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

3

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38 **Running head:** Diversification of *Amazophrynella*

39

40 **Author contributions:** A.F, L.J.C.L.M. and F.P.W. conceived the study. A.F., M.T.R.,
41 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
42 sampling. A.F., A.R. and L.D. generated DNA sequence data. L.J.C.L.M., A.R. and A.F.
43 analysed the data; L.J.C.L.M. and A.F. led the writing; and all authors reviewed the final
44 draft.

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

63

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

66 INTRODUCTION

67 Amazonia has experienced dramatic geomorphologic and climatic changes leading to major
68 landscape changes over the Cenozoic (Hoorn *et al.*, 2010; Albert *et al.*, 2018). Those
69 changes have been invoked to explain this region's high species richness and current
70 biological distributional patterns (Antonelli & Sanmartín, 2011; Leite & Rogers, 2013;
71 Bicudo *et al.*, 2019). However, despite major improvements in our comprehension of these
72 landscape changes (Hoorn *et al.*, 2010; Antonelli *et al.*, 2018), the timing of these changes
73 and how they have affected the diversification of organisms remain unclear. For example,
74 most studies investigating diversification processes within Amazonia focused on birds,
75 which mostly diversified over a relatively recent timeframe (< 5 Ma) within this region
76 (Silva *et al.*, 2019). Other studies have focused on current patterns of community
77 composition in vertebrate groups but did not investigate historical processes (Oliveira *et al.*,
78 2017; Godinho & da Silva, 2018; Vacher *et al.*, 2020). One of the reasons for the scarcity
79 of biogeographic studies in other animal groups comes from challenges of obtaining a
80 comprehensive spatial sampling due to the difficulty to access many Amazonian regions
81 (Vacher *et al.*, 2020). In fact, the few biogeographic studies investigating the diversification
82 of small terrestrial Amazonian vertebrates such as amphibians and squamates, which
83 supposedly disperse less efficiently than birds and large mammals, generally unraveled
84 much older events on comparable spatial scales, which may suggest a role of ancient
85 historical landscape changes in their diversification (Fouquet *et al.*, 2012a, b, 2014; Kok *et*
86 *al.*, 2017, 2018; Marques-Souza *et al.*, 2020; Moraes *et al.*, 2020; Réjaud *et al.*, 2020).

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

90 mountain range notably led to the closing of an estuary at the western end of a
91 hydrographic system running towards the Pacific Ocean some 23 Myr (Hoorn *et al.*, 2010;
92 Bicudo *et al.*, 2019). Consequently, western Amazonia has probably been covered by an
93 enormous lacustrine ecosystem that drained into the Caribbean sea to the north (the current
94 Orinoco drainage) until ca. 9 Myr (the “Pebas System”; Wesselingh & Salo, 2006; Hoorn *et*
95 *al.*, 2010, 2017). Recent evidence completed the picture by proposing a watershed in
96 western Amazonia segregating a deep aquatic system along the Andes from a central
97 fluviotidal basin covered by extensive seasonally flooded habitats to the east (Bicudo *et al.*,
98 2019). About 9 Myr, the orogeny of the northern Andes and continuous sedimentation of
99 these aquatic systems, mainly with the young Andean sediments, have ultimately led to a
100 shift of the flow of this proto-Amazon River system toward the Atlantic Ocean (Hoorn *et*
101 *al.*, 2010). A biologically diverse mega-wetland (the “Acre System”; Latrubesse *et al.*,
102 2010) has apparently persisted in southwestern Amazonia for ca. 3 million years after the
103 establishment of this eastward flow. Subsequent late Miocene erosion favored the
104 progressive development of non-flooded (*terra firme*) forests and frequent hydrological
105 changes in this region, ultimately leading to the modern configuration of the Amazon River
106 and its tributaries (Albert *et al.*, 2018). It is noteworthy that the timeframe and the
107 amplitude of these changes are still discussed (Hoorn *et al.*, 2010, 2017; Latrubesse *et al.*,
108 2010) and their consequences on biotic diversification remain elusive.

109 Anuran amphibians often display distinct biogeographic patterns as compared to
110 other vertebrate taxa because they have finely tuned environmental preferences and
111 functional characteristics often associated with limited dispersal ability (Moraes *et al.*,
112 2016; Wollenberg-Valero *et al.*, 2019). These characteristics make their populations
113 particularly sensitive to the aforementioned historical events, which ultimately lead to

114 striking spatial and temporal signatures in their distributional patterns and phylogenetic
115 relationships (Fouquet *et al.*, 2012a, 2014). The tiny toads of the pan-Amazonian genus
116 *Amazophrynella* Fouquet *et al.*, 2012a,b perfectly falls into this description since they all
117 are similarly small-bodied, mostly associated with the leaf-litter of *terra firme* forests, and
118 breed in small temporary ponds (Fouquet *et al.*, 2012a; Rojas *et al.*, 2018). The entire genus
119 appears to display highly conserved ecology, morphology and habitat use (Rojas *et al.*,
120 2018). Its external morphology is so conserved that until the 1990s, only one described
121 species was supposed to occupy the entire Amazonia. However, the taxonomic knowledge
122 has increased rapidly in recent years, with the description of eleven new species over the
123 past decade (e.g., Rojas *et al.*, 2018; Kaefer *et al.*, 2019; Mângia *et al.*, 2020). Thirteen taxa
124 are currently recognized, which led to the realization that all species of *Amazophrynella*
125 have in fact small and almost completely allopatric ranges (Rojas *et al.*, 2018).
126 Furthermore, phylogenetic analyses based on molecular data suggest that the genus started
127 to diversify as early as 25 Myr with an initial divergence between two major clades largely
128 restricted to geomorphologically and climatically distinct western and eastern Amazonian
129 regions (Fouquet *et al.*, 2012a; Rojas *et al.*, 2018). Subsequent divergences between
130 northern vs. southern lineages in each of these major clades suggest a role of the
131 transcontinental Amazon River as a geographical barrier (Rojas *et al.*, 2018). However,
132 some knowledge gaps persist regarding the actual species richness and phylogenetic
133 relationships within *Amazophrynella*, as numerous recently identified mitochondrial DNA
134 (mtDNA) lineages (Vacher *et al.*, 2020) were not yet included in a phylogenetic
135 reconstruction of the whole genus. In addition, many undersampled areas in Amazonia
136 probably harbor additional species (Fouquet *et al.*, 2012a; Rojas *et al.*, 2018).

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

157 MATERIAL AND METHODS

158 INPUT DATA CONSTRUCTION

159 We focused on the 16S mitochondrial gene for the species delimitation analyses, because
160 this gene has been widely used in studies targeting *Amazophrynella* (e.g., Rojas *et al.*,
161 2018) and is recognized as one of the universal barcodes for Neotropical amphibians
162 (Vences *et al.*, 2005b). We gathered geolocalized 16S sequences from 286 specimens (69
163 newly acquired and 217 that were previously deposited in GenBank) covering almost the
164 entire distributional range of the genus (Supporting Information, Fig. S1). Details of the
165 16S sequencing process for newly generated data are presented in the Supporting
166 Information, Appendix A. Regarding previously published data, we found missing blocks
167 and several instances of incongruences among voucher numbers, accession numbers,
168 species labels, and geographic coordinates for GenBank sequences (mostly from Rojas *et*
169 *al.*, 2018). Therefore, we excluded most of these sequences, keeping only the unambiguous
170 ones and those representing taxa not sampled by us (Supporting Information, Table S1). A
171 summary (most likely conservative) of these incongruences is presented in Supporting
172 Information, Table S2.

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

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

193

194 SPECIES DELIMITATION

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

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

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

213 The ASAP delimitation was performed on the online server
214 (<https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html>) considering a simple distance model
215 to compute the distances between samples, and default parameters. We kept the
216 delimitation scheme supported by the lowest ASAP score (Puillandre *et al.*, 2021). For the
217 mPTP delimitation, we first reconstructed a maximum likelihood (ML) phylogenetic tree
218 with RAxML 8.2.4 (Stamatakis, 2014), running 1,000 nonparametric bootstrap replicates to
219 assess nodal support. The best-fitted model for our dataset was GTR+G+I according to the
220 Bayesian Information Criterion (BIC; Hurvich & Tsai, 1989) in a PARTITIONFINDER 2.1.1
221 (Lanfear *et al.*, 2017) analysis. However, for the ML inference, we did not consider the
222 estimation of Invariable Sites proportion (*I* parameter) as it prevents reliable estimates of
223 the other parameters (Stamatakis, 2014). We rooted the tree with 18 outgroups, including
224 12 of the 14 New-World genera of Bufonidae (*Atelopus*, *Dendrophryniscus*, *Frostius*,
225 *Melanophryniscus*, *Rhinella*, *Anaxyrus*, *Incilius*, *Nannophryne*, *Oreophrynella*,
226 *Osornophryne*, *Peltophryne* and *Rhaebo*) and eight Old-World genera (*Bufo*, *Bufotes*,
227 *Duttaphrynus*, *Epidalea*, *Leptophryne*, *Parapelophryne*, *Strauchbufo* and *Pedostibes*)
228 (Supporting Information, Table S1). This sampling accounts for all genera with available

229 molecular data from the paraphyletic group of “atelopodids” (i.e. taxa branching near the
230 base of the Bufonidae tree; Kok *et al.*, 2018). Using the resulting ML tree, we ran the
231 mPTP delimitation on the EDB-calc cluster (Toulouse, France), with 50 million Markov
232 chain Monte Carlo (MCMC) iterations, sampling every 100,000 iterations and discarding
233 10% of initial burn-in.

234 For the GMYC delimitation, we obtained an ultrametric phylogeny by
235 reconstructing a time-calibrated Bayesian tree using the software BEAST 2.6.3 (Bouckaert *et*
236 *al.*, 2014) with the GTR+G+I substitution model, and using only unique haplotypes. We
237 used a birth-death process to model speciation and extinction (Gernhard, 2008), and an
238 uncorrelated relaxed clock to model evolutionary rate variation among branches
239 (Drummond *et al.*, 2006). In the absence of fossil records for *Amazophrynella* and closely
240 related genera, we calibrated the tree using two secondary node constraints based on time-
241 calibrated anuran phylogenies inferred from comprehensive genomic datasets and fossil
242 calibrations (Feng *et al.*, 2017; Hime *et al.*, 2021). These dates were constrained with a
243 normal prior distribution and included: (1) the crown age of Bufonidae (Mean = 48.0 Myr,
244 SD = 2.5), and (2) the divergence time of *Amazophrynella* + *Dendrophryniscus* vs.
245 remaining bufonids (Mean = 35.4 Myr, SD = 2.4). MCMC parameters were set to four
246 parallel runs with 100 million iterations, 10,000 of thinning and 10% of initial burn-in. We
247 checked the convergence of parameters (ESS > 200) of the combined log file of four runs
248 with TRACER 1.7 (Bouckaert *et al.*, 2014; Rambaut *et al.*, 2018) and extracted the
249 maximum clade credibility tree using TREE ANNOTATOR 2.6.3 (Bouckaert *et al.*, 2014). We
250 performed the multiple threshold GMYC delimitation using only the *Amazophrynella* clade
251 of this ultrametric tree using the GMYC function of the ‘splits’ R package (Ezard *et al.*,
252 2014) with a threshold interval between 0–10 Myr.

253 We defined the distinct Operational Taxonomic Units (OTUs) based on the
254 combined evidence of these three delimitation methods (i.e., congruence between the
255 results of at least two of them), and the stability of the current taxonomic knowledge of the
256 genus (i.e., by considering each of the currently valid species as distinct OTUs). Some of
257 these OTUs included specimens from the type series of nominal species; others specimens
258 could be attributed to nominal taxa because the range of corresponding OTUs spanned the
259 type localities of these taxa (see Supporting Information, Appendix C). Lastly, with MEGA
260 7 (Kumar *et al.*, 2016), we estimated the mean uncorrected genetic distances (p-distances)
261 among OTUs.

262

263 **TIME-CALIBRATED PHYLOGENETIC RELATIONSHIPS**

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

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

284

285 **BIOGEOGRAPHIC ANALYSES**

286 The time-calibrated mitogenomic tree obtained from the BEAST analysis was used to
287 perform an ancestral area reconstruction using the ‘BioGeoBEARS’ R package (Matzke,
288 2013), which infers the geographic distribution of ancestral species and speciation events.
289 As this package requires an attribution of species distributions as proxies of biogeographic
290 regions, we performed two analyses considering different partitioning schemes of
291 Amazonia. Under the combined evidence of these two approaches, we aim at identifying
292 broad and refined geographic patterns of the group diversification, allowing the testing of
293 hypotheses related to ancient and more recent Amazonian landscape changes. First, we
294 considered a broad delimitation of biogeographic regions based on the Wallacean districts
295 (Wallace, 1854), defined as wide units following main geological compartments and
296 landscape features of this region: 1) western Amazonia (WA), corresponding to the
297 sedimentary Solimões basin, and 2) Guiana Shield (GS) and 3) Brazilian Shield (BS),
298 corresponding to the crystalline shields. These units were delimited by the large rivers
299 Negro, Madeira and the lower course of the Amazon, known to correspond to major breaks
300 in amphibian community composition across Amazonia (Godinho & da Silva, 2018;

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

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

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

331 Finally, we tested if and when the diversification rate of *Amazophrynella* varied
332 through time using Lineage Through Time (LTT) analyses. We conducted a Monte Carlo
333 Constant Rate (MCCR) analysis using the ‘LASER’ 2.4.1 R package (Pybus & Harvey,
334 2000; Rabosky, 2006) to test if the observed diversification pattern is significantly different
335 from the expected from a Yule pure-birth model while accounting for randomly distributed
336 missing taxa. Using the same package, we compared the fit of seven models of
337 diversification: two constant-rate Yule models (pure-birth and birth-death), two density-
338 dependent models (DDX and DDL), and the Yule-n-rate model accounting for two, three
339 and four changes in speciation rates across the tree. We determined the best-fit model by
340 comparing AIC values. Using the ‘ape’ 5.3 R package (Paradis *et al.*, 2004), we plotted the
341 empirical lineage accumulation through time relative to that expected under a Yule pure-
342 birth model with a 95% confidence interval.

343

344 **DATA AVAILABILITY**

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

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

350 RESULTS

351 SPECIES DELIMITATION

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

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

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

381

382 **PHYLOGENETIC RELATIONSHIPS AND TEMPO OF CLADOGENESIS**

383 The mitogenomic phylogeny was based on an alignment of 13,888 nucleotide sites and 55
384 terminals. This phylogenetic inference yielded a strongly supported topology with the
385 majority of nodes showing posterior probabilities support > 0.95 (Fig. 2).
386 *Melanophryniscus* was inferred as the sister of all other bufonid genera, followed by a
387 strongly supported clade formed by (*Atelopus* + *Oreophrynella*) and (*Frostius* +
388 *Osornophryne*) that dated back to the Eocene, ca. 37.3 Myr (95% Height Posterior Density
389 [95% HPD] = 32–42.6) (Fig. 2). The Atlantic Forest genus *Dendrophryniscus* was
390 recovered as sister to *Amazophrynella*. In turn, this clade was inferred as sister to a clade
391 encompassing all the remaining bufonid genera, with the divergence between them dating
392 back to the late Eocene (ca. 36.8 Myr, 95% HPD = 32.6–40.9). The results corroborate an
393 ancient history of diversification for *Amazophrynella*, dating back to 23 Myr (95% HPD =
394 19.3–26.6) (Fig. 2).

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

398 recovered as nested within the western clade. The divergence of the western and eastern
399 clades coincides with the Paleogene–Neogene transition (Oligocene–Miocene ca. 23 Myr;
400 95% HPD = 19.3–26.6), which is relatively older than most crown ages of other bufonid
401 genera (Fig. 2). The crown ages of the two major *Amazophrynella* clades both date back to
402 the middle Miocene, at ca. 14.9 Myr (95% HPD 12.2–17.7) for the western clade and 16.1
403 Myr (95% HPD = 12.7–19.5) for the eastern clade.

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

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

420

421 **HISTORICAL BIOGEOGRAPHY**

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

430 The most recent common ancestor (MRCA) of the genus *Amazophrynella* (ca. 23
431 Myr, 95% HPD 19.3–26.6) likely occupied the southwestern Amazonia, more specifically
432 in the interface of the Inambari and Rondonia, which currently corresponds to the area
433 delimited northward by the upper Amazon River and eastward by the Tapajós River. An
434 ancient dispersal/vicariance event led to the split between the western and eastern major
435 clades within the genus. These clades subsequently diversified within distinct areas, along
436 the Andean foothills in southwestern Amazonia (Inambari) and at the interface of the
437 Inambari and Rondonia, respectively (Fig. 3; Supporting Information, Fig. S3). Lineages
438 from the western clade have secondarily dispersed twice northward during the Miocene,
439 reaching the Napo and ultimately the Imeri regions. This clade has also dispersed toward
440 the Guiana Shield some 7 Myr (Fig. 3; Supporting Information, Fig. S3). In turn, lineages
441 within the eastern clade dispersed from around the Inambari and Rondonia regions (western
442 Brazilian Shield) toward the Guiana Shield during the Miocene (ca. 13.4 Myr, 95% HPD
443 10.5–16.5), and twice eastward: an older dispersal event at ca. 5.6 Myr (95% HPD 3.8–7.5),
444 and a more recent event at ca. 3 Myr (95% HPD 1.1–5.8) (Fig. 3; Supporting Information,
445 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

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

476

477 **DIVERSIFICATION THROUGH TIME**

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

492 **DISCUSSION**

493 **SPECIES DELIMITATION**

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

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

515 2011; Miller *et al.*, 2021), but also to the variable effort undertaken on their taxonomic
516 resolution (Fouquet *et al.*, 2021).

517 Our species delimitation analyses also led to ambiguous OTU boundaries in several
518 instances, especially among some recently diverging lineages from northwestern
519 Amazonia. Such discrepancy was likely influenced by the small size of our focal mtDNA
520 locus and the absence of nuDNA, morphological, and acoustic data (see Miralles & Vences,
521 2013). Based on these limitations, we advocate caution over strict interpretation of our
522 delimitation results in the case of such ambiguous boundaries. Nevertheless, the results of
523 our ancestral area reconstruction analyses should be reliable because most of the conflicting
524 OTU boundaries involved geographically close populations that occur within the same
525 broad biogeographic region.

526

527 **LANDSCAPE EVOLUTION INFERRED FROM THE *AMAZOPHRYNELLA* DIVERSIFICATION**

528 The ancestors of the western and eastern major clades of *Amazophrynella* were most likely
529 respectively isolated along the eastern foothills of the Andes and on the western Brazilian
530 Shield some 23 Myr. This ancient division of this genus into two major clades at the
531 Oligocene–Miocene transition was also suggested by previous divergence time analyses
532 (Rojas *et al.*, 2018). This timeframe is concomitant with the emergence of a vast lacustrine
533 system and other mega wetlands in western Amazonia, due to the combined effect of the
534 uplift of the Andean mountain range and a western depression of the continental plate
535 (Hoorn *et al.*, 2010; Bicudo *et al.*, 2019). These mega wetland systems may have acted as
536 relevant geographical barriers by segregating populations strongly associated with *terra*
537 *firme* forests and narrow ecological niche breadth (Hoorn *et al.*, 2010), as is the case of
538 *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

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

601 Conversely, the Eastern subclade broadly expanded its range to the east during the
602 same timeframe as the western clade (< 10 Myr). Instead of a greater geomorphological
603 influence controlling the development of *terra firme* forests, major changes in vegetation
604 cover in this region have been especially affected by climatic variations over time, with
605 drier glacial periods likely changing the structure of forests (Cheng *et al.*, 2013).
606 *Amazophrynella* have possibly dispersed eastward following humid forest development
607 during favorable climatic conditions. However, they maintained low diversification rates
608 until ca. 6–5 Myr, when the putative combined influence of drainage rearrangements of the
609 tributaries of the Amazon River (Latrubesse, 2002; Rossetti, 2014; Hayakawa & Rossetti,
610 2015; Moraes *et al.*, 2020) and cyclical unfavorable climatic conditions (Cheng *et al.*,

611 2013) may have promoted a rapid accumulation of new lineages. These factors may also
612 explain a higher stasis on the diversification of the Guiana Shield clade over time, as this
613 region currently presents a lower occurrence of large tributaries of the Amazon River and
614 has been geomorphologically more stable over time (Bicudo *et al.*, 2019).

615

616 **ECOLOGICAL CONSERVATISM AND DIVERSIFICATION RATES**

617 Increasing diversification rates are generally associated with the acquisition of evolutionary
618 novelties that allow the exploration of new ecological opportunities (Erwin, 2015).

619 Therefore, the nearly continuous and stable diversification rate seen in *Amazophrynella* is
620 in accordance with its extreme phenotypic conservatism and overall conserved ecology
621 (Rojas *et al.*, 2018). Even with the putative new ecological opportunities arising from the
622 dynamic Amazonian landscape evolution of the Miocene, such conservatism most likely
623 limited *Amazophrynella* dispersal and possibly fostered lineage extinction in unsuitable
624 regions (Rabosky, 2009; Peterson *et al.*, 2011).

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

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

641

642 **CONCLUSION**

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

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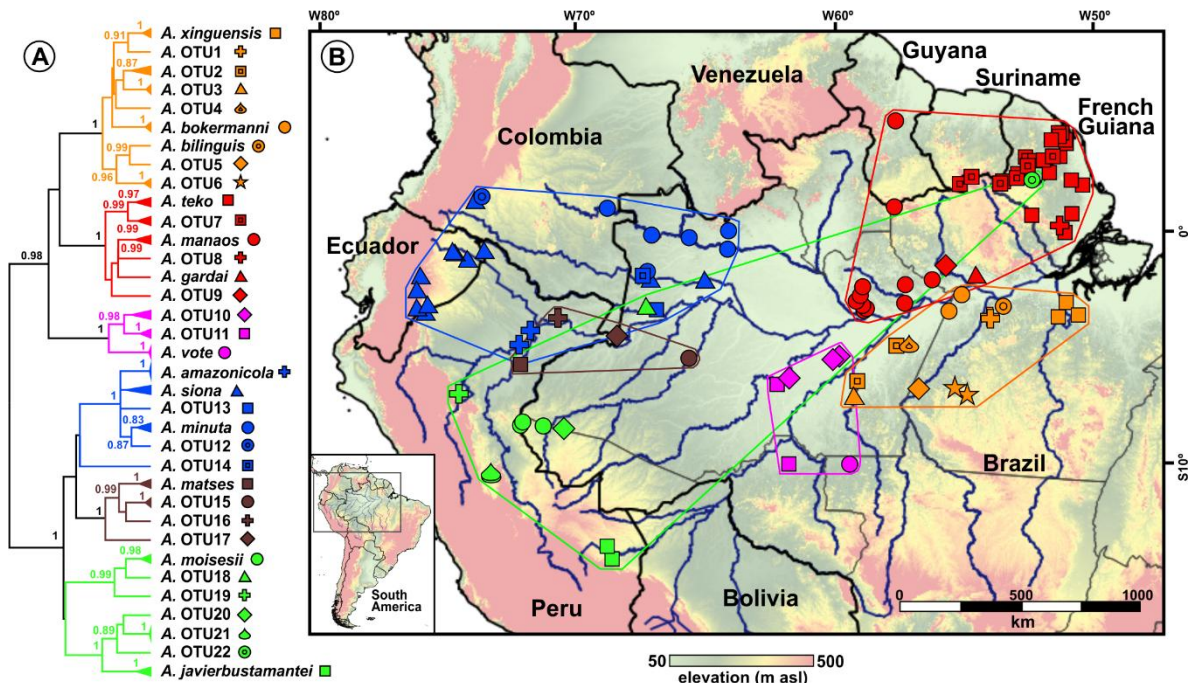
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990 FIGURES

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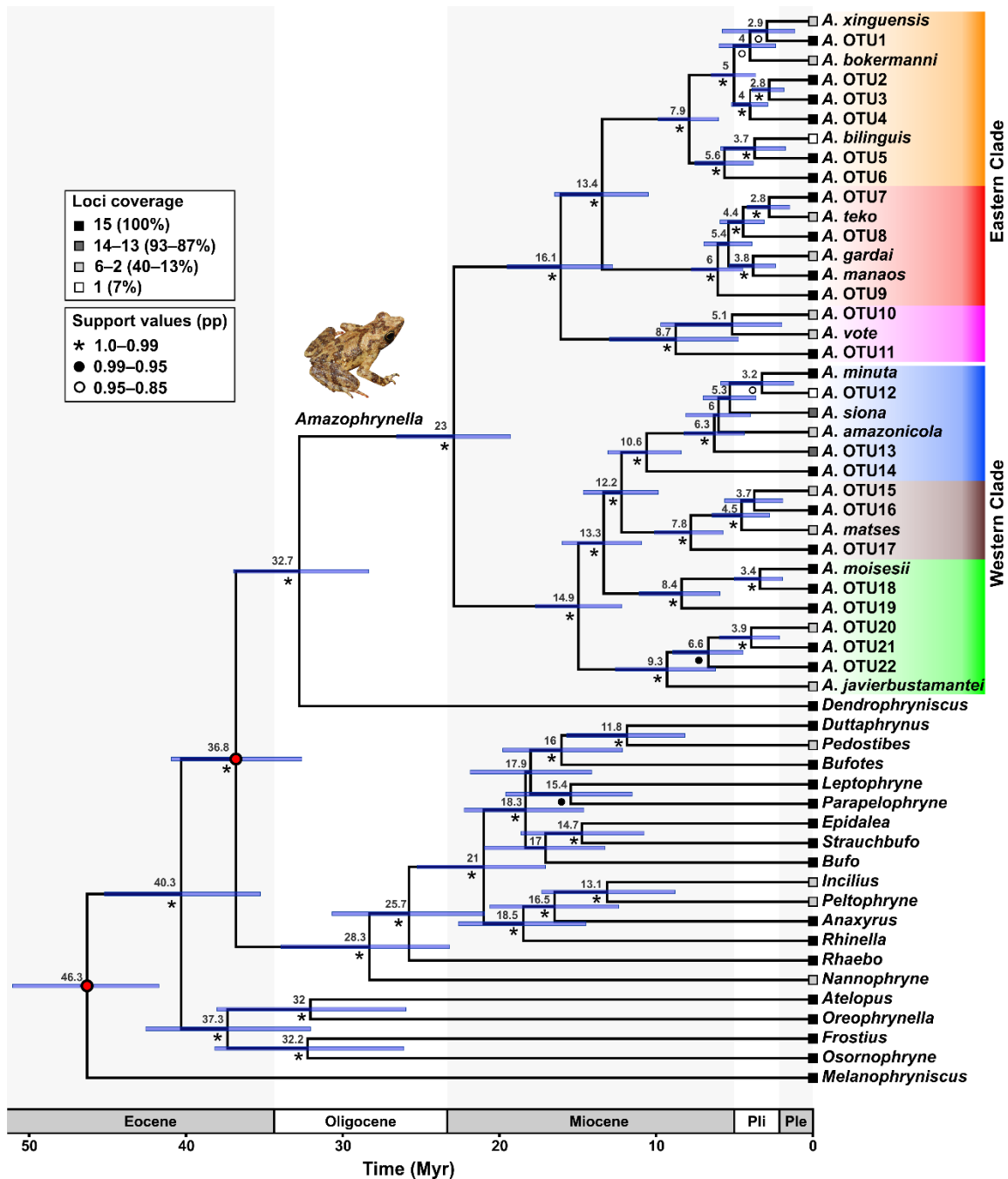
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993 **Figure 1.** Phylogenetic relationships (A) and geographic distributions (B) of Operational994 Taxonomic Units (OTUs) inferred within *Amazophrynella*. The phylogenetic tree was

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

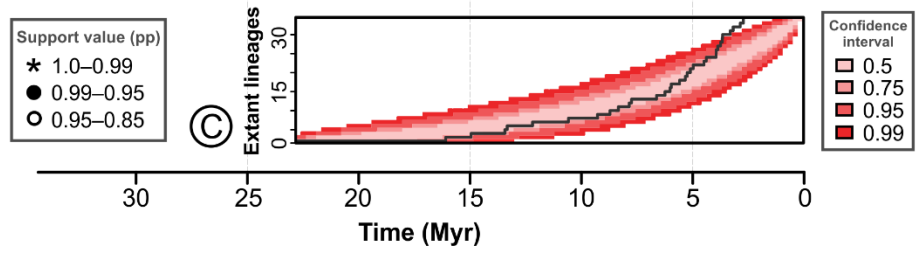
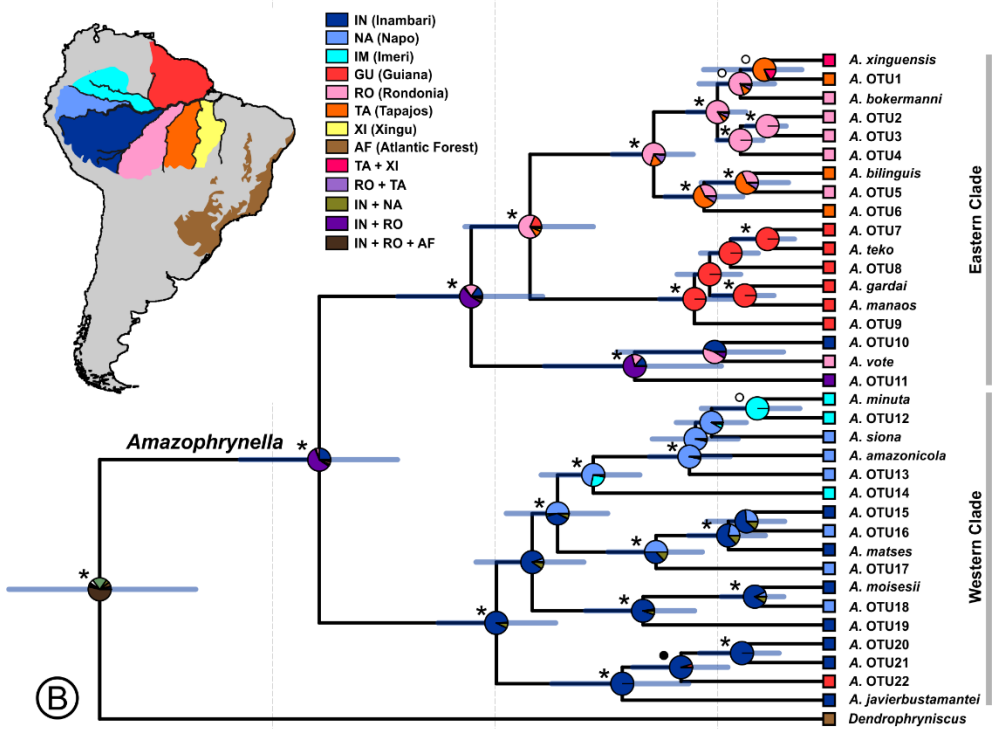
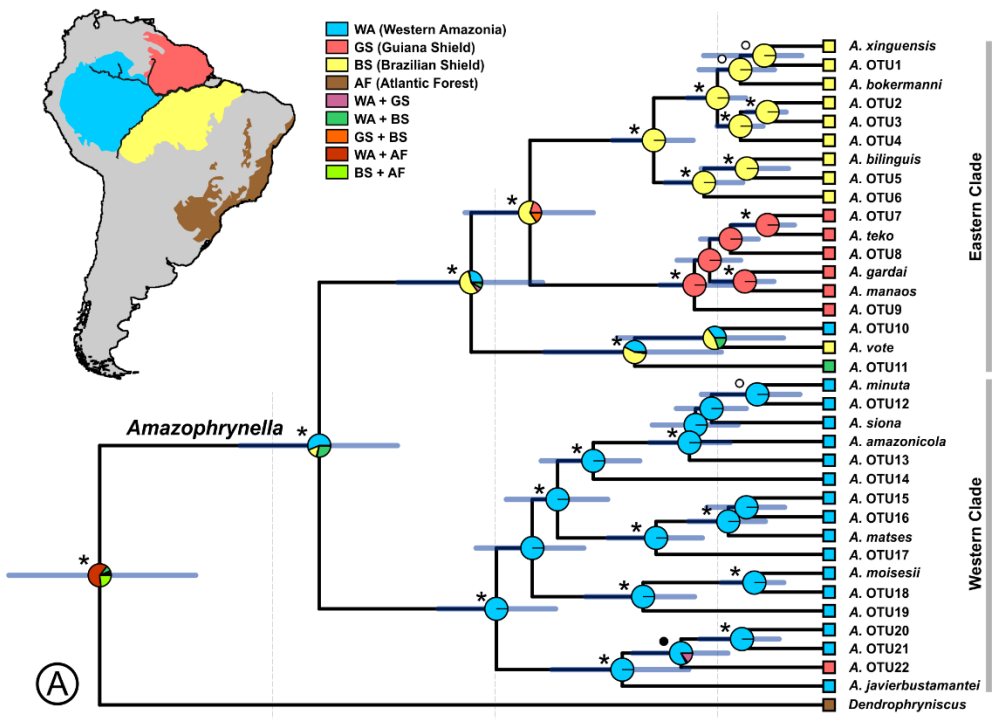
997 (symbols in B) are colored according to the main genetic clusters (colors in A).



998

999 **Figure 2.** Bayesian mitogenomic time-calibrated phylogenetic tree of the family Bufonidae,
 1000 with a focus on relationships within *Amazophrynella*. Nodal support values are shown in
 1001 symbols below branches (posterior probabilities below 0.85 omitted), and loci coverage for
 1002 each terminal is shown in the gray-scale squares on the tips of the tree; both are detailed in
 1003 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.



1008 **Figure 3.** Biogeographic history of the genus *Amazophrynella* inferred from
1009 ‘BioGeoBEARS’ optimization on the mitogenomic Bayesian chronogram (Fig. 2). The
1010 best-fit models were DIVALIKE+J for the broad spatial partitioning (Wallacean districts)
1011 (A) and DEC+J for the refined one (Areas of Endemism) (B). Most likely ancestral areas
1012 shown as likelihood pie charts on nodes. The current distribution of Operational Taxonomic
1013 Units is depicted as squares at the tips of the trees, colored according to the inset legends.
1014 The inset maps show the biogeographic areas used; for details on their riverine boundaries,
1015 see text. Combinations of areas are considered in the respective analyses but not depicted
1016 on the maps, and only the most likely area is presented. Nodal support values are shown as
1017 symbols above branches, detailed in the inset legend (posterior probabilities below 0.85
1018 omitted). Blue horizontal bars on nodes correspond to the 95% HPD of time estimates. In
1019 (C), the temporal pattern of lineage accumulation within *Amazophrynella*, inferred with a
1020 Lineage Through Time (LTT) plot using the same Bayesian chronogram (Fig. 2). The red
1021 gradient in (C) indicates the confidence intervals of expected lineage accumulation under a
1022 Yule pure-birth diversification model, and the grey line represents the empirical data.

1023 **SUPPORTING INFORMATION**

1024 **Appendix A.** DNA extraction and 16S data acquisition

1025 **Appendix B.** Mitogenome sequencing, assembling and annotation

1026 **Appendix C.** Taxonomic considerations

1027 **Table S1.** Samples included in the analyses

1028 **Table S2.** Summary of incongruences in GenBank data

1029 **Table S3.** Mean uncorrected pairwise genetic distances among OTUs

1030 **Table S4.** Summary statistics for each of the models fit to *Amazophrynella* diversification

1031 in ‘BioGeoBEARS’ analyses

1032 **Table S5.** Summary results for number and types of dispersal events during

1033 *Amazophrynella* diversification according to Biogeographical Stochastic Mapping analyses

1034 **Table S6.** Summary statistics for each of the models fit to *Amazophrynella* diversification

1035 in Lineage Through Time analyses

1036 **Figure S1.** Known distribution records of *Amazophrynella*

1037 **Figure S2.** Results of species delimitation analyses

1038 **Figure S3.** Hypothetical reconstruction of *Amazophrynella* diversification based on

1039 ancestral area reconstruction analyses and geomorphological changes in Amazonian

1040 landscape during the Neogene–Quaternary

1041 **Figure S4.** Biogeographic history of the genus *Amazophrynella* from ‘BioGeoBEARS’

1042 optimization on the mitogenomic Bayesian chronogram without considering the ‘J’

1043 parameter