

Diversification of tiny toads (Bufonidae: *Amazophrynella*) sheds light on ancient landscape dynamism in Amazonia

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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 continues to be 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 (286 samples, all nominal species), we delimited operational taxonomic units (OTUs) from mitochondrial DNA 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, probably isolated from one other by lacustrine ecosystems in western Amazonia during the Miocene, display a pattern of northward and eastward dispersals throughout the Miocene–Pliocene. Given the ecological association of *Amazophrynella* with non-flooded forests, our results reinforce the perception that ancient Amazonian landscape changes had a major impact on the diversification of terrestrial vertebrates.

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INTRODUCTION

Amazonia has experienced dramatic geomorphological 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 the 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 understanding 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 have focused on birds, which mostly diversified over a relatively recent timeframe (<5 Mya) within this region (Silva *et al.*, 2019). Other studies have focused on current patterns of community composition, as opposed to historical processes, in vertebrate groups (Oliveira *et al.*, 2017; Godinho & da Silva, 2018; Vacher *et al.*, 2020). One of the reasons for the scarcity of biogeographical studies in other animal groups comes from challenges of obtaining a comprehensive spatial sampling due to the difficulty in accessing many Amazonian regions (Vacher *et al.*, 2020). In fact, the few biogeographical 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 revealed 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; Kók *et al.*, 2017, 2018; Marques-Souza *et al.*, 2020; Moraes *et al.*, 2020; Réjaud *et al.*, 2020).

During the Neogene (~23–2.5 Mya), 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 Mya (Hoorn *et al.*, 2010; Bicudo *et al.*, 2019). Consequently, western Amazonia was probably covered by an enormous lacustrine ecosystem that drained into the Caribbean sea to the north (the current Orinoco drainage) until ~9 Mya (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 fluviotidal basin

covered by extensive seasonally flooded habitats to the east (Bicudo *et al.*, 2019). About 9 Mya, the orogeny of the northern Andes and continuous sedimentation of these aquatic systems, mainly with young Andean sediments, 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 megawetland (the 'Acre System'; Latrubesse *et al.*, 2010) has apparently persisted in south-western Amazonia for ~3 Myr after the establishment of this eastward flow. Subsequent late Miocene erosion favoured 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 being discussed (Hoorn *et al.*, 2010, 2017; Latrubesse *et al.*, 2010) and their consequences on biotic diversification remain elusive.

Anuran amphibians often display distinct biogeographical 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 led 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 fall perfectly 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 a 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 considered to occupy the entire Amazonia. However, taxonomic knowledge of the genus has increased rapidly in recent years, with the description of 11 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 has led to the realization that all species of *Amazophrynella* in fact have 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 Mya 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 and 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*, and numerous recently identified mitochondrial DNA (mtDNA) lineages (Vacher *et al.*, 2020) have not yet been included in a phylogenetic reconstruction of the genus as a whole. In addition, many undersampled areas in Amazonia probably harbour 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 Amazonia during the 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 clade 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 shield areas (Guiana and Brazilian shields) to the east, respectively, where they diversified in isolation until ~9 Mya. We also hypothesize that the diversification of the western clade after the demise of these lacustrine systems (9 Mya onwards) was linked to the progressive expansion of *terra firme* forests and a dynamic riverine system (Hoorn *et al.*, 2010; Pupim *et al.*, 2019). Additionally, 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 pre-dates 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 re-evaluated 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

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 Appendix A. Regarding previously published data, we found missing blocks and several instances of incongruences among voucher numbers, accession numbers, species labels and geographical 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 (Table S1). A summary of these incongruences is presented in Table S2.

We selected a representative terminal for most of the delimited operational taxonomic units (OTUs) to build a mitogenomic dataset and reconstruct a time-calibrated phylogenetic hypothesis. Complete mitogenomes were obtained through low-coverage shotgun sequencing for 22 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, only keeping the ones 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*) (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 Appendix B.

SPECIES DELIMITATION

Aiming to circumvent potential ambiguities on current species boundaries in *Amazophrynella*, we relied on a molecular delimitation of 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 an approximate 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 for use of the E-INS-i strategy, which is indicated for data with multiple conserved domains and long gaps (Kato & 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 1000 non-parametric bootstrap replicates to assess nodal support. The best-fitting 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 the proportion of invariable sites (*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 (*Anaxyrus*, *Atelopus*, *Dendrophryniscus*, *Frostius*, *Incilius*, *Melanophryniscus*, *Nannophryne*, *Oreophrynella*, *Osornophryne*, *Peltophryne*, *Rhaebo* and *Rhinella*,) and eight Old World genera (*Bufo*, *Bufotes*, *Duttaphrynus*, *Epidalea*, *Leptophryne*, *Parapelophryne*, *Pedostibes* and *Strauchbufo*) (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% 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 Mya, SD = 2.5) and (2) the divergence time of *Amazophrynella* + *Dendrophryniscus* vs. remaining bufonids (mean = 35.4 Mya, SD = 2.4). MCMC parameters were set to four parallel runs with 100 million iterations, 10 000 thinning intervals and 10% initial burn-in. We checked the convergence of parameters (Effective Sample Size - 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 considering only the *Amazophrynella* clade of this ultrametric tree using the GMYC function of the 'splits' R package (Ezard *et al.*, 2009) with a threshold interval between 0–10 Mya.

We defined the OTUs based on the combined evidence of these three delimitation methods (i.e. congruence between the results for 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 a distinct OTU). Some of these OTUs included specimens from the type series of nominal species; other 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 coding sequences (CDS), which is recommended for sequences with global homology (Katoh & Standley, 2013). Coding regions were realigned considering reading frames, 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-fitting substitution models, we reconstructed a time-calibrated Bayesian phylogenetic tree with BEAST. Parameters for the analysis and 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 with sampling 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 nuclear DNA (nuDNA) information.

BIOGEOGRAPHICAL 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 geographical distribution of ancestral species and speciation events. As this package requires an attribution of species distributions as proxies of biogeographical regions, we performed two analyses considering different partitioning schemes of Amazonia. Under the combined evidence of these two approaches, we aimed to identify broad and refined geographical patterns of the group diversification, allowing us to test hypotheses related to ancient and more recent Amazonian landscape changes. First, we considered a broad delimitation of biogeographical regions based on Wallacean districts (Wallace, 1854), defined as wide units following main geological compartments and landscape features of the 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 biogeographical studies of birds and primates (Cracraft, 1985). These areas are mostly limited by the large rivers of the region, as follows (riverine boundaries in parentheses): 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), Tapajós (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 the Tocantins River), at high elevations in the Pantepui region or from the easternmost Solimões–Negro interfluve (Jaú Area of Endemism *sensu* 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 discuss the differences between the results of best-fitting models considering or omitting this parameter. Model fit was assessed under the Akaike Information Criterion (AIC). To further investigate the frequency and geographical context of biogeographical 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 biogeographical scenarios accounting for the same pattern of diversification as the best-fitting model to obtain an estimate of event frequencies across simulations (mean \pm SD). 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 that 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.

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 differences 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 (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* (Fig. S2). We delimited those three nominal species as distinct OTUs given their segregation in geographical 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 three-fold increase in richness.

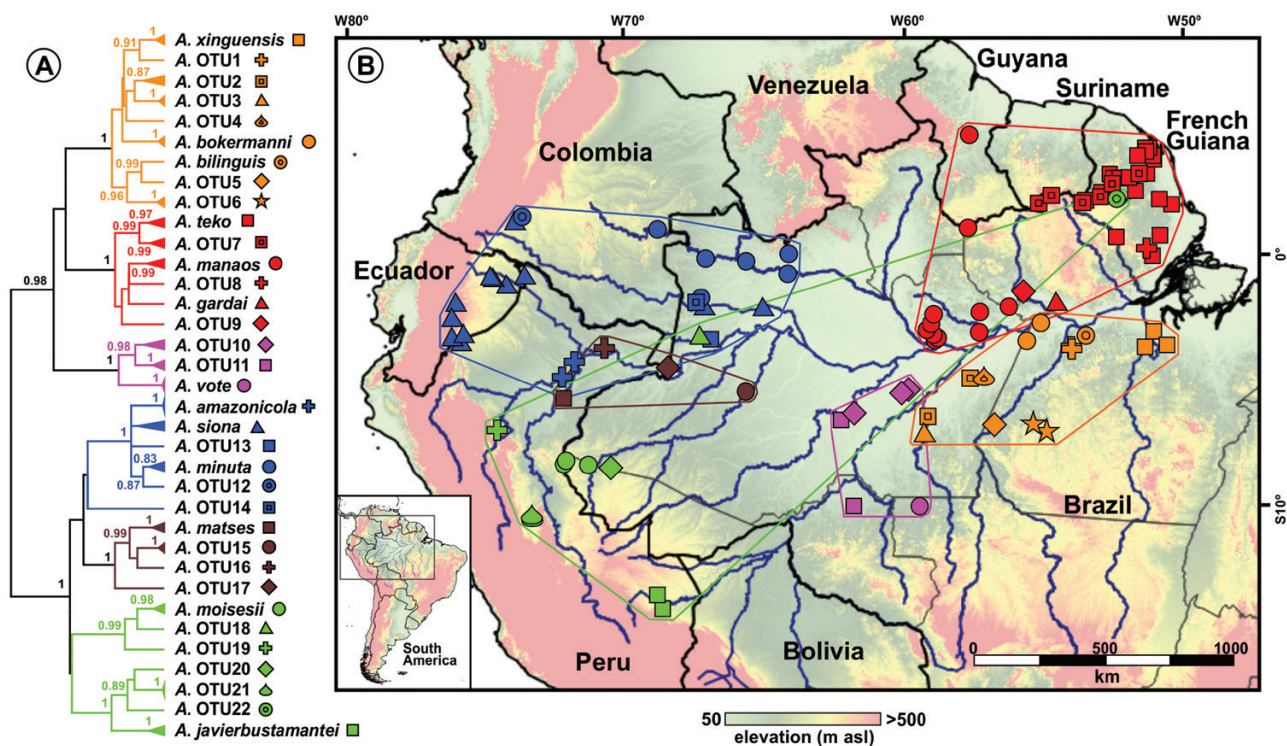


Figure 1. Phylogenetic relationships (A) and geographical distributions (B) of operational taxonomic units (OTUs) inferred within *Amazonophrynella*. The phylogenetic tree was inferred through Bayesian optimality criteria. Nodal support values are shown close to the branches (posterior probabilities < 0.8 have been omitted). The geographical distributions of OTUs (symbols in B) are coloured according to the main genetic clusters (colours in A).

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). Geographical 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 north-western 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 (43/53) of nodes showing posterior probabilities > 0.95 (Fig. 2). Lower support values were more common among recently diverging *Amazophrynella* terminals, but not necessarily those represented by less mitogenomic data (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, ~37.3 Mya [95% highest 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 (~36.8 Mya, 95% HPD = 32.6–40.9). The results corroborate an ancient history of diversification for *Amazophrynella*, dating back to 23 Mya (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 Palaeogene–Neogene transition (Oligocene–Miocene ~23 Mya; 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 ~14.9 Mya (95% HPD 12.2–17.7) for the

western clade and 16.1 Mya (95% HPD = 12.7–19.5) for the eastern clade.

In the western clade, northernmost OTUs are nested in a subclade with representatives of mid-western and south-western Amazonia, which is sister of a subclade exclusively composed of OTUs from the south-western region (Fig. 2). This pattern is similar within the eastern clade, with OTUs from north-eastern Amazonia more closely related to one of the two subclades distributed in the south-eastern 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 ~12.2 Mya (95% HPD 9.8–14.6) for the western clade and 13.4 Mya (95% HPD 10.5–16.5) for the eastern clade.

Diversification rates differed slightly 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 Mya), mostly within two subclades (Fig. 2). The most recent divergences occurred in the eastern clade, during the late Pliocene (~2.8–2.9 Mya). In the western clade, recent divergences are overall older, during the Miocene–Pliocene transition, and concentrated in north-western Amazonia (Figs 1, 2).

HISTORICAL BIOGEOGRAPHY

Of the six biogeographical 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; 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 (Table S4).

Considering the best-fit models (i.e. including the ‘J’ parameter), the most recent common ancestor (MRCA) of the genus *Amazophrynella* (~23 Mya, 95% HPD 19.3–26.6) probably occupied south-western 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/vicariant 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 south-western Amazonia (Inambari) and at the interface

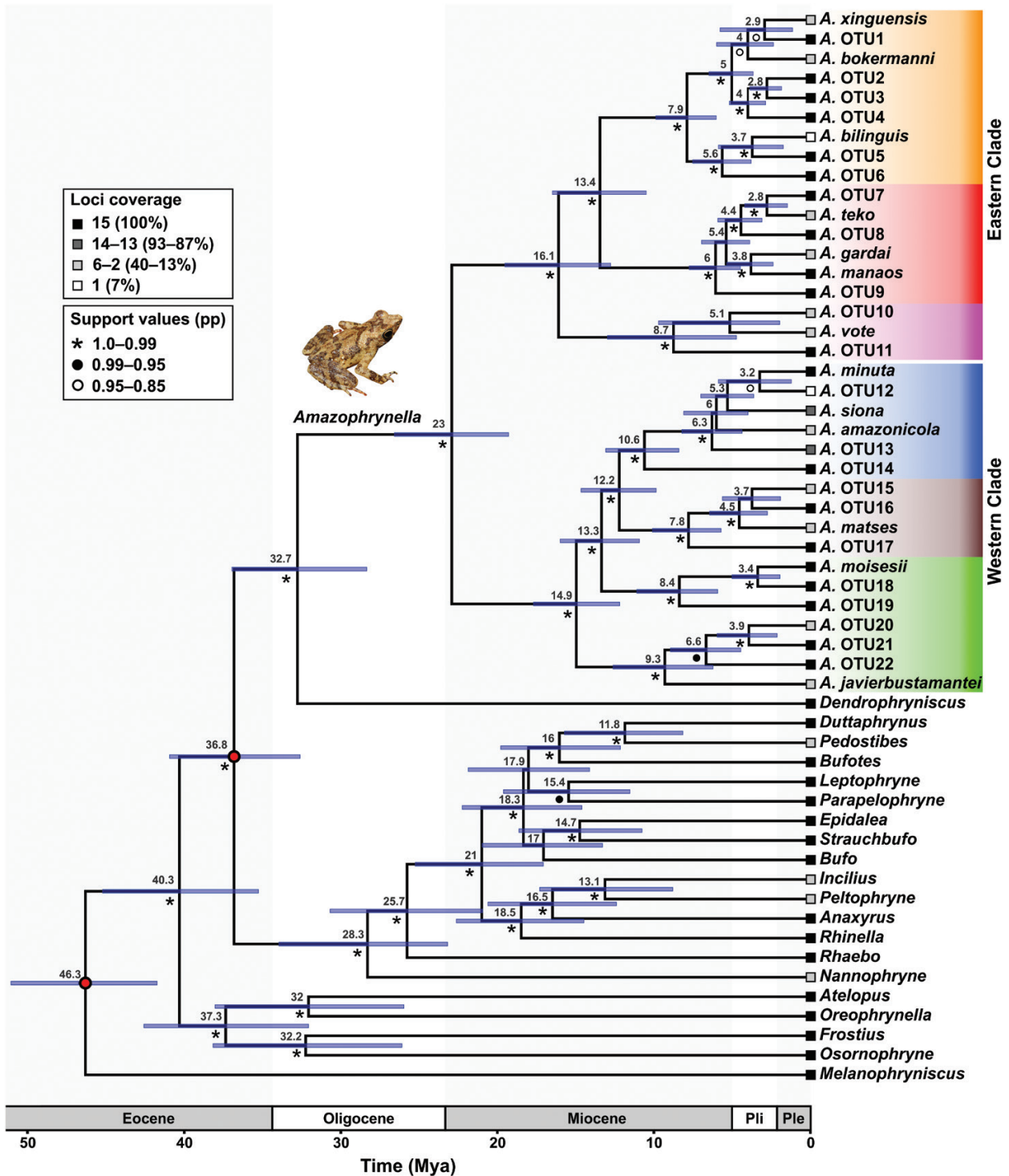


Figure 2. Bayesian mitogenomic time-calibrated phylogenetic tree of the family Bufonidae, with a focus on relationships within *Amazophrynella*. Nodal support values are shown in symbols below branches (posterior probabilities < 0.85 have been omitted), and locus coverage for each terminal is shown in the greyscale squares on the tips of the tree; both are detailed in the inset legends. The mean value of estimated time for cladogenetic events is presented above branches, and blue horizontal bars on nodes correspond to their 95% HPD. Red dots highlight the calibrated nodes (see Material and Methods). Colours of OTUs correspond to those in Figure 1. Geological epochs: Pli, Pliocene; Ple, Pleistocene–Holocene.

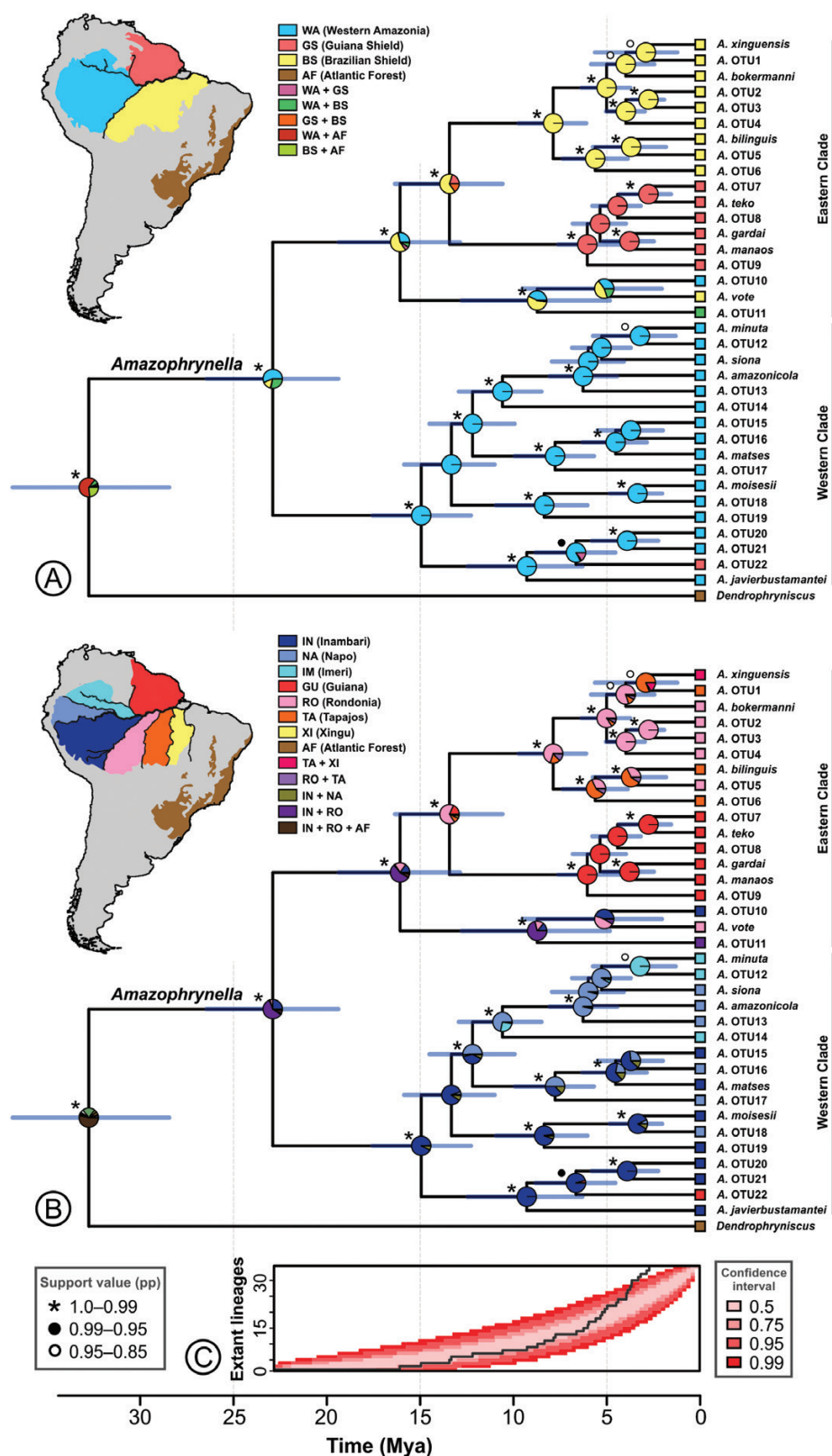


Figure 3. Biogeographical history of the genus *Amazophrynella* inferred from ‘BioGeoBEARS’ optimization on the mitogenomic Bayesian chronogram (Fig. 2). The best-fit models were DIVALIKE+J for the broad spatial partitioning

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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. This clade also dispersed toward the Guiana some 7 Mya (Fig. 3; Fig. S3). In turn, lineages within the eastern clade dispersed from around the Inambari and Rondonia (western Brazilian Shield) toward Guiana during the Miocene (~13.4 Mya, 95% HPD 10.5–16.5), and twice eastward: an older dispersal event at ~5.6 Mya (95% HPD 3.8–7.5), and a more recent event at ~3 Mya (95% HPD 1.1–5.8) (Fig. 3; Fig. S3). This last dispersal phase displays a stepping-stone pattern, with lineages dispersing from the Rondonia toward the Tapajos, and subsequently east into the Xingu (Fig. 3; Fig. S3).

Results of the best-fit models not including the jump dispersal parameter ('J') inferred more ambiguous ancestral areas relative to those incorporating this parameter (Supporting Information, Fig. S4). Despite this, those models also inferred that the MRCA of *Amazophrynella* probably occupied the south-western Amazonia region (Fig. S4), and that a most likely vicariant event triggered the split between western and eastern major clades of *Amazophrynella* in the early Miocene. Vicariant events were also implied in those models not including 'J' for the split between the Guiana Shield and Brazilian Shield clades (within the eastern clade), as well as in the diversification events in north-western Amazonia, involving the Napo and Imeri (Fig. S4).

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

Considering the refined spatial partitioning, the Inambari and Napo harboured the highest number

of *in situ* speciation events (5.4 ± 1.4 and 2.9 ± 0.9 , respectively). Fewer speciation events occurred in the eastern clade, in which most *in situ* speciation occurred within the Brazilian Shield (9.4 ± 0.9 events). Within the Brazilian Shield, most *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) probably 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 were probably the major cores of dispersal events (0.3 ± 0.3 and 0.2 ± 0.2 events, respectively) (Table S5).

DIVERSIFICATION THROUGH TIME

Diversification of *Amazophrynella* differed significantly from the expected accumulation of lineages under a constant diversification model over time ($\gamma = -2.72$, $P < 0.01$) (Fig. 3C). Nevertheless, the overall diversification pattern as illustrated by an LTT graph (Fig. 3C) indicates constant diversification (matching the null model) until ~4 Mya, 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 ~4 Mya (Supporting Information, Table S6) and a decrease after ~3 Mya ($r = 0.01$). However, the shift in the diversification rate detected ~4 Mya 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 artefactual, and to the absence of nominal species that formed recently, which could be also related to coarse-grained taxonomy.

(Wallacean districts) (A) and DEC+J for the refined one (Areas of Endemism) (B). The most likely ancestral areas are shown as likelihood pie charts on nodes. The current distribution of operational taxonomic units is depicted as squares at the tips of the trees, coloured according to the inset legends. The inset maps show the biogeographical 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 are presented in. Nodal support values are shown as symbols above branches, as detailed in the inset legend (posterior probabilities < 0.85 have been omitted). Blue horizontal bars on nodes correspond to the 95% HPD of time estimates. 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.

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 south-western Amazonia and in the Brazilian Shield, where more than 70% and 66% of the respective diversity may not yet be formally described. These observations, along with a pattern of narrow and mostly non-overlapping geographical ranges, suggest that remaining sampling gaps probably harbour additional candidate species, notably in Bolivia, Venezuela, Colombia, and south-western Brazilian Amazonia (Supporting Information, Fig. S1). Bolivian populations (see De la Riva, 1999) are of particular 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* (~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 north-western Amazonia. Such discrepancy was probably 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 biogeographical region.

LANDSCAPE EVOLUTION INFERRED FROM THE
Amazophrynella DIVERSIFICATION

The ancestors of the western and eastern major clades of *Amazophrynella* were, respectively, probably isolated along the eastern foothills of the Andes and on the western Brazilian Shield some 23 Mya. This ancient division of the 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 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 for *Amazophrynella*. Such a pattern of ancient lineage segregation between western and eastern lineages is also evident in the diversification history of other Amazonian groups, such as the anuran genera *Allobates* (Réjaud *et al.*, 2020) and *Adenomera* (Fouquet *et al.*, 2014), and lizard genera *Alopoglossus* (Ribeiro-Júnior *et al.*, 2020), *Kentropyx* (Sheu *et al.*, 2020) and *Chatogecko* (Geurgas & Rodrigues, 2010).

Within each of the two major clades of *Amazophrynella*, we found a consistent pattern of northern Amazonian subclades originating through dispersal events from southern Amazonia, before the establishment of the transcontinental Amazon River (10–9 Mya according to Hoorn *et al.*, 2010, 2017; or 5–3 Mya according to Latrubesse *et al.*, 2010). This contradicts a previous biogeographical interpretation that attributed these splits to a vicariant event triggered by the emergence of the Amazon River (Rojas *et al.*, 2018). Nevertheless, the diversification history of *Amazophrynella* still suggests a major role of the Amazon River as a secondary geographical barrier, as the establishment of this river probably limited the dispersion and genetic interchange between northern and southern populations after the middle Miocene. This timing is therefore consistent with the ‘old origin’ hypothesis for the establishment of the Amazon River (Hoorn *et al.*, 2010, 2017). This riverine barrier may have favoured *in situ* diversification throughout northern and southern Amazonia, mirroring what has been suggested for the effects of large rivers on the diversification of *terra firme* Amazonian birds (Naka & Brumfield, 2018).

During the middle Miocene (~15–10 Mya), the western clade of *Amazophrynella* underwent an initial diversification along the southern part of the Andean foothills and progressively dispersed and

diversified northward. This is concomitant with the uplift of the Fitzcarrald Arch and the south-western to north-western perimontane accumulation of Andean sediment as a result of continuous orogeny (Espurt *et al.*, 2010; Hoorn *et al.*, 2010). Both events may have facilitated *Amazophrynella* range expansion due to the development of *terra firme* forests in westernmost Amazonia. At the same time, the eastern clade also expanded its range to the east and north into the Guiana Shield, the latter probably via an upland route connecting these regions (Purus Arch) (Hoorn *et al.*, 2010). The west–east inversion of the Amazon watershed (Hoorn *et al.*, 2010, 2017; Latrubesse *et al.*, 2010) ultimately prevented any further dispersals between the Guiana Shield and Brazilian Shield. Similar to the western clade, east- and northward range expansions within the eastern clade were concomitant with the demise of mega wetlands and the development of *terra firme* forests (Bicudo *et al.*, 2019).

Subsequent diversification events within *Amazophrynella* (< 10 Mya) include the unexpected dispersal of a lineage embedded within the western subclade into the Guiana Shield during the late Miocene (~7 Mya). This apparently long dispersion event may be explained by possible extinction of intervening populations, or simply by a bias of undersampling. Either way, it implies a trans-Amazonian dispersal, considering that this river was already established at that time (*sensu* Hoorn *et al.*, 2010, 2017). Given that internal areas of the western sedimentary basin have only recently become more suitable for the expansion of *terra firme* lineages such as *Amazophrynella* (Pupim *et al.*, 2019), this dispersal event may have occurred via a north-western route, possibly through the Vaupes Arch, a concomitant northern watershed connecting the uplands of western and eastern Amazonia (Mora *et al.*, 2010). In fact, given the strong habitat association of this genus, the diversification of the western clade is consistent with progressive development of *terra firme* forests towards the east, as a result of continuous Andean sediment influx and lowering of the river channels after the demise of the lacustrine systems (Pupim *et al.*, 2019). This is also supported by the fact that the most recent lineages of the western clade are confined to 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 interfluvium (Jaú Area of Endemism; Borges & Silva, 2012) (Fig. S1). 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 (~1000 years ago; Ruokolainen *et al.*, 2019), may have prevented the range expansion of the western clade of *Amazophrynella* into the innermost north-western Amazonia.

Conversely, the Eastern subclade broadly expanded its range to the east during the same timeframe as the western clade (< 10 Mya). 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 probably changing the structure of forests (Cheng *et al.*, 2013). *Amazophrynella* species have possibly dispersed eastward following humid forest development during favourable climatic conditions. However, they maintained low diversification rates until ~6–5 Mya, 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 unfavourable climatic conditions (Cheng *et al.*, 2013) may have promoted 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 has fewer 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 probably limited *Amazophrynella* dispersal and possibly fostered lineage extinction in unsuitable regions (Rabosky, 2009; Peterson *et al.*, 2011).

Biogeographical 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, 2021; Sá *et al.*, 2019; Réjaud *et al.*, 2020), 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 re-evaluation of species richness within *Amazophrynella* and their respective distributions. Moreover, they provide insights into the historical biogeography of these tiny toads, which is consistent with proposed landscape changes in Amazonia throughout the 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* agrees largely with a progressive transition of lacustrine and fluviotidal systems to *terra firme* forest habitats in 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.

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DATA AVAILABILITY

Newly generated DNA sequences are available from GenBank (for accession numbers, see [Supporting Information, Table S1](#)). R scripts used for the phylogenetic reconstruction and the biogeographical inferences were the same as in [Réjaud *et al.* \(2020\)](#). Additional data underlying this study are available in the Supporting Information, or directly from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Appendix A. DNA extraction and 16S data acquisition.

Appendix B. Mitogenome sequencing, assembly 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 fitted 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 fitted 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 the Amazonian landscape during the Neogene–Quaternary.

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