species over the past decade (e.g., Rojas *et al.*, 2018; Kaefer *et al.*, 2019; Mângia *et al.*, 2020). Thirteen taxa are currently recognized, which led to the realization that all species of *Amazophrynella* have in fact small and almost completely allopatric ranges (Rojas *et al.*, 2018). Furthermore, phylogenetic analyses based on molecular data suggest that the genus started to diversify as early as 25 Myr with an initial divergence between two major clades largely restricted to geomorphologically and climatically distinct western and eastern Amazonian regions (Fouquet *et al.*, 2012a; Rojas *et al.*, 2018). Subsequent divergences between northern vs. southern lineages in each of these major clades suggest a role of the transcontinental Amazon River as a geographical barrier (Rojas *et al.*, 2018). However, some knowledge gaps persist regarding the actual species richness and phylogenetic relationships within *Amazophrynella*, as numerous recently identified mitochondrial DNA (mtDNA) lineages (Vacher *et al.*, 2020) were not yet included in a phylogenetic reconstruction of the whole genus. In addition, many undersampled areas in Amazonia probably harbor additional species (Fouquet *et al.*, 2012a; Rojas *et al.*, 2018).

Given the ancient origin of *Amazophrynella*, its striking ecological conservatism, and the dynamic landscape of the Amazonia during Neogene, we hypothesize that major geomorphological changes have fragmented and imposed barriers to dispersal for these toads. More specifically, given the early split between a western and an eastern clades within the genus (Rojas *et al.*, 2018), we hypothesize that the development of broad lacustrine ecosystems across western Amazonia has isolated the ancestors of these two major clades along the Andean foothills to the west and the crystalline shields (Guiana and Brazilian shields) to the east, respectively, where they diversified in isolation until ca. 9 Myr. We also hypothesize that the diversification of the western clade after the demise of these lacustrine systems (9 Myr onwards) was linked to the progressive expansion of *terra firme* forests and a dynamic riverine system (Hoorn *et al.*, 2010; Pupim *et al.*, 2019). Conversely, we hypothesize that the diversification of the eastern clade, notably the divergence between species from the Guiana Shield to the north and Brazilian Shield to the south, either predates or is concomitant with the establishment of the transcontinental Amazon River (Hoorn *et al.*, 2010). To test these hypotheses, we gathered an unprecedented spatial sampling throughout Amazonia (286 sequences of the 16S gene), including all of the extant species of *Amazophrynella* plus sequences from newly sampled regions, and reevaluated their boundaries. We then gathered mitogenomic data for most of the delimited species to investigate phylogenetic relationships and historical biogeography within the genus based on ancestral area reconstruction and diversification analyses.

MATERIAL AND METHODS

INPUT DATA CONSTRUCTION

We focused on the 16S mitochondrial gene for the species delimitation analyses, because this gene has been widely used in studies targeting *Amazophrynella* (e.g., Rojas *et al.*, 2018) and is recognized as one of the universal barcodes for Neotropical amphibians (Vences *et al.*, 2005b). We gathered geolocalized 16S sequences from 286 specimens (69 newly acquired and 217 that were previously deposited in GenBank) covering almost the entire distributional range of the genus (Supporting Information, Fig. S1). Details of the 16S sequencing process for newly generated data are presented in the Supporting Information, Appendix A. Regarding previously published data, we found missing blocks and several instances of incongruences among voucher numbers, accession numbers, species labels, and geographic coordinates for GenBank sequences (mostly from Rojas *et al.*, 2018). Therefore, we excluded most of these sequences, keeping only the unambiguous ones and those representing taxa not sampled by us (Supporting Information, Table S1). A summary (most likely conservative) of these incongruences is presented in Supporting Information, Table S2.

We selected a representative terminal for most of the delimited OTUs to build a mitogenomic dataset and reconstruct a time-calibrated phylogenetic hypothesis. Complete mitogenomes were obtained through low-coverage shotgun sequencing for X OTUs. Due to the low coverage in some regions, two of the OTUs were represented by only 13–14 loci from the complete mitogenome (15 loci). For the remaining 13 *Amazophrynella* OTUs for which complete mitogenomes were not available, we gathered all available mitochondrial loci (12S, 16S, COI) from GenBank with unambiguous metadata (Supporting Information, Appendix B). We also incorporated the same outgroups used in the species delimitation

analyses. Complete mitogenomes were already available for nine outgroup bufonid genera in GenBank (*Anaxyrus*, *Bufo*, *Bufotes*, *Duttaphrynus*, *Epidalea*, *Leptophryne*, *Parapelophryne*, *Rhinella* and *Strauchbufo*), and we generated novel complete mitogenome data for seven genera (*Atelopus*, *Dendrophryniscus*, *Frostius*, *Melanophryniscus*, *Oreophrynella*, *Osornophryne* and *Rhaebo*). Lastly, to complete the mitogenomic matrix for the outgroups, we retrieved all available mitochondrial loci (12S, 16S, COI, ND1, ND2 and Cytb) for the remaining four bufonid genera (*Incilius*, *Nannophryne*, *Pedostibes* and *Peltophryne*) (Supporting Information, Table S1). After discarding the d-loop region and tRNAs from mitogenomes, we extracted the rDNA (12S, 16S) and protein-coding genes (ND1, ND2, COI, COII, ATP6, COIII, ND3, ND4L, ND4, ND5, ND6, Cytb). Additional details of the mitogenome sequencing, assembling and annotation are available in the Supporting Information, Appendix B.

SPECIES DELIMITATION

Aiming to circumvent potential ambiguities on current species boundaries in *Amazoprhynella*, we relied on a molecular delimitation of Operational Taxonomic Units (OTUs). We acknowledge that integrative taxonomy, i.e., the integration of multiple lines of evidence such as morphological and acoustic data along with DNA, would be preferable to delimit species (Padial *et al.*, 2010). However, those data are largely missing, and molecular data can provide a yet approximative but effective overview of the species diversity existing in a focal clade (Vences *et al.*, 2005a; Fouquet *et al.*, 2007; Paz & Crawford, 2012).

We aligned the 16S sequences on the MAFFT online server with default parameters except by the use of the E-INS-i strategy, which is indicated for data with multiple

conserved domains and long gaps (Katoh & Standley, 2013).The resulting alignment was used to delimit *Amazophrynella* OTUs based on the combined analysis of three molecular-based species delimitation methods, each with distinct advantages and limitations in recognizing evolutionary lineages (Ratnasingham & Hebert 2013; Luo *et al.*, 2017). These methods included the distance-based Assemble Species by Automatic Partitioning (ASAP; Puillandre *et al.*, 2021), and two tree-based methods, the multi-rate Poisson Tree Processes model (mPTP; Kapli *et al.*, 2017), and the Generalized Mixed Yule Coalescent approach (GMYC; Fujisawa & Barraclough, 2013).

The ASAP delimitation was performed on the online server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) considering a simple distance model to compute the distances between samples, and default parameters. We kept the delimitation scheme supported by the lowest ASAP score (Puillandre *et al.*, 2021). For the mPTP delimitation, we first reconstructed a maximum likelihood (ML) phylogenetic tree with RAxML 8.2.4 (Stamatakis, 2014), running 1,000 nonparametric bootstrap replicates to assess nodal support. The best-fitted model for our dataset was GTR+G+I according to the Bayesian Information Criterion (BIC; Hurvich & Tsai, 1989) in a PartitionFinder 2.1.1 (Lanfear *et al.*, 2017) analysis. However, for the ML inference, we did not consider the estimation of Invariable Sites proportion (*I* parameter) as it prevents reliable estimates of the other parameters (Stamatakis, 2014). We rooted the tree with 18 outgroups, including 12 of the 14 New-World genera of Bufonidae (*Atelopus*, *Dendrophryniscus*, *Frostius*, *Melanophryniscus*, *Rhinella*, *Anaxyrus*, *Incilius*, *Nannophryne*, *Oreophrynella*, *Osornophryne*, *Peltophryne* and *Rhaebo*) and eight Old-World genera (*Bufo*, *Bufotes*, *Duttaphrynus*, *Epidalea*, *Leptophryne*, *Parapelophryne*, *Strauchbufo* and *Pedostibes*) (Supporting Information, Table S1). This sampling accounts for all genera with available

molecular data from the paraphyletic group of "atelopodids" (i.e. taxa branching near the base of the Bufonidae tree; Kok *et al.*, 2018). Using the resulting ML tree, we ran the mPTP delimitation on the EDB-calc cluster (Toulouse, France), with 50 million Markov chain Monte Carlo (MCMC) iterations, sampling every 100,000 iterations and discarding 10% of initial burn-in.

For the GMYC delimitation, we obtained an ultrametric phylogeny by reconstructing a time-calibrated Bayesian tree using the software BEAST 2.6.3 (Bouckaert *et al.*, 2014) with the GTR+G+I substitution model, and using only unique haplotypes. We used a birth-death process to model speciation and extinction (Gernhard, 2008), and an uncorrelated relaxed clock to model evolutionary rate variation among branches (Drummond *et al.*, 2006). In the absence of fossil records for *Amazophrynella* and closely related genera, we calibrated the tree using two secondary node constraints based on time-calibrated anuran phylogenies inferred from comprehensive genomic datasets and fossil calibrations (Feng *et al.*, 2017; Hime *et al.*, 2021). These dates were constrained with a normal prior distribution and included: (1) the crown age of Bufonidae (Mean = 48.0 Myr, SD = 2.5), and (2) the divergence time of *Amazophrynella* + *Dendrophryniscus* vs. remaining bufonids (Mean = 35.4 Myr, SD = 2.4). MCMC parameters were set to four parallel runs with 100 million iterations, 10,000 of thinning and 10% of initial burn-in. We checked the convergence of parameters (ESS > 200) of the combined log file of four runs with TRACER 1.7 (Bouckaert *et al.*, 2014; Rambaut *et al.*, 2018) and extracted the maximum clade credibility tree using TREE ANNOTATOR 2.6.3 (Bouckaert *et al.*, 2014). We performed the multiple threshold GMYC delimitation using only the *Amazophrynella* clade of this ultrametric tree using the GMYC function of the 'splits' R package (Ezard *et al.*, 2014) with a threshold interval between 0–10 Myr.

We defined the distinct Operational Taxonomic Units (OTUs) based on the combined evidence of these three delimitation methods (i.e., congruence between the results of at least two of them), and the stability of the current taxonomic knowledge of the genus (i.e., by considering each of the currently valid species as distinct OTUs). Some of these OTUs included specimens from the type series of nominal species; others specimens could be attributed to nominal taxa because the range of corresponding OTUs spanned the type localities of these taxa (see Supporting Information, Appendix C). Lastly, with MEGA 7 (Kumar *et al.*, 2016), we estimated the mean uncorrected genetic distances (p-distances) among OTUs.

TIME-CALIBRATED PHYLOGENETIC RELATIONSHIPS

We aligned each locus of the mitogenomes independently using the MAFFT online server with default parameters, except for the use of E-INS-i strategy for rDNA, with multiple conserved domain and long gaps, and the G-INS-i strategy for CDS, which is recommended for sequences with global homology (Katoh & Standley, 2013). Coding regions were realigned considering reading frame, and individual alignments were concatenated using GENEIOUS 9.1.8 (Kearse *et al.*, 2012). We estimated the best-fitting partition scheme and model of evolution for each partition comparing the BIC in a PARTITIONFINDER analysis. Our predefined division of this dataset considered a single partition for rDNA and one for each codon position of the protein coding genes (CDS1, CDS2, CDS3). Best-fitted substitution models were GTR+I+G for rDNA, CDS1 and CDS2, and TNR+I+G for CDS3. Using the final alignment, resulting partition schemes and best-fitted substitution models, we reconstructed a time-calibrated Bayesian phylogenetic tree with BEAST. Parameters of the analysis, MCMC runs, and ages for node calibrations

were identical to those described in GMYC species delimitation analysis. We are aware that incorporating mtDNA only in our phylogenetic analyses may lead to overestimation of divergence times (McCormack *et al.*, 2011). However, given the challenges to sample Amazonian organisms comprehensively, we focus on obtaining greater taxonomic and geographical breadth rather than genomic coverage. Our resulting hypotheses can be explicitly tested with the accumulation of knowledge from the integration of future nuDNA information.

BIOGEOGRAPHIC ANALYSES

The time-calibrated mitogenomic tree obtained from the BEAST analysis was used to perform an ancestral area reconstruction using the 'BioGeoBEARS' R package (Matzke, 2013), which infers the geographic distribution of ancestral species and speciation events. As this package requires an attribution of species distributions as proxies of biogeographic regions, we performed two analyses considering different partitioning schemes of Amazonia. Under the combined evidence of these two approaches, we aim at identifying broad and refined geographic patterns of the group diversification, allowing the testing of hypotheses related to ancient and more recent Amazonian landscape changes. First, we considered a broad delimitation of biogeographic regions based on the Wallacean districts (Wallace, 1854), defined as wide units following main geological compartments and landscape features of this region: 1) western Amazonia (WA), corresponding to the sedimentary Solimões basin, and 2) Guiana Shield (GS) and 3) Brazilian Shield (BS), corresponding to the crystalline shields. These units were delimited by the large rivers Negro, Madeira and the lower course of the Amazon, known to correspond to major breaks in amphibian community composition across Amazonia (Godinho & da Silva, 2018;

Vacher *et al.*, 2020). The second partitioning considered a refined delimitation based on "Areas of Endemism", historically defined in Amazonian biogeographic studies of birds and primates (Cracraft, 1985). These areas are mostly limited by the large rivers of the region, as follows (riverine boundaries in parenthesis): Inambari (IN; Huallaga–Madeira), Napo (NA; Japurá–Amazon), Imeri (IM; Japurá–Negro), Guiana (GU; Negro–Amazon, corresponding to the Guiana Shield), Rondonia (RO; Madeira–Tapajós), Tapajos (TA; Tapajós–Xingu) and Xingu (XI; Xingu–Tocantins). Members of *Amazophrynella* are not known to occur in other classic Areas of Endemism, such as Belem (eastwards Tocantins River), at high elevations in the Pantepui region, or from the easternmost Solimões–Negro interfluve (Jaú Area of Endemism) (Borges & Silva, 2012) (Supporting Information, Fig. S1). These areas of endemism were thus not included in our analyses. Considering that OTUs within *Amazophrynella* were spatially restricted, mostly occurring within single areas, we set the maximum number of ancestral areas to two for the broad-partitioning analysis and three for the refined one, and excluded non-adjacent ancestral distributions to narrow down ancestral states.

In 'BioGeoBEARS', three diversification models with distinct premises were compared: Dispersal Extinction Cladogenesis (DEC; Ree & Smith, 2008), Dispersal-Vicariance (DIVALIKE; Ronquist, 1997), and BayArea (BAYAREA; Landis *et al.*, 2013). We also considered those three models including founder-event speciation (J parameter; Matzke, 2013), but because the utility of this parameter has been debated (Ree & Sanmartin, 2018; Klaus & Matzke, 2020), we discussed the differences between the results of best-fitted models considering or not this parameter. Model fit was assessed under the Akaike Information Criterion (AIC). To further investigate the frequency and geographic context of biogeographic events (i.e., vicariance, dispersal, and sympatric speciation), we

conducted a Biogeographical Stochastic Mapping (BSM) analysis implemented in 'BioGeoBEARS' (Dupin *et al.*, 2017). With BSM, we simulated 50 possible biogeographic scenarios accounting for the same pattern of diversification as the best-fit model to obtain an estimate of event frequencies across simulations (mean ± standard deviation). To perform this analysis, we used the same dataset and distinct schemes of spatial partitioning (broad and refined) used in the ancestral area reconstruction analysis.

Finally, we tested if and when the diversification rate of *Amazophrynella* varied through time using Lineage Through Time (LTT) analyses. We conducted a Monte Carlo Constant Rate (MCCR) analysis using the 'LASER' 2.4.1 R package (Pybus & Harvey, 2000; Rabosky, 2006) to test if the observed diversification pattern is significantly different from the expected from a Yule pure-birth model while accounting for randomly distributed missing taxa. Using the same package, we compared the fit of seven models of diversification: two constant-rate Yule models (pure-birth and birth-death), two density-dependent models (DDX and DDL), and the Yule-n-rate model accounting for two, three and four changes in speciation rates across the tree. We determined the best-fit model by comparing AIC values. Using the 'ape' 5.3 R package (Paradis *et al.*, 2004), we plotted the empirical lineage accumulation through time relative to that expected under a Yule pure-birth model with a 95% confidence interval.

DATA AVAILABILITY

Newly generated DNA sequences will be submitted to GenBank upon acceptance of this study (Supporting Information, Table S1). R scripts used for the phylogenetic reconstruction and the biogeographic inferences were the same from Réjaud *et al.* (2020).

Additional data underlying this study are available in the Supporting Information, or directly from the corresponding author upon request.

RESULTS

SPECIES DELIMITATION

The resulting 16S alignment consisted of 491 nucleotide sites. The partitioning from the three species delimitation methods (ASAP, mPTP and GMYC) display rare hard incongruences but extensive difference in their subdivisions. The mPTP method was the most conservative, recovering 15 OTUs, whereas the ASAP and the GMYC methods recovered 39 and 40 OTUs, respectively (Supporting Information, Fig. S2). The mPTP method can be considered overconservative, since nine nominal species were lumped within three OTUs. By contrast, in a few instances the ASAP and GMYC methods split geographically adjacent populations and even samples from the type series of a single nominal species (*A. matses*) into distinct OTUs (Supporting Information, Fig. S2). We therefore attempted to maximize the consensus across these three methods while minimizing seemingly spurious splits by considering the current taxonomic knowledge of the genus and distributions of taxa. Delimitation of OTUs was notably conflictual across methods for the subclade formed by *A. minuta*, *A. siona* and *A. amazonicola* (see below) (Supporting Information, Fig. S2). We delimited those three nominal species as distinct OTUs given their segregation in geographic space and morphological distinctiveness (Rojas *et al.*, 2018). Our delimitation approach resulted in 35 OTUs (Fig. 1) distributed equally across the two major clades, with 18 (western) and 17 (eastern) OTUs (Fig. 1A). With 13 nominal species currently recognized in the genus, this delimitation corresponds to almost a threefold increase in richness.

The minimum mean genetic distance between OTUs according to this delimitation is 2.7 % (corresponding to the comparison between *A. vote* and *A.* OTU10). Intraspecific distances only slightly surpassed this minimum threshold in the case of *A.* OTU2 (3.1 %),

but were mostly below 2.1 % (Supporting Information, Table S3). Geographic distributions of the delimited OTUs revealed a striking allopatric pattern, with very limited overlap among closely related OTUs. Cases of spatial overlap among related OTUs seem more common in eastern Amazonia (a region better represented in our sampling), but most OTUs were found to be micro-endemic or narrowly distributed (Fig. 1B). *Amazophrynella siona* and *A. minuta* from northwestern Amazonia, and *A. manaos* and *A. teko* from the Guiana Shield region, display the widest ranges (Fig. 1B).

PHYLOGENETIC RELATIONSHIPS AND TEMPO OF CLADOGENESIS

The mitogenomic phylogeny was based on an alignment of 13,888 nucleotide sites and 55 terminals. This phylogenetic inference yielded a strongly supported topology with the majority of nodes showing posterior probabilities support > 0.95 (Fig. 2). *Melanophryniscus* was inferred as the sister of all other bufonid genera, followed by a strongly supported clade formed by (*Atelopus* + *Oreophrynella*) and (*Frostius* + *Osornophryne*) that dated back to the Eocene, ca. 37.3 Myr (95% Height Posterior Density [95% HPD] = 32–42.6) (Fig. 2). The Atlantic Forest genus *Dendrophryniscus* was recovered as sister to *Amazophrynella*. In turn, this clade was inferred as sister to a clade encompassing all the remaining bufonid genera, with the divergence between them dating back to the late Eocene (ca. 36.8 Myr, 95% HPD = 32.6–40.9). The results corroborate an ancient history of diversification for *Amazophrynella*, dating back to 23 Myr (95% HPD = 19.3–26.6) (Fig. 2).

Our phylogenetic results also recovered the monophyly of *Amazophrynella* and two major clades within the genus (Fig. 2), largely restricted to the western and eastern portions of Amazonia. The only and noteworthy exception is *A.* OTU22 from the Guiana Shield,

recovered as nested within the western clade. The divergence of the western and eastern clades coincides with the Paleogene–Neogene transition (Oligocene–Miocene ca. 23 Myr; 95% HPD = 19.3–26.6), which is relatively older than most crown ages of other bufonid genera (Fig. 2). The crown ages of the two major *Amazophrynella* clades both date back to the middle Miocene, at ca. 14.9 Myr (95% HPD 12.2–17.7) for the western clade and 16.1 Myr (95% HPD = 12.7–19.5) for the eastern clade.

In the western clade, northernmost OTUs are nested in a subclade with representatives of midwestern and southwestern Amazonia, which is sister of a subclade exclusively composed of OTUs from the southwestern region (Fig. 2). This pattern is similar within the eastern clade, with OTUs from northeastern Amazonia more closely related to one of the two subclades distributed in the southeastern region (Fig. 2). Cases of reciprocal monophyly between OTUs from the northern and southern banks of the Amazon River were found within both the eastern and western clades. These divergences date back to the middle Miocene, at ca. 12.2 Myr (95% HPD 9.8–14.6) for the western clade and 13.4 Myr (95% HPD 10.5–16.5) for the eastern clade.

Diversification rates slightly differed between the western and eastern clades. Cladogenesis within the western clade seems to have taken place at a relatively constant pace, whereas the eastern clade seems to have diversified during a more recent period (late Miocene; < 10 Myr), mostly within two subclades (Fig. 2). The most recent divergences occurred in the eastern clade, during the late Pliocene (ca. 2.8–2.9 Myr). In the western clade, recent divergences are overall older, during the Miocene-Pliocene transition, and concentrated in northwestern Amazonia (Figs. 1, 2).

HISTORICAL BIOGEOGRAPHY

Of the six biogeographic models compared in the 'BioGeoBEARS' analyses, the DIVALIKE+J and DEC+J produced the best statistical fit to the data for the broader and refined regionalization schemes, respectively (Supporting Information, Table S4). We interpret the historical biogeography of *Amazophrynella* based on the combination of these two results (Fig. 3; Supporting Information, Fig. S3). Nevertheless, we also compared the results obtained by the best-fit models for each partitioning approach without the consideration of parameter 'J', namely the DIVA model for the broader partitioning and DEC for the refined one (Supporting Information, Table S4).

The most recent common ancestor (MRCA) of the genus *Amazophrynella* (ca. 23 Myr, 95% HPD 19.3–26.6) likely occupied the southwestern Amazonia, more specifically in the interface of the Inambari and Rondonia, which currently corresponds to the area delimited northward by the upper Amazon River and eastward by the Tapajós River. An ancient dispersal/vicariance event led to the split between the western and eastern major clades within the genus. These clades subsequently diversified within distinct areas, along the Andean foothills in southwestern Amazonia (Inambari) and at the interface of the Inambari and Rondonia, respectively (Fig. 3; Supporting Information, Fig. S3). Lineages from the western clade have secondarily dispersed twice northward during the Miocene, reaching the Napo and ultimately the Imeri regions. This clade has also dispersed toward the Guiana Shield some 7 Myr (Fig. 3; Supporting Information, Fig. S3). In turn, lineages within the eastern clade dispersed from around the Inambari and Rondonia regions (western Brazilian Shield) toward the Guiana Shield during the Miocene (ca. 13.4 Myr, 95% HPD 10.5–16.5), and twice eastward: an older dispersal event at ca. 5.6 Myr (95% HPD 3.8–7.5), and a more recent event at ca. 3 Myr (95% HPD 1.1–5.8) (Fig. 3; Supporting Information, Fig. S3). This last dispersal phase displays a stepping-stone pattern, with lineages

446 dispersing from the Rondonia toward the Tapajos, and subsequently east into the Xingu
447 (Fig. 3; Supporting Information, Fig. S3).

448 Results of the best-fitted models not including the 'J' (jump dispersal) parameter
449 inferred similar ancestral areas relative to models incorporating this parameter (Supporting
450 Information, Fig. S4). However, by removing the possibility of jump dispersal, the most
451 probable ancestral areas became more ambiguous. Models without 'J' also inferred that the
452 MRCA of *Amazophrynella* most likely occupied the southwestern Amazonia region
453 (Supporting Information, Fig. S4), and that a vicariant event led to the split between its
454 western and eastern major clades in the early Miocene. Vicariant events were also implied
455 in the split between the Guiana Shield and Brazilian Shield clades (within the eastern
456 clade), as well as in the diversifications events in northwestern Amazonia involving the
457 Napo and Imeri (Supporting Information, Fig. S4).

458 According to BSM analyses, most of the speciation events within *Amazophrynella*
459 occurred *in-situ* (i.e., within biogeographic areas), considering both the broad and refined
460 spatial partitioning (29.8 ± 0.4 and 20 ± 0.8 events, respectively). For the broad scale
461 scheme, 15.4 ± 0.9 *in-situ* speciation events have occurred within western Amazonia.
462 Dispersal events mostly occurred from the Brazilian Shield toward western Amazonia
463 (Purus–Madeira interfluve; 3.3 ± 0.9 events). Nevertheless, the Guiana Shield was found to
464 be the major dispersal receiver resulting in speciation (FE: 0.8 ± 0.1).

465 Considering the refined spatial partitioning, the Inambari and Napo harbored the
466 highest number of *in-situ* speciation events (5.4 ± 1.4 and 2.9 ± 0.9 events, respectively).
467 Fewer speciation events occurred in the eastern clade, in which most *in-situ* speciation
468 occurred within the Brazilian Shield (9.4 ± 0.9 events). Within the Brazilian Shield, most
469 *in-situ* speciation events occurred in its westernmost part (Rondonia; 4.2 ± 1.2 events), with

a decrease in speciation toward the east, reaching 1.5 ± 0.6 events within the Tapajos and no speciation within the Xingu (Supporting Information, Table S5). The Andean foothills (Inambari and Napo) likely acted as the major source of dispersal events within the western clade (0.3 ± 0.3 and 0.3 ± 0.1 events, respectively). Within the eastern clade, the Rondonia and Tapajos likely were the major cores of dispersal events (0.3 ± 0.3 and 0.2 ± 0.2 events, respectively) (Supporting Information, Table S5).

DIVERSIFICATION THROUGH TIME

Diversification of *Amazophrynella* differed significantly from the expected accumulation of lineages under a constant diversification model over time (γ = -2.72, $p < 0.01$) (Fig. 3c). Nevertheless, the overall diversification pattern as illustrated by a Lineage Through Time graph (Fig. 3c) indicates constant diversification (matching the null model) until ca. 4 Myr, when an increase in cladogenesis occurred. Supporting this pattern of nearly constant diversification followed by a rapid and relatively recent increase, we found the best-fit diversification model to be the Yule pure-birth four-rate model. This model showed a constant rate of lineage accumulation (r = 0.17) followed by an increase in diversification rate (r = 0.64) at ca. 4 Myr (Supporting Information, Table S6) and a decrease after ca. 3 Myr (r = 0.01). However, the shift in the diversification rate detected ca. 4 Myr is probably produced by the absence of terminal branches during that time frame which is at least partly inherent to the DNA-based method of delimitation and thus artifactual, and to the absence of nominal species that formed recently which could be also related to coarse-grained taxonomy.

DISCUSSION

SPECIES DELIMITATION

Our DNA-based species delimitation, which resulted in 22 OTUs on top of the 13 described species, suggests a vast underestimation of the species richness in *Amazophrynella*. This underestimation is especially evident in southwestern Amazonia and in the Brazilian Shield, where more than 70% and 66% of the respective diversity may be not yet formally described. These observations, along with a pattern of completely non overlapping narrow geographic ranges, suggest that remaining sampling gaps probably harbor additional candidate species, notably in Bolivia, Venezuela, Colombia, and southwestern Brazilian Amazonia (Supporting Information, Fig. S1). Bolivian populations (see De la Riva, 1999) are of special interest because this region may have acted as a dispersal route between Amazonia (which eventually gave origin to *Amazophrynella*) and the coastal Atlantic Forest (*Dendrophryniscus*).

The underestimation of species diversity has been repeatedly highlighted in studies of Amazonian amphibians. A recent estimate found that about 40–50% of the species inhabiting this region remain to be named and described (Vacher *et al.*, 2020). According to the present study, this number is even higher for *Amazophrynella* (ca. 62 %). Relative to other amphibian genera widespread in lowland Amazonia that also started to diversify during the early Neogene, undescribed diversity within *Amazophrynella* is comparable to that in *Adenomera* (57%, Fouquet *et al.*, 2014), higher than in *Allobates* (21%; Réjaud *et al.*, 2020) and lower than in *Synapturanus* (83%; Fouquet *et al.*, 2021). Such variation in unrecognized diversity in clades of similar age may be related to differential ecological factors and dispersal ability affecting their diversification (Rabosky, 2009; Peterson *et al.*,

2011; Miller *et al.*, 2021), but also to the variable effort undertaken on their taxonomic resolution (Fouquet *et al.*, 2021).

Our species delimitation analyses also led to ambiguous OTU boundaries in several instances, especially among some recently diverging lineages from northwestern Amazonia. Such discrepancy was likely influenced by the small size of our focal mtDNA locus and the absence of nuDNA, morphological, and acoustic data (see Miralles & Vences, 2013). Based on these limitations, we advocate caution over strict interpretation of our delimitation results in the case of such ambiguous boundaries. Nevertheless, the results of our ancestral area reconstruction analyses should be reliable because most of the conflicting OTU boundaries involved geographically close populations that occur within the same broad biogeographic region.

LANDSCAPE EVOLUTION INFERRED FROM THE *AMAZOPHRYNELLA* DIVERSIFICATION

The ancestors of the western and eastern major clades of *Amazophrynella* were most likely respectively isolated along the eastern foothills of the Andes and on the western Brazilian Shield some 23 Myr. This ancient division of this genus into two major clades at the Oligocene–Miocene transition was also suggested by previous divergence time analyses (Rojas *et al.*, 2018). This timeframe is concomitant with the emergence of a vast lacustrine system and other mega wetlands in western Amazonia, due to the combined effect of the uplift of the Andean mountain range and a western depression of the continental plate (Hoorn *et al.*, 2010; Bicudo *et al.*, 2019). These mega wetland systems may have acted as relevant geographical barriers by segregating populations strongly associated with *terra firme* forests and narrow ecological niche breadth (Hoorn *et al.*, 2010), as is the case of *Amazophrynella* spp. Such a pattern of ancient lineage segregation between western and

539 eastern lineages is also evident in the diversification history of other Amazonian groups,
540 like the anuran genera *Allobates* (Réjaud *et al.*, 2020) and *Adenomera* (Fouquet *et al.*,
541 2014) and lizard genera *Alopoglossus* (Ribeiro-Júnior *et al.*, 2020), *Kentropyx* (Sheu *et al.*,
542 2020), and *Chatogekko* (Geurgas & Rodrigues, 2010).

543 Within each of the two major clades of *Amazophrynella*, we found a consistent
544 pattern of northern Amazonian subclades originating through dispersal events from
545 southern Amazonia, before the establishment of the transcontinental Amazon River (10–9
546 Myr according to Hoorn *et al.*, 2010, 2017 or 5–3 Myr according to Latrubesse *et al.*,
547 2010). This contradicts a previous biogeographic interpretation that attributed these splits to
548 a vicariant event triggered by the emergence of the Amazon River (Rojas *et al.*, 2018).
549 Nevertheless, the diversification history of *Amazophrynella* still suggests a major role of
550 the Amazon River as a secondary geographical barrier, as the establishment of this river
551 likely limited the dispersion and genetic interchange between northern and southern
552 populations since the middle Miocene. This timing is therefore consistent with the "old
553 origin" hypothesis for the establishment of the Amazon River (Hoorn *et al.*, 2010, 2017).
554 This riverine barrier may have favored *in-situ* diversification throughout northern and
555 southern Amazonia, mirroring what has been suggested for the effects of large rivers on the
556 diversification of *terra firme* Amazonian birds (Naka & Brumfield, 2018).

557 During the middle Miocene (ca. 15–10 Myr), the western clade of *Amazophrynella*
558 underwent an initial diversification along the southern part of the Andean foothills and
559 progressively dispersed toward the north and diversified northward. This is concomitant
560 with the uplift of the Fitzcarrald Arch and the southwestern to northwestern perimontane
561 accumulation of Andean sediment as a result of continuous orogeny (Espurt *et al.*, 2010;
562 Hoorn *et al.*, 2010). Both events may have facilitated *Amazophrynella* range expansion due

563 to the development of *terra firme* forests in westernmost Amazonia. At the same time, the
564 eastern clade also expanded its range to the east and north into the Guiana Shield, the latter
565 probably via an upland route connecting these regions (Purus Arch) (Hoorn *et al.*, 2010).
566 The west-east inversion of the Amazon watershed (Hoorn *et al.*, 2010, 2017; Latrubesse *et*
567 *al.*, 2010) ultimately prevented any further dispersals between the Guiana Shield and
568 Brazilian Shield. Similar to the western clade, east- and northward range expansions within
569 the eastern clade were concomitant with the demise of mega wetlands and the development
570 of *terra firme* forests (Bicudo *et al.*, 2019).

571 Posterior diversification events within *Amazophrynella* (< 10 Myr) include the
572 unexpected dispersal of a lineage embedded within the western subclade into the Guiana
573 Shield during the late Miocene (ca. 7 Myr). This apparently long dispersion event may be
574 explained by possible extinction of intervening populations, or simply by a bias of
575 undersampling. Either way, it implies a trans-Amazonian dispersal, considering that this
576 river was already established at that time (sensu Hoorn *et al.*, 2010, 2017). Considering that
577 internal areas of the western sedimentary basin are only recently more suitable for the
578 expansion of *terra firme* lineages such as *Amazophrynella* (Pupim *et al.*, 2019), this
579 dispersal event may have occurred via a northwestern route, possibly through the Vaupes
580 Arch, a concomitant northern watershed connecting the uplands of western and eastern
581 Amazonia (Mora *et al.*, 2010). In fact, given the strong habitat association of this genus, the
582 western clade diversification is consistent with progressive development of *terra firme*
583 forests towards the east, as a result of continuous Andean sediment influx and lowering of
584 the river channels after the demise of the lacustrine systems (Pupim *et al.*, 2019). This is
585 also supported by the fact that the most recent lineages of the western clade are confined to
586 the region once filled by the mega wetland systems, corroborating the pattern observed in

other amphibian diversification histories (Fouquet *et al.*, 2014; Réjaud *et al.*, 2020) and a concentration of recent and phylogenetically closer bird lineages in this region (Bicudo *et al.*, 2019; Crouch *et al.*, 2019). It is also noteworthy that, even though the undersampling of Amazonia hampers firm interpretation (Vacher *et al.*, 2020), *Amazophrynella* populations are possibly absent from the innermost western Amazonia sedimentary basin (Supporting Information, Fig. S1), where *terra firme* forests are more recent (Pupim *et al.*, 2019). In addition, no *Amazophrynella* population has been reported to date from the Branco River basin and the easternmost Solimões-Negro interfluve (Supporting Information, Fig. S1), the latest being considered as an area of endemism for birds (Jaú; Borges & Silva, 2012). A combination of recent development of *terra firme* forests and prevalence of more open habitats in this region (Adeney *et al.*, 2016), as well as the existence of a riverine barrier connecting the Japurá River to the Negro River until very recently (ca. 1,000 years ago; Ruokolainen *et al.*, 2019), may have prevented the range expansion of the western clade of *Amazophrynella* into the innermost northwestern Amazonia.

Conversely, the Eastern subclade broadly expanded its range to the east during the same timeframe as the western clade (< 10 Myr). Instead of a greater geomorphological influence controlling the development of *terra firme* forests, major changes in vegetation cover in this region have been especially affected by climatic variations over time, with drier glacial periods likely changing the structure of forests (Cheng *et al.*, 2013). *Amazophrynella* have possibly dispersed eastward following humid forest development during favorable climatic conditions. However, they maintained low diversification rates until ca. 6–5 Myr, when the putative combined influence of drainage rearrangements of the tributaries of the Amazon River (Latrubesse, 2002; Rossetti, 2014; Hayakawa & Rossetti, 2015; Moraes *et al.*, 2020) and cyclical unfavorable climatic conditions (Cheng *et al.*,

2013) may have promoted a rapid accumulation of new lineages. These factors may also explain a higher stasis on the diversification of the Guiana Shield clade over time, as this region currently presents a lower occurrence of large tributaries of the Amazon River and has been geomorphologically more stable over time (Bicudo *et al.*, 2019).

ECOLOGICAL CONSERVATISM AND DIVERSIFICATION RATES

Increasing diversification rates are generally associated with the acquisition of evolutionary novelties that allow the exploration of new ecological opportunities (Erwin, 2015). Therefore, the nearly continuous and stable diversification rate seen in *Amazophrynella* is in accordance with its extreme phenotypic conservatism and overall conserved ecology (Rojas *et al.*, 2018). Even with the putative new ecological opportunities arising from the dynamic Amazonian landscape evolution of the Miocene, such conservatism most likely limited *Amazophrynella* dispersal and possibly fostered lineage extinction in unsuitable regions (Rabosky, 2009; Peterson *et al.*, 2011).

Biogeographic studies using Amazonian amphibians as model systems commonly support ancient timeframes for their initial diversification (e.g., Santos *et al.*, 2009; Castroviejo-Fisher *et al.*, 2014; Fouquet *et al.*, 2014; Sá *et al.*, 2019; Réjaud *et al.*, 2020; Fouquet *et al.*, 2021), and this is also the case for *Amazophrynella*. Such ancient timeframes are somewhat incongruent with the relatively more recent ones reported for other vertebrates, such as some birds (e.g., Silva *et al.*, 2019) and primates (e.g., Alfaro *et al.*, 2015), but also for other amphibians (e.g., Jaramillo *et al.*, 2020). Changes in the permeability of riverine barriers until recently (Plio-Pleistocene) may have led to a relatively higher frequency of dispersal events in the evolutionary history of vertebrate groups with higher vagility, which may also have involved continuous adaptation to

different habitats (Smith *et al.*, 2014; Pirani *et al.*, 2019). These processes may explain higher and more recent lineage accumulation during the Neogene for these groups compared to ecologically conserved and dispersal-limited amphibians. Therefore, based on the evidence for *Amazophrynella*, we suggest that a combination of narrow habitat associations and greater dispersal limitation led to stronger signatures of ancient landscape changes on the history of biological diversification.

CONCLUSION

In summary, our results provide a reevaluation of species richness within *Amazophrynella* and their respective distributions. Moreover, they provide insights on the historical biogeography of these tiny toads, which is consistent with proposed landscape changes in Amazonia throughout Neogene. Given the extreme ecological association of *Amazophrynella* with *terra firme* forests, our results corroborate most of the hypothesized spatial and temporal evolution of these habitats across the Amazonian landscape. The historical biogeography of *Amazophrynella* largely agrees with a progressive transition of lacustrine and fluviotidal systems to *terra firme* forest habitats at western Amazonia during the Neogene, as well as to a Miocene origin of the transcontinental Amazon River. These results reinforce the perception that ancient Amazonian landscape changes, such as the emergence of broad western lacustrine ecosystems and the longitudinal drainage transition, had a major impact on the diversification of terrestrial vertebrates.

ACKNOWLEDGEMENTS

This study benefited from an "Investissement d'Avenir" grant managed by the Agence Nationale de la Recherche (CEBA, ANR-10-LABX-25-01; TULIP, ANR-10-LABX-0041; ANAEE-France, ANR-11-INBS-0001), and the French/Brazilian GUYAMAZON program (IRD, CNRS, CTG, CIRAD and Fundação de Amparo à Pesquisa do Estado do Amazonas-FAPEAM; #062.00962/2018), co-coordinated by AF and FPW. LJCLM thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES for a scholarship (#88887.630472/2021-00). MTR thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; grants: #2003/10335-8, #2011/50146-6), and NSF-FAPESP Dimensions of Biodiversity Program (grants: BIOTA #2013/50297-0, NSF-DEB #1343578) and NASA. SRR acknowledges a grant from SENESCYT (Arca de Noé Initiative). FPW thanks CNPq (Productivity Fellowship), CAPES (Visiting Professor Fellowship), FAPEAM, and the L'Oréal-UNESCO for Women in Science Program. PJRK thanks the Fonds voor Wetenschappelijk Onderzoek (grants FWO12A7614N and FWO12A7617N). We thank B. Noonan (UM), T. Grant (USP), D. Dittmann (LSU), J. Gomes (MPEG), T. C. Ávila-Pires (MPEG), S. Katanova (AMNH), D. A. Kizirian (AMNH), A. Crawford (UNIANDES), J. M. Padial (AMNH), A. A Silva, C. C. Ribas and M. Freitas (INPA), and S. Castroviejo-Fisher (PUCRS) for their contribution with material used in this work, and S. Manzi and U. Suescun for their help in the laboratory procedures. We are also thankful to L. Pinheiro (UFPA), Amy Lathrop (ROM), C. Spencer (MVZ), A. Acosta Galvis (IAvH-Am), J. Courtois (MNHN), and A. Ohler (MNHN) for providing relevant information on museum specimens; and to Igor L. Kaefer (UFAM) for kindly sharing the original material sequenced for *A. bilinguis*.

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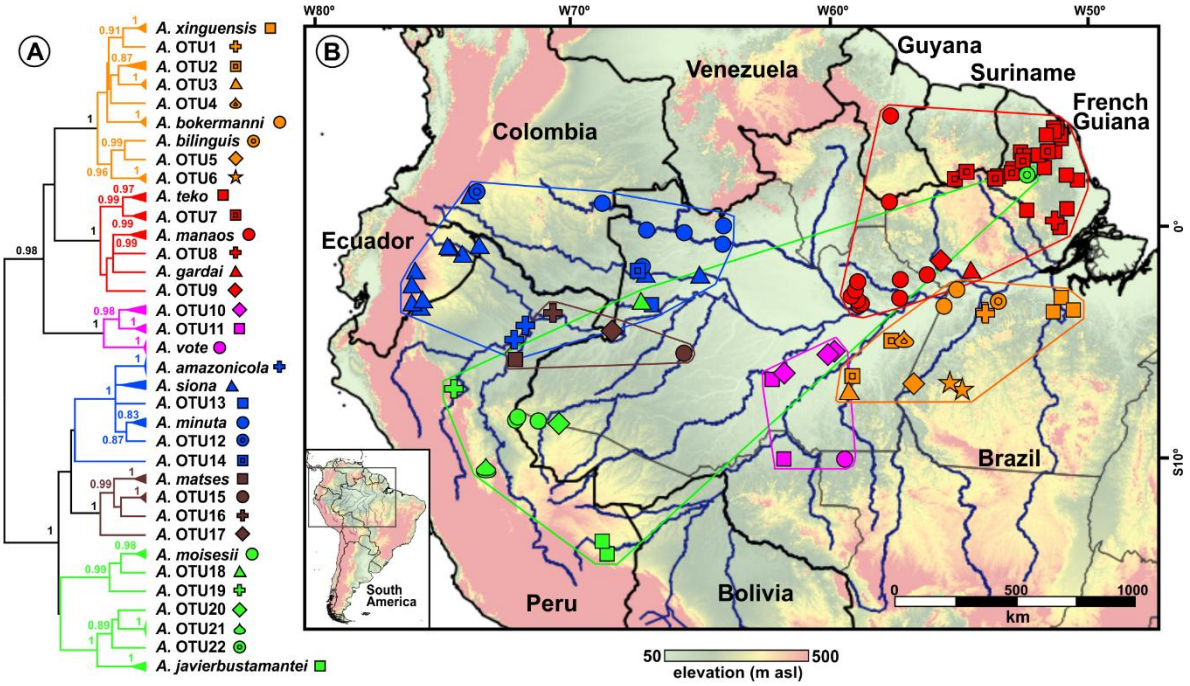
990 **FIGURES**

991



992

993 **Figure 1.** Phylogenetic relationships (A) and geographic distributions (B) of Operational

994 Taxonomic Units (OTUs) inferred within *Amazophrynella*. The phylogenetic tree was

995 inferred through Bayesian optimality criteria. Nodal support values are shown close to the

996 branches (posterior probabilities below 0.8 omitted). The geographic distributions of OTUs

997 (symbols in B) are colored according to the main genetic clusters (colors in A).

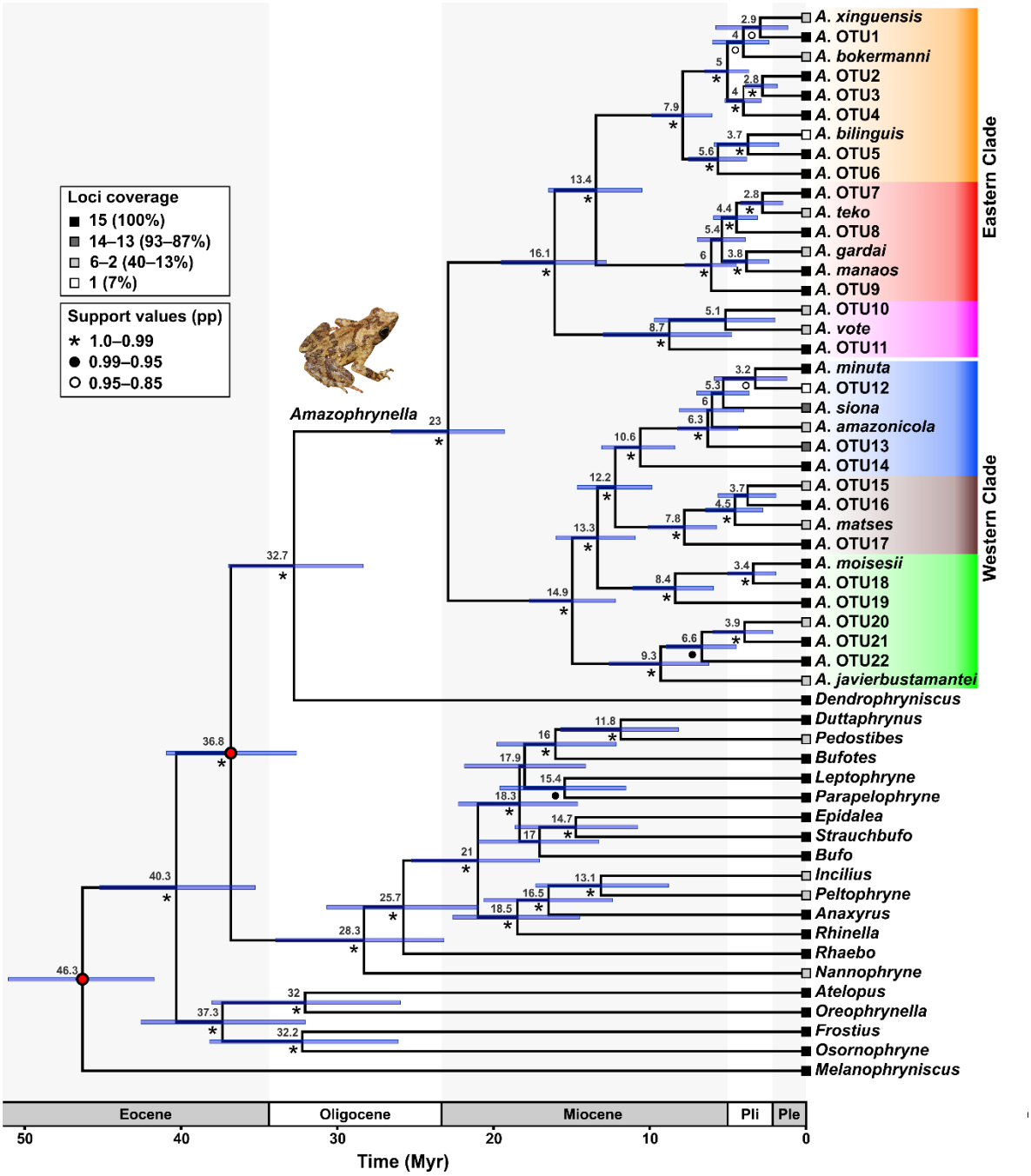
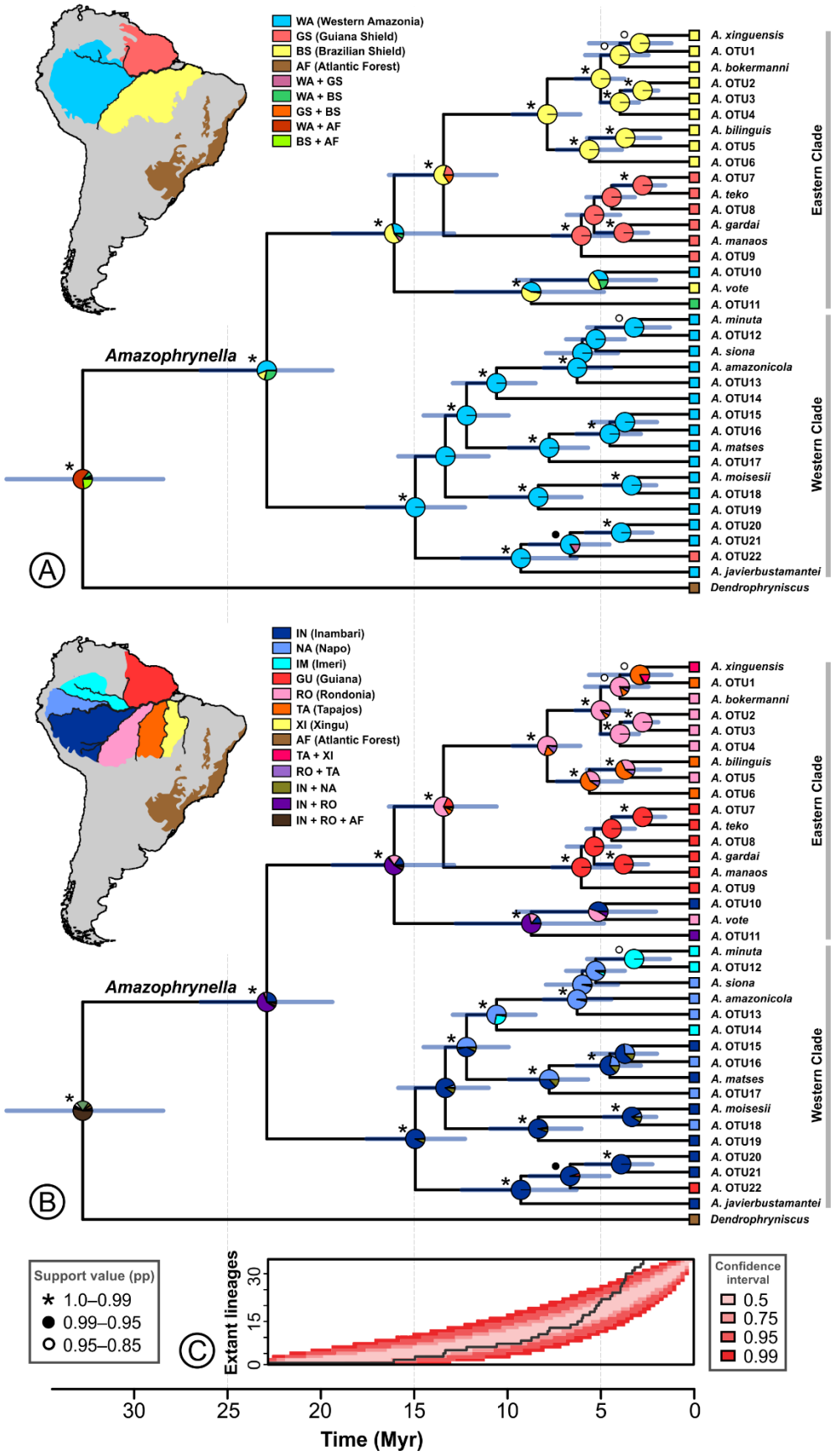


Figure 2. Bayesian mitogenomic time-calibrated phylogenetic tree of the family Bufonidae, with a focus on relationships within *Amazophrynella*. Nodal support values are shown in symbols below branches (posterior probabilities below 0.85 omitted), and loci coverage for each terminal is shown in the gray-scale squares on the tips of the tree; both are detailed in the inset legends. Mean value of estimated time for cladogenetic events is presented above

1004 branches, and blue horizontal bars on nodes correspond to their 95% HPD. Red dots
1005 highlight the calibrated nodes (see Material and Methods). Colors of OTUs correspond to
1006 those in Fig. 1. Geological epochs: (Pli) Pliocene; (Ple) Pleistocene-Holocene.


WA (Western Amazonia)
GS (Guiana Shield)
BS (Brazilian Shield)
AF (Atlantic Forest)
WA + GS
WA + BS
GS + BS
WA + AF
BS + AF
A. xinguensis
A. OTU1
A. bokermanni
A. OTU2
A. OTU3
A. OTU4
A. bilinguis
A. OTU5
A. OTU6
A. OTU7
A. teko
A. OTU8
A. gardai
A. manaos
A. OTU9
A. OTU10
A. vote
A. OTU11
A. minuta
A. OTU12
A. siona
A. amazonicola
A. OTU13
A. OTU14
A. OTU15
A. OTU16
A. matses
A. OTU17
A. moisesii
A. OTU18
A. OTU19
A. OTU20
A. OTU21
A. OTU22
A. javierbustamantei
Dendrophryniscus
Eastern Clade
Western Clade
Amazophrynella
A
IN (Inambari)
NA (Napo)
IM (Imeri)
GU (Guiana)
RO (Rondonia)
TA (Tapajos)
XI (Xingu)
AF (Atlantic Forest)
TA + XI
RO + TA
IN + NA
IN + RO
IN + RO + AF
B
Support value (pp)
1.0–0.99
0.99–0.95
0.95–0.85
C
Extant lineages
0
15
30
Confidence interval
0.5
0.75
0.95
0.99
30
25
20
15
10
5
0
Time (Myr)

1007

Figure 3. Biogeographic history of the genus *Amazophrynella* inferred from 'BioGeoBEARS' optimization on the mitogenomic Bayesian chronogram (Fig. 2). The best-fit models were DIVALIKE+J for the broad spatial partitioning (Wallacean districts) (A) and DEC+J for the refined one (Areas of Endemism) (B). Most likely ancestral areas shown as likelihood pie charts on nodes. The current distribution of Operational Taxonomic Units is depicted as squares at the tips of the trees, colored according to the inset legends. The inset maps show the biogeographic areas used; for details on their riverine boundaries, see text. Combinations of areas are considered in the respective analyses but not depicted on the maps, and only the most likely area is presented. Nodal support values are shown as symbols above branches, detailed in the inset legend (posterior probabilities below 0.85 omitted). Blue horizontal bars on nodes correspond to the 95% HPD of time estimates. In (C), the temporal pattern of lineage accumulation within *Amazophrynella*, inferred with a Lineage Through Time (LTT) plot using the same Bayesian chronogram (Fig. 2). The red gradient in (C) indicates the confidence intervals of expected lineage accumulation under a Yule pure-birth diversification model, and the grey line represents the empirical data.

SUPPORTING INFORMATION

Appendix A. DNA extraction and 16S data acquisition

Appendix B. Mitogenome sequencing, assembling and annotation

Appendix C. Taxonomic considerations

Table S1. Samples included in the analyses

Table S2. Summary of incongruences in GenBank data

Table S3. Mean uncorrected pairwise genetic distances among OTUs

Table S4. Summary statistics for each of the models fit to *Amazophrynella* diversification in 'BioGeoBEARS' analyses

Table S5. Summary results for number and types of dispersal events during *Amazophrynella* diversification according to Biogeographical Stochastic Mapping analyses

Table S6. Summary statistics for each of the models fit to *Amazophrynella* diversification in Lineage Through Time analyses

Figure S1. Known distribution records of *Amazophrynella*

Figure S2. Results of species delimitation analyses

Figure S3. Hypothetical reconstruction of *Amazophrynella* diversification based on ancestral area reconstruction analyses and geomorphological changes in Amazonian landscape during the Neogene–Quaternary

Figure S4. Biogeographic history of the genus *Amazophrynella* from 'BioGeoBEARS' optimization on the mitogenomic Bayesian chronogram without considering the 'J' parameter