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Research Article

Integrative species delimitation and biogeography of the *Rhinella margaritifera* species group (Amphibia, Anura, Bufonidae) suggest an intense diversification throughout Amazonia during the last 10 million years

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The accumulation of studies delimiting species in Amazonia has not only shed light on the patterns of its outstanding species richness but also allowed a better understanding of the processes of diversification within this immense region. Nevertheless, vast knowledge gaps remain even for prominent anuran species complexes, such as the *Rhinella margaritifera* species group. This clade of toads comprises 23 valid species-level taxa, mainly distributed in Amazonia but also in South America's Dry Diagonal and Atlantic and trans-Andean rainforests. Species boundaries and taxonomy in this group are notoriously complex, with studies suggesting the existence of several unnamed species. Available phylogenetic information suggests an Andean-western Amazonian origin of the group with subsequent diversification within Amazonian lowlands during the last 10 Myr and secondary dispersals into other Neotropical regions. To further test this biogeographic scenario and improve knowledge on species diversity, we used an unprecedentedly large mtDNA sampling (>800 16S sequences) across the clade's distribution and comprising all but one described species. We delimited 54 Molecular Operational Taxonomic Units, which we tested further based on patterns of variation of a nuclear locus and acoustic and morphological data. This approach confirmed the existence of at least 25 candidate species, 19 of which correspond to currently recognized taxa whereas 30 remained 'unconfirmed'. Our results clarify the taxonomic status of some species but also suggest multiple introgression events that blur some mtDNA-based species boundaries. Lastly, to provide a temporal framework for the clade's diversification, we generated a time-calibrated phylogenetic tree based on a mitogenomic matrix, which confirmed a Miocene (~9 Ma) western Amazonian origin and six major clades in the group, each having initially diversified in different regions within Amazonia. Most of these clades have later dispersed throughout Amazonia during the establishment of the modern Amazonian hydrographic system, i.e., in the last 6 Myr.

Key words: Bioacoustics, cryptic species, DNA, morphometrics, Neotropics, phylogenetics

Introduction

The Neotropics harbour an immense number of species (Jenkins et al., 2013), with more vertebrates than any other world region. Amazonia has apparently played a central role in the Neotropics' high species richness by acting as a source of diversity for the entire continent (Antonelli et al., 2018). However, the processes responsible for species diversification within Amazonia are still poorly understood, notably because basic information on the number and distribution of species within its >6 million km^2 remains elusive, even for terrestrial vertebrates such as amphibians (Giam et al., 2012; Vacher et al., 2020). More than 200 species of Amazonian amphibians have been described between 1999 and 2009 (World Wildlife Fund [WWF], 2009), and almost all DNA-based studies focusing on particular groups have revealed many lineages corresponding to unnamed species, which are often deeply rooted in geological time (e.g., Fouquet, Leblanc, et al., 2021; Moraes et al., 2022). Using either species description trends (Giam et al., 2012) or the number of mitochondrial DNA (mtDNA) lineages (Vacher et al., 2020) as proxies of undescribed species numbers, more than 2000 species of amphibians can be expected to occur in Amazonia, i.e., more than three times the ca. 600 described species known to occur in the region (Godinho & Da Silva, 2018 from IUCN data). Considering current threats to the Amazonian biome (Albert et al., 2023; Gomes et al., 2019), it is urgent to improve our understanding of species diversity and distributions in this region.

Many described species of Amazonian anurans previously thought to be widespread have later been found to correspond to diverse species complexes (e.g., Ferrão, Hanken, et al., 2022; Fouquet et al., 2014; Fouquet, Marinho, et al., 2021; Funk et al., 2012; Gehara et al., 2014). Accurately delimiting species within clades spanning a region as vast and diverse as Amazonia requires a spatially dense and taxonomically complete sampling of molecular, morphological, and acoustic data. Another significant challenge is to unambiguously link taxa erected decades ago with recently collected specimens and data (Raxworthy & Smith, 2021). This challenge recurrently thwarts efforts to describe new species because many older descriptions are superficial or brief, type material is frequently difficult to access, and several morphologically similar species can occur at the same locality, leading to ambiguity in their correspondence with the name-bearing types (e.g., Lavilla et al., 2013; Sturaro & Peloso, 2014).

The infamously difficult *Rhinella margaritifera* species group (hereafter, *R.* gr. *margaritifera*) combines all of these challenges. Several studies have shown the existence of many unnamed species (Ávila et al., 2020; Dos Santos et al., 2015; Ferrão et al., 2020; Ferrão, de Souza, et al., 2022; Fouquet, Gilles, et al., 2007; Pereyra et al., 2021) and highlighted the group's large intraspecific and interspecific morphological variation, as well as widespread sympatry with 4–5 species typically co-occurring at a given locality. Moreover, pronounced sexual dimorphism, extensive ontogenetic variation and brief acoustic activity windows (i.e., explosive breeding) further hinder the identification and description of species in this group (Fouquet, Gaucher, et al., 2007; Hass et al., 1995; Hoogmoed, 1986, 1990; Lavilla et al., 2013, 2017; Vélez-Rodriguez, 2004). Currently, *R.* gr. *margaritifera* is composed of 23 nominal species considered valid, distributed from Panama to southern Brazil. Most of those taxa ($n = 15$) occur in

Amazonia (including *R. gildae* and *R. martyi*; considered synonyms by Pereyra et al., 2021), one occurs in the Andes (*R. iserni*), two in trans-Andean regions (*R. sclerocephala* and *R. alata*), four in the Dry Diagonal (*R. ocellata*, *R. sebbeni*, *R. scitula*, and *R. paraguayensis*, the latter considered a synonym of *R. scitula* by Pereyra et al., 2021), and one in the Atlantic Forest (*R. hoogmoedi*), even though some Amazonian species extend into those two last regions. The most recent study focusing on the phylogenetic relationships within the genus *Rhinella* (Pereyra et al., 2021) hypothesized the existence of 11 undescribed species in *R.* gr. *margaritifera*, mostly from Amazonia (herein considered to include forested lowlands ca. < 1000 m elevation, following Olson et al. (2001)). Assigning extant populations to the names *R. margaritifera*, *R. proboscidea*, *R. dapsilis*, *R. roqueana*, and *R. acutirostris* (all described before 1950) has been notoriously difficult because of the aforementioned issues, leading to a chaotic taxonomic history. This is particularly true for the name *R. margaritifera*, a taxon described by Laurenti (1768) based on the illustrations of Seba (1734) of a specimen from 'Brasilia' (= Brazil). This taxon was subsequently used to identify many populations that in fact belong to other species in the group. Aiming at resolving confusion, and because the type specimen was thought to be lost, a neotype from Humaitá (Amazonas, Brazil) was designated by Lavilla et al. (2013). However, this decision was later invalidated by Lavilla et al. (2017) because Milto and Barabanov (2011) reported the existence of the specimens used by Seba in the collection of the Zoological Institute, St. Petersburg Museum (Russia). One of these specimens has been subsequently designated as lectotype by Pereyra et al. (2021). However, the location of the type locality remains vague, and thus the assignment of recently collected data to this name remains difficult.

These profound knowledge gaps have also obscured the group's evolutionary history. Nevertheless, some available information on (1) the diversity and distribution of the *R.* gr. *margaritifera* species and (2) previous analyses of phylogenetic relationships and the timing of diversification within *Rhinella* (Pereyra et al., 2021; Pramuk et al., 2008; Van Bocxlaer et al., 2010) pose some conjectural hypotheses. *Rhinella* gr. *margaritifera* forms a clade with *R.* gr. *festae*, altogether forming the sister group of *R.* gr. *veraguensis*. These two last groups occupy the Andes, whereas the earliest known divergence within *R.* gr. *margaritifera* separates a lineage from Amazonian Ecuador and all the other species (Pereyra et al., 2021). Moreover, the crown age of *R.* gr. *margaritifera* has been estimated to date back to ~13 Ma (Van Bocxlaer et al., 2010), although that multi-locus-based estimate appears overestimated relative to those of recent phylogenomic analyses (Feng et al., 2017; Hime et al., 2021). As such, we might expect an Andean-western Amazonian origin of the group with subsequent lowland diversification within Amazonia and secondary dispersal into other Neotropical regions (trans-Andes, Atlantic Forest, Dry Diagonal) during the last 10 Myr. Diversification within Amazonia during the last 10 Myr would imply one of two scenarios: (1) multiple dispersal events across a changing hydrographic system and climate, or (2) *in situ* diversification within Amazonian subregions (Guiana Shield, Brazilian Shield, Western Amazonia; see definitions in Materials and Methods) separated by major rivers that have potentially acted as barriers to dispersal (Albert et al., 2021; Bicudo et al., 2019; Campbell et al., 2006; Hoorn et al., 2017, 2022; Latrubesse et al., 2010). These scenarios would share similarities with the biogeographic histories inferred for other anuran lineages of similar ages, such as extensive dispersal in *Ameerega* (Guillory et al., 2020), *Boana* gr. *albopunctata* (Fouquet, Marinho, et al., 2021) and the *Allobates trilineatus* clade (Réjaud et al., 2020); or the prevalence of *in situ* diversification (i.e., within Amazonian subregions) as inferred in *Adenomera* (de Carvalho et al., 2021); *Synapturanus* (Fouquet, Leblanc, et al., 2021); *Pristimantis* (Fouquet et al., 2022) and *Amazophrynella* (Moraes et al., 2022). The large to medium-sized members of the *R.* gr. *margaritifera* are essentially forestial (even though they can marginally occupy ecotones with open habitats) but differ in breeding sites, with some species reproducing (1) directly in streams (*R. proboscidea*; APL pers. obs.) or more often in small ponds formed along the banks of rivers and streams (e.g., *R. acutirostris*, *R. margaritifera*, *R. lescurei*, *R. roqueana*, *R. proboscidea*, *R. exostosica*, *R. ocellata*, *R. alata*, *R. hoogmoedi*, *R. stanlaii*, *R. scitula*); (2) in small cavities filled with water in trunks, palms, and nut husks (e.g., *R. magnussoni*, *R. castaneotica*); or (3) directly in small pools formed on *terra firme* ground (*R. teotoniensis*, *R. iserni*, *R. margaritifera*). These distinct breeding ecologies probably influence the ability of different species to disperse (Fouquet et al., 2015). In particular, river-associated species appear to have larger ranges.

Herein, we gathered a spatially dense sampling combining molecular (841 sequenced specimens for mtDNA and 55 selected ones for a nuDNA locus), morphological (2,018 examined specimens), and acoustic data (104 recorded specimens) encompassing the entire range of *R.* gr. *margaritifera* to improve knowledge on species boundaries and distributions. This sampling includes specimens from or nearby the type localities of most described species. Moreover, we examined the

morphology of types of most taxa in an attempt to assign our delimited candidate species to existing names. Finally, we gathered mitogenomic data for selected representatives of most delimited species to obtain a time-calibrated phylogeny. Based on this temporal framework, we tested how successive dispersals throughout Amazonia or *in situ* speciation within Amazonian subregions (i.e., interfluves) have contributed to species diversity and distributions in this ubiquitous yet challenging toad clade.

Materials and methods

Species delimitation

Aiming at investigating the species boundaries within *Rhinella* gr. *margaritifera*, we initially delimited Molecular Operational Taxonomic Units (MOTUs) based on a fragment of the 16S rRNA gene. Given limitations pertaining to the use of mtDNA evidence alone to delimit species (Chan et al., 2022; Funk & Omland, 2003), we interpret the MOTUs cautiously as a first approximation of the extant diversity and putative species boundaries in the group, which we use to inform downstream analyses. Moreover, we secondarily test the congruence of the delimited MOTUs based on patterns of variation in nuclear DNA (nuDNA), morphological characters (quantitative and qualitative), and acoustic data. We then assess the robustness of delimited species based on the congruence among these four lines of evidence.

mtDNA sampling and analyses

Our 16S rRNA sampling included sequences from 841 specimens of *Rhinella* gr. *margaritifera* (Supplemental Table S1), spanning the entire geographic distribution of the group and including all 23 currently valid nominal species except for the recently described *Rhinella angeli* (Rojas et al., 2022). Our sampling also included most of the synonymized names. These samples were obtained through fieldwork and loans complemented with sequences from GenBank (Supplemental Table S1). Newly acquired sequences were obtained from both Illumina MiSeq sequencing and Sanger sequencing (methodological details available in Supplemental Appendix 1). We also included 55 sequences (both newly sequenced and from GenBank) from taxa in the sister group *R.* gr. *festae* (Pereyra et al., 2021), totalling 896 sequences. These sequences were aligned on the MAFFT online server under the E-INS-i option with default parameters (Katoh et al., 2017). The final matrix after trimming sequence ends was 543 base pairs (bp) long.

Based on this matrix, we applied three single-locus species delimitation approaches: (a) the distance-based method Automated Barcode Gap Discovery (ABGD; Puillandre et al., 2012), (b) the multi-rate coalescent-based method multi-rate Poisson Tree Processes (mPTP; Kapli et al., 2017), and (c) the single-threshold coalescent-based method Generalized Mixed Yule Coalescent (GMYC; Pons et al., 2006; Monaghan et al., 2009). ABGD was implemented using the online web server (available at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with a prior of intraspecific divergences (K80) between 0.001 and 0.1 ($p = 0.001–0.1$), a proxy for minimum relative gap width of 1 ($X = 1$), and a number of steps equal to 30 ($n = 30$). For mPTP, we first inferred a ML tree with RAxML v.8.2.4 (Stamatakis, 2014) using the GTR + I + Γ model, which was estimated as the best-fitting model of evolution using Modelfinder in IQTREE (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017). We ran mPTP using the rooted tree (outgroups removed) and 5 million Markov chain Monte Carlo (MCMC) iterations sampling every 10,000th iteration and discarding the initial 20% iterations as burn-in. For GMYC, we inferred a time-calibrated phylogeny using BEAST 2.5.2 (Bouckaert et al., 2014) using a birth-death population model. We used a single partition under the GTR + I + Γ substitution model. An uncorrelated relaxed lognormal clock was used to model rate variation among branches (Drummond et al., 2006). Furthermore, we used the estimated age of the most recent common ancestor (MRCA) of *R. marina* and *R. acrolopha* from Hime et al. (2021) as a calibration point, assuming a normal prior distribution with mean = 19.3 Ma (95% highest posterior density (HPD) = 16.5–22.1). This analysis used two independent MCMC chains of 20 million iterations each, recording every 1000th iteration. We combined the log and tree files of the two runs, discarded the first 25% iterations as burn-in, and resampled 10% of the resulting trees using LogCombiner 2.5 (Bouckaert et al., 2014). We confirmed parameter convergence based on ESS above 100 (except for the birth and death rates). We then extracted the maximum clade credibility tree (based on 3500 posterior trees) using TreeAnnotator 2.5 (Bouckaert et al., 2014). We performed single-threshold GMYC delimitation on this tree using the GMYC function of the splits R package (Ezard et al., 2009), with a threshold interval between 0 and 10 Ma.

Final MOTUs were defined based on a majority-rule consensus from the results of those three delimitation methods, i.e., a lineage was considered a MOTU when recovered by at least two of the three methods. The strict application of this criterion was not possible in three instances: (1) within the taxon *R. acutirostris*,

where contradicting partitions between the three approaches prevented finding any consensus; (2) among several morphologically distinctive taxa that were found lumped into a single MOTU when applying a majority-rule consensus, which we consider unlikely given pronounced morphological disparity (e.g., *R. exostosica* + *R. stanlaii* + *R. scitula*; *R. dapsilis* + *R. margaritifera* + *R. sebbeni*; *R. proboscidea* + *R. teotoniensis* + *R. castaneotica*); and (3) within the taxon *R. hoogmoedi*, where two groups of populations were lumped together despite having disjunct geographic ranges, in the Atlantic forest and the Guiana Shield. In these cases, we followed a finer delimitation (nested partitions) that is justified in more detail in Supplemental Appendix 2. Lastly, to obtain an estimate of genetic divergences among the resulting MOTUs, we calculated mean genetic p-distances using MEGA X (Kumar et al., 2018).

nuDNA sampling and analysis

To further assess the evolutionary divergence between delimited MOTUs, we examined nuDNA allele sharing and network cohesion between selected representatives of the main MOTUs, particularly those that appear to overlap geographically. We selected 33 samples to generate a 542 bp-long fragment of the POMC gene (see Fouquet et al., 2022 for primers and PCR conditions) that were combined with sequences from GenBank ($n = 22$, Supplemental Table S2), totalling 55 individuals representing 28 mtDNA-based MOTUs. These sequences were aligned using the 'translation align' option and the Geneious alignment algorithm in Geneious R9 (https://www.geneious.com). To determine the most probable alleles for heterozygous individuals, we used PHASE (Stephens et al., 2001; Stephens & Donnelly, 2003) implemented in DnaSP 5 (Librado & Rozas, 2009). We performed 100 iterations after a burn-in of 100 iterations, using a thinning interval of 1 and default cut-off thresholds.

Based on this nuclear sequence matrix, we reconstructed a Median-Joining network (Bandelt et al., 1999) using PopArt 1.7 (Leigh & Bryant, 2015). We considered the absence of nuDNA allele sharing among specimens assigned to closely related MOTUs as supportive of their speciation.

Morphology and morphometrics

We analysed the external morphology of 2,018 specimens, including the type specimens of most nominal species deposited in 14 herpetological collections (Supplemental Table S3). Non-type specimens were assigned to MOTUs based on molecular, acoustic, geographic range, or a combination of those data. We also analysed high-quality photographs of some holotypes that were available on GBIF (https://www.gbif.org) or provided by museum curators. Sex was determined by examination of dark gular and chest colour in males, nuptial excrescences (Ávila et al., 2020; Caramaschi & de Niemeyer, 2003; Hoogmoed, 1990; Leao & Cochran, 1952; Lima et al., 2007), and the presence of vocal slits in males.

Nineteen morphometric measurements from 1191 specimens (773 adult males and 418 adult females, excluding juveniles, damaged specimens, and those not clearly assigned to any MOTUs) were taken with digital callipers to the nearest 0.1 mm following Ávila et al. (2020) by a sole measurer (RA): SVL (snout–vent length); HL (head length); HW (head width); IND (internarial distance); END (eye to nostril distance); ED (eye diameter); UEW (upper eyelid width); IOD (interorbital distance); POCH (postorbital crest height); POCL (postorbital crest length); HTD (horizontal tympanum diameter); VTD (vertical tympanum diameter); PGL (parotoid gland length); HAL (hand length); FAL (forearm length); THL (thigh length); TL (tibia length); TAL (tarsal length); and FL (foot length). To assess morphometric differentiation among 15 MOTUs (excluding MOTUs with limited morphometric data and focusing on targeted clades *R. proboscidea*, *R. margaritifera* and *R. acutirostris*; see Results), we performed principal component analysis (PCA) based on both the entire dataset (1,191 measured specimens) and partitions focusing on specific subclades (i.e., closely related MOTUs) using both raw data and log-transformed ratios (Claude, 2013). We considered an absence of or marginal overlap among MOTUs along the different PCA axes as supportive of their delimitation as distinct species. These analyses were conducted using the MorphoInd R (Baylac, 2008) and Vegan R packages (Oksanen et al., 2013).

Besides the quantitative characters above, we considered the following 12 qualitative morphological characters for 31 MOTUs (terminology for cephalic characters follows Heyer et al., 1990) as scored by a sole measurer (RA): (1) development of the apical ridge of snout (low or high, observing the formation of a proboscis) (Fig. 1); presence and development of (2) canthal, (3) preocular, (4) supraocular, (5) supratympanic, (6) postocular, and (7) parietal cranial crests (absent, inconspicuous with markings or tubercles only, low, high, or hypertrophied); (8) parotoid gland morphology (triangular or elongated), (9) presence of tubercles on the parotoid gland edge (present or absent); (10) presence and development of neural spines (absent, inconspicuous with markings or tubercles only, low, or high); (11) presence


(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)

and development of the protrusion at the mouth angles (absent, developed, or hypertrophied); and (12) morphology of the dorsolateral line (tuberculated fold with scattered tubercles only, tuberculated fold with distinctive round tubercles, or spinose with developed pointed tubercles). To assess variation of qualitative morphological characters, we carefully examined the 12 characters described above of 10 adult males and 10 adult females of each available MOTU.

Bioacoustics

We analyzed advertisement call recordings of 104 male specimens representing 22 MOTUs (Supplemental Table S4). New calls were recorded with digital recorders equipped with external directional microphones. Digital recordings were sampled at 44.1 kHz sampling rate and a 16 bit resolution, saved as uncompressed .wav files, and deposited in the Banco de Registros Bioacústicos of the Laboratório de Herpetologia do Instituto de Biociências da Universidade Federal de Mato Grosso (LH; Cuiabá, Mato Grosso, Brazil), Fonoteca Neotropical Jacques Vielliard (FNJV; Campinas, São Paulo, Brazil), Centro de Estudos Integrados da Biodiversidade Amazônica (CENBAM), Muséum National d'Histoire Naturelle Sonothèque (MNHN; Paris, France), and Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ in BIOWEB). We visualized the spectrograms using Raven Pro 1.3 (www.birds.cornell.edu/raven), with the following settings: DFT size 256-point samples, time grid overlaps 50%, and Hanning window. Calls of the species of *R.* gr. *margaritifera* are either emitted in long (>20 calls) or short series (<10 calls, e.g., *R. roqueana*), and each call is generally formed by a series of notes, either single or organized in clusters. The variation of this complex call structure across species is challenging to describe with a single set of variables. We measured the following 11 temporal parameters (in ms) from the waveform and one spectral parameters (in Hz) from a spectral trace extracted from entire calls (terminology follows the call-centered definition of Köhler et al., 2017): (1) maximum number of calls per series (CS; when >20 we set that measure to 20, as the number of calls can vary with specimen excitement); (2) call length (CL); (3) inter-call interval (CI); (4) number of notes per call (NN; delimited as either single pulses or clusters of pulses separated by silences longer than silences between pulses groups; in the case of *R. lescurei*, each note is single-pulsed); (5) shortest note duration per call (SN; usually the first); (6) longest note duration per call (LN; usually the last); (7) minimum inter-note interval (SI; usually the last); (8) maximum inter-note interval (LI; usually the first); (9) number of pulses in the first note (NPF); (10) number of pulses in the last note (NPL); (11) pulse interval (PI; time between two peaks taken between the first pair of pulses); (12) dominant frequency (DF).

Recordings were assigned to each of 22 MOTUs based on (1) direct assignment of genotyped recorded males, (2) co-occurrence of genotyped specimens with calling males, and (3) co-occurrence of lineages where the recording was taken (i.e., when call types from a locality could be unambiguously assigned to a given MOTU, then other call types were assumed to correspond to a different and co-occurring MOTU). We used these variables to perform a PCA first using all the data. We excluded *R. magnussoni* from the analysis because its call consists of tonal notes, contrasting with the rest of the group. Some MOTUs had clearly distinct calls, but three groups of MOTUs overlapped in the multidimensional call space. We thus performed additional PCA focusing on these three clusters separately. These PCA were conducted using the FactoMineR R package (Lê et al., 2008; R Development Core Team, 2021).

Fig. 1. Photographs showing some diagnostic characters and their variation among representatives of *Rhinella* gr. *margaritifera* (see Supplemental Fig. S1 for photographs of most MOTUs). Diagnostic characters: (1) snout apical ridge; (2–7) cranial crests; (8) parotoid gland shape; (9) tubercles on parotoids; (10) neural spines; (11) protrusion at the mouth angles; and (12) dorsolateral folds. (A) *R. proboscidea* (UFMT-A 13354, male from Urucará, Amazonas State) with a snout apical ridge developed into proboscis, preocular crests absent, supraocular crests absent, no protrusion at the mouth angles and dorsolateral folds with small tubercles; (B) *R. stanlaii* (UFMT-A 6246, male from Cuiabá, Mato Grosso State) with supratympanic crests undeveloped, neural spines absent, and dorsolateral folds with enlarged tubercles; (C) *R. margaritifera* (UFMT-A 11670, female from Primavera, Para State) with low snout apical ridge, preocular crests undeveloped, neural spines low; (D) *R. roqueana* (INPA-H 33112, female from Canutama, Amazonas State) with canthal crests absent, supratympanic crests hypertrophied, tubercles on parotoids present, neural spines prominent and no protrusion at the mouth angles; (E) *R.* sp. Itoupe 1 (MNHN-RA-2022.0003, female from Itoupe, French Guiana) with canthal crests absent, supratympanic undeveloped, parietal crests absent, and parotoids elongated; (F) *R.* sp. Purus (INPA-H 25624, male from BR-319 highway, Ipixuna, Amazonas State) with canthal and supraocular crests hypertrophied and parotoids elongated; (G) *R.* sp. Aripuana (UFMT-A 11412, female from Aripuanã, Mato Grosso State) with canthal and parietal crests developed, parotoids triangular, dorsolateral folds with spinose tubercles; (H) *R. exostosica* (paratype INPA-H 41318, female from Jaci, Porto Velho, Rondonia State) with preocular and postocular crests developed and hypertrophied protrusion at the mouth angles.

Integrative support for candidate species

Based on the integration of genetic, acoustic, and morphological data, we assigned each MOTU to the category of confirmed candidate species (CCS), unconfirmed candidate species (UCS), or deep conspecific lineage (DCL), following the framework of Vieites et al. (2009). We considered as 'confirmed' any MOTU for which there was at least one congruent difference in any character other than the primary mtDNA divergence criterion between close relatives. We considered as 'unconfirmed' any MOTU for which additional data were lacking (i.e., if only genetic data were available to support a MOTU's status as a candidate species).

Phylogeny and molecular dating

We selected one representative of each delimited MOTU ($n = 54$, see Results) to estimate phylogenetic relationships and divergence times. To this goal, we obtained whole mitogenomic data via shotgun sequencing for representatives of 33 MOTUs in *R.* gr. *margaritifera* (one from GenBank) and three representatives in *R.* gr. *festae* (one from GenBank) (Supplemental Table S5; methodological details are available in Supplemental Appendix 3). We complemented this mtDNA matrix for the remaining MOTUs by incorporating GenBank sequences of the 12S, 16S, COI, and Cytb genes (Supplemental Table S5). We also retrieved from GenBank mitogenomes of 15 outgroups representing most major bufonid lineages (Supplemental Fig. S5). DNA sequences were realigned using the MAFFT online server under the E-INS-i option for 12S and 16S and considering reading frames for each coding sequence (CDS) with default parameters (Katoh et al., 2017). The control region and tRNA were discarded, as well as flanking regions that were not available for most terminals. The final alignment consisted of 14,057 bp (12S–16S: 2,721 bp; CDS: 11,336 bp). Eleven MOTUs had only 16S, five had only 16S and COI, and 15 had only 12–16S. All the other terminals had at least 12S–16S and COI or complete mitogenomes (Supplemental Table S4).

We predefined four nucleotide partitions, one for the rRNA genes (12S–16S) and one for each codon position of the concatenated protein-coding genes. We then selected the best-fit partition combination scheme and model of evolution for each partition using Modelfinder in IQTREE (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017), according to the Bayesian Information Criterion (BIC). The best-fit scheme included one partition corresponding to the two ribosomal RNA genes and three additional partitions for the protein-coding genes, each of these partitions corresponding to a codon position (combined across genes).

We inferred a time-calibrated phylogenetic tree in BEAST 2.5.2 using a birth-death tree prior substitution models and clock models across nucleotide partitions were unlinked. Trees were linked. For divergence time estimation, we implemented an uncorrelated relaxed log-normal clock model on each partition to account for rate heterogeneity among branches (Drummond et al., 2006). We relied on four secondary calibrations based on the dated phylogeny of Hime et al. (2021), which incorporated genome-wide nuclear markers (220 loci, 291 kilobases): (1) the crown age of Bufonidae with mean = 48.2 Ma (95% HPD 44.1–52.3); (2) the crown age of Bufonidae except for *Melanophryniscus* at 37.4 Ma (95% HPD 33.6–41.2); (3) the crown age of the 'toad phenotype' (*sensu* Van Bocxlaer et al., 2010), corresponding to *Rhinella* + *Anaxyrus* + *Incilius* + all the non-American bufonids, at 24.1 Ma (95% HPD 21.3–26.9); and (4) the crown age of *Rhinella* at 19.3 Ma (95% HPD 16.5–22.1).

We set four independent MCMC runs of 10 million iterations each, recording every 1,000th iteration and discarding the first 10% of iterations as burn-in. We combined the log and the tree files and the resulting posterior samples of trees of the two independent runs using LogCombiner 2.5 and checked the convergence of model parameters via time-series plots. Chain mixing was considered adequate when parameters achieved an effective sample size above 100 (obtained for all parameters). We extracted a maximum clade credibility tree (based on the 36,002 resulting trees) using TreeAnnotator 2.5.

In order to estimate the reliability of the obtained time estimates for our focal group we also undertook complementary analyses using: (1) only the two oldest calibration points (#1 and 2); only the most recent calibration point (#4) and an upper bound for Bufonidae set as 66 Ma, considering the KPg boundary has a well-established period of early diversification of Hyloidea; (3) using two fossil calibrations providing minimum ages for the crown *Rhinella* (Estes and Wassersug (1963) identified a middle Miocene (min. 11.6 Ma) fossil as *Rhinella marina*) and for the crown of the toad-like phenotype *sensu* Van Bocxlaer et al. (2010) i.e., the clade including *Rhaebo* and *Rhinella* in our tree following Baez & Nicoli (2004) who identified bufonid fossils that they considered to be closely related to extant *Rhinella*, from the late Oligocene (min. 25.5 Ma). Additional justifications for the fossil calibrations are available in Supplemental Appendix 4. For this last analysis we also set the upper bound for Bufonidae as 66 Ma. These analyses consisted of MCMC runs of 20 million iterations each, recording every 1,000th iteration and discarding the first 20% of iterations as burn-in.

Biogeographic analyses

We used the subtree including *Rhinella* gr. *margaritifera* and *R.* gr. *festae* (i.e., pruning the other bufonids) from the time-calibrated phylogeny to infer ancestral areas and biogeographic events using the BioGeoBEARS R package (Matzke, 2013). We compared three models: (i) a likelihood version of the Dispersal-Vicariance model (DIVALIKE; Ronquist, 1997) (ii) a likelihood version of the BayArea (BBM) model (Landis et al., 2013), and (iii) the Dispersal-Extinction Cladogenesis model (DEC; Ree & Smith, 2008). We also compared versions of these models allowing jump-dispersal as described by the J parameter (Klaus & Matzke, 2020; Matzke, 2013; Ree & Sanmartín, 2018). Models were compared using the Akaike Information Criterion (AIC).

To infer the history of lineage diversification in a spatial context, we outlined seven biogeographic areas: Guiana Shield (GS), Western Amazonia (WA), Brazilian Shield (BS), Atlantic Forest (AF), Dry Diagonal (DD), Andes (AN) and a Trans-Andean region (TA). The three areas corresponding to Amazonia (GS, WA, and BS) were defined based on major geological features in this region (Hoorn et al., 2010) and their correspondence to distinctive biogeographic provinces historically known as Wallace's districts (Wallace, 1854). These provinces are roughly delimited by modern riverine barriers, namely the Madeira River, the Negro River, and the lower course of the Amazon River. This spatial partitioning allows us to investigate the putative historical connectivity across Neotropical regions and within Amazonia (Fouquet, Marinho, et al., 2021; Réjaud et al., 2020). Altogether the three Amazonian areas correspond to a broad definition of the Amazonian biome, matching the boundaries of the Terrestrial Ecoregions of the World by Olson et al. (2001), which correspond to a ca. 1000 m elevation limit along the Andes on the west, the boundary with the Dry Diagonal on the east, the Atlantic Ocean on the north, and including patches of open vegetation within Amazonia. Pantepui was not considered because it does not harbour species in our focal group.

Results

MtDNA species delimitation

The phylogenetic trees obtained from the ML and the Bayesian analyses of the 16S rDNA gene strongly supported both *Rhinella* gr. *margaritifera* and *R.* gr. *festae* as monophyletic (Supplemental Fig. S2). *Rhinella* gr. *margaritifera* is structured into seven major clades: the *R. margaritifera* clade (Amazonia and Atlantic Forest), the *R. acutirostris* clade (western Amazonia and Trans-Andean region), the *R. ocellata* clade (western Amazonia and Dry Diagonal), the *R. proboscidea* clade, *R. parecis* (Amazonia; note that *R. parecis* is strongly supported as sister to the *R. proboscidea* clade based on mitogenomic data; see Fig. 2 and below), the *R. magnussoni* clade (Amazonia), and the *Rhinella* 'western' clade (western Amazonia) (Fig. 2, Supplemental Fig. S2).

Of the three species delimitation methods, mPTP was the most conservative, delimiting 24 MOTUs. ABGD resulted in an intermediate level of partitioning by delimiting 41 MOTUs. ABGD allows the user to select the most plausible species partition threshold among a set of nested partitions; we kept the 7–8th partitions ($p = 0.00304$) because higher thresholds clustered all the species of *R.* gr. *margaritifera* in a single partition, whereas lower thresholds led to unrealistically large numbers of MOTUs. Similarly, GMYC delimited a seemingly unreasonably high number of MOTUs, 85 (Fig. 2; Supplemental Table S1). The majority rule consensus across these delimitations led to 40 MOTUs, including three MOTUs within *R. acutirostris* that display conflicting boundaries across the three methods (Supplemental Table S1; Supplemental Appendix 2). We also found several instances of incongruences between the DNA-based partitioning and the morphologically diagnosable species currently recognized in taxonomy, for instance the lumping of taxa that are unambiguously distinct morphologically, which we interpret as false negatives. These putative false negatives were particularly prevalent within the *R. margaritifera* and the *R. proboscidea* clades. Therefore, to derive a more plausible picture of species diversity in the group, we departed from the strict application of the consensus across the three mtDNA-based delimitation methods and used nested partitions, following considerations extensively developed in Supplemental Appendix 2. With that, we outlined a total of 54 MOTUs that we considered candidate species in the group.

Mean p-distances among many of these MOTUs (based on 431 bp of 16S that overlap across most samples) were low, below 2% among closely related MOTUs in general but notably so among members of the *R. proboscidea* clade. Such low genetic distances separate taxa such as *R. alata* and *R. sclerocephala*, *R. castaneotica* and *R. proboscidea*. Slightly higher distances, 2–3%, separate nominal species in four instances: *R. stanlaii* and *R. exostosica*; *R. margaritifera* and *R. martyi*; *R. teotoniensis* and *R. castaneotica*; and *R. teotoniensis* and *R. proboscidea* (Supplemental Table S6). Other nominal species were separated by genetic distances above 3%, typically considered indicative of species-level divergences for 16S (Fouquet, Gilles, et al., 2007; Vences et al., 2005).

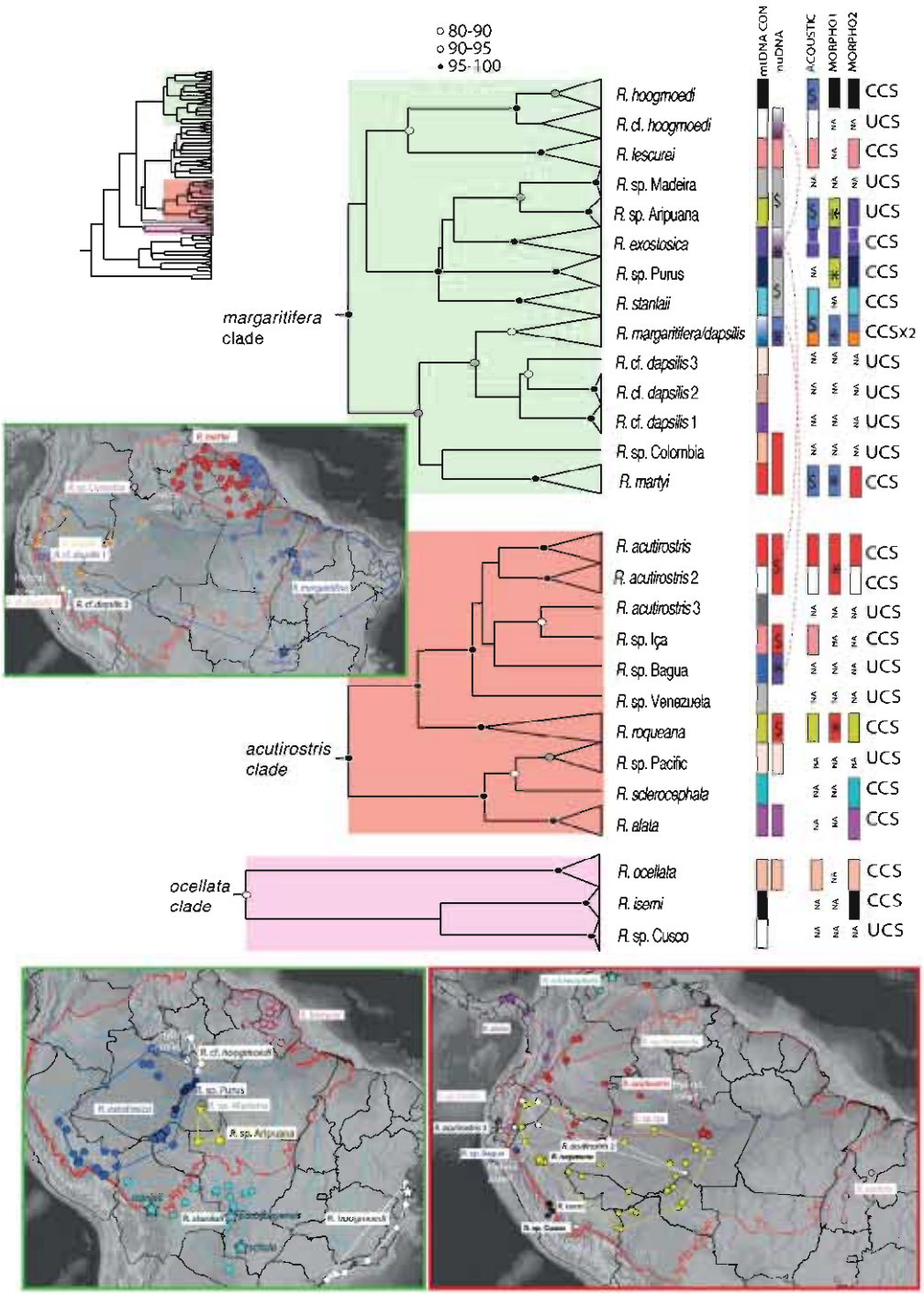

Fig. 2. Results of the species delimitation analysis of *Rhinella* gr. *margaritifera* (first: *R. margaritifera*, *R. acutirostris*, *R. ocellata* clades; second: *R. proboscidea*, *R. magnussoni* 'western' clades). On the upper left, an ultrametric subtree (with outgroups removed) obtained from Bayesian phylogenetic analysis of the 16S rRNA sequences (posterior probabilities are indicated with filled circles on each node). The branches are collapsed according to the delimited MOTUs (see Results). Major clades (confirmed by mitogenomic phylogenetic analysis; Fig. 6) are depicted with distinct background colours, applied also in subsequent figures. The vertically aligned coloured boxes illustrate the partitions found using (from left to right) (1) the DNA-based species delimitation consensus (after adaptations explained in the taxonomic accounts in Supplemental Appendix 2), (2) nuDNA network, with red dotted lines indicating incongruences with mtDNA, (3) acoustic data, (4) morphometric data, (5) qualitative morphological data and (6) the integrative diagnostic. (*) and ($) indicate unique partitions that are scattered in the tree. Distribution maps show the range of the delimited MOTUs using the corresponding colours as in the mtDNA partitioning. Type localities are depicted by filled stars and minimum convex polygons depict the distribution boundaries. The limits of the biome Amazonia sensu lato are depicted in red.

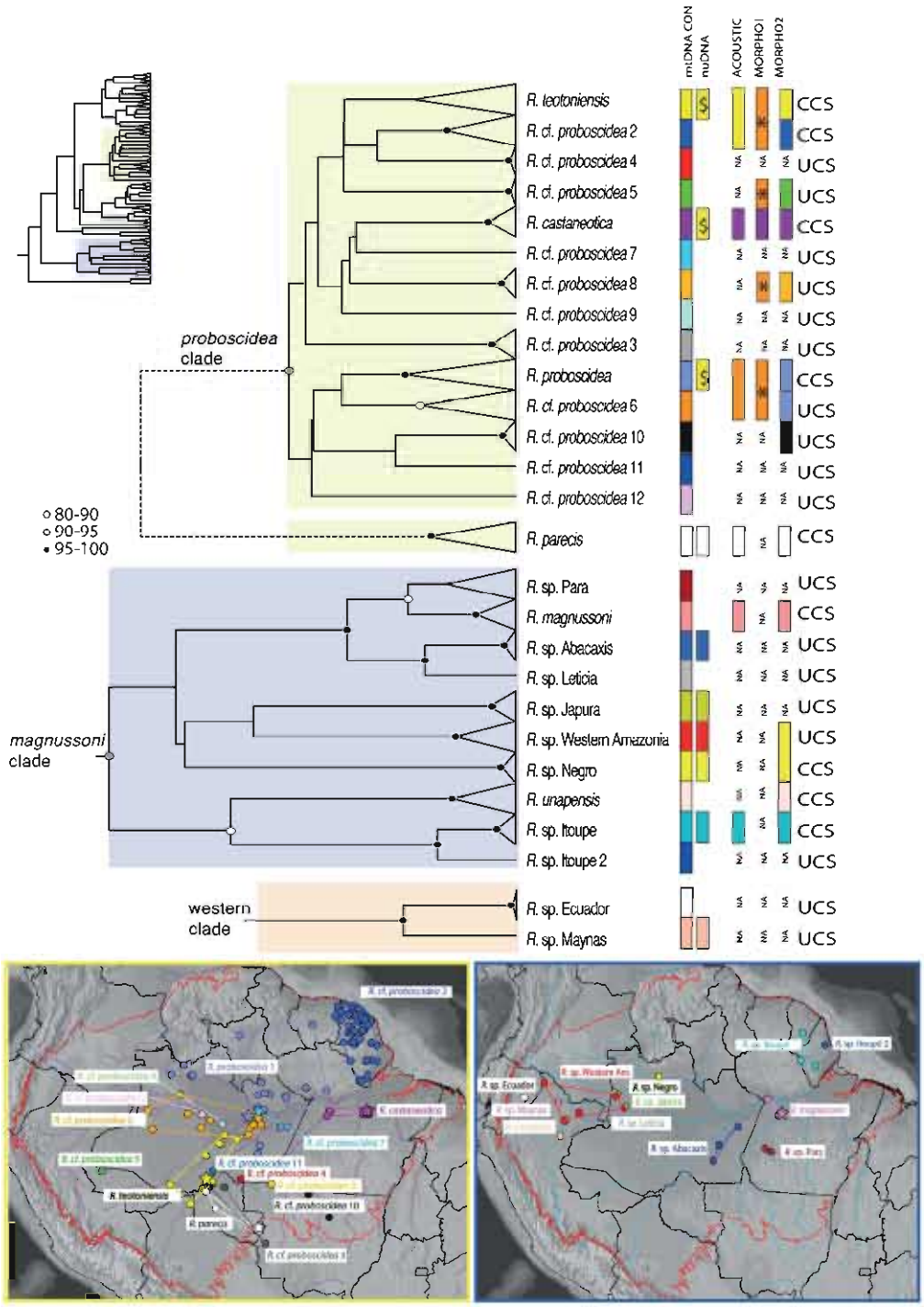

Fig. 2. Continued.

nuDNA

We obtained 51 distinct phased alleles (110 total) from 55 POMC sequences (Supplemental Table S1). Given the low number of samples included and the low variation on this single locus, the obtained median-joining network does not provide much information regarding the differentiation among closely related MOTUs (Fig. 3). Nevertheless, it supports the distinctiveness of *R. lescurei*, *R. parecis*, *R.* sp. Maynas, *R.* sp. Abacaxis, *R.* sp. Negro, *R.* sp. Japura, *R.* sp. Itoupé, *R.* sp. Western Amazonia, and *R. ocellata*. Moreover, the network is overall congruent with the major mtDNA clades, because their corresponding nuDNA alleles form non-overlapping clusters. Nevertheless, there were a few striking discrepancies. First, *R.* sp. Bagua shares one allele with both the co-occurring *R. dapsilis* and the geographically distant *R. exostosica* (from the Japura River region); by contrast, mtDNA inferred *R.* sp. Bagua to be nested in the *R. acutirostris* clade, whereas *R. dapsilis* and *R. exostosica* are nested in the *R. margaritifera* clade. The second allele of *R.* sp. Bagua is similar to that of *R. dapsilis* within the *R. margaritifera* cluster. A second conflict between nuclear alleles clusters and mtDNA clades involves one sample of *R. exostosica* from the Negro River; one of its alleles was nested in the *R. margaritifera* cluster (shared with *R.* sp. Colombia and concordant with mtDNA-based results), while the other one is shared with *R. acutirostris* 1. Another sample from Negro River assigned to *R. exostosica* on the basis of mtDNA has both alleles identical, being nested in the *R. acutirostris* cluster. This allele is shared with the geographically adjacent *R.* cf. *hoogmoedi*. The second allele of *R.* cf. *hoogmoedi* is however nested in the *R. margaritifera* cluster (as with mtDNA), being shared with the geographically neighbouring *R. martyi* and *R.* sp. Purus. The clustering of nuclear alleles from phylogenetically distant lineages may be indicative of extant or recent genetic introgression across species, particularly in the westernmost Amazonia (involving *R.* sp. Bagua and *R. dapsilis*) and along the lower Negro River basin (between *R.* cf. *hoogmoedi*, *R. exostosica*, *R. acutirostris*, and *R. martyi*; see Discussion). Finally, the haplotypes of *R.* sp. Western Amazonia (from the *R. magnussoni* clade) are also scattered in two groups within the nuDNA network, one being more similar to the co-occurring *R.* sp. Negro and the other more similar to the remaining species of the *R. magnussoni* clade.

Quantitative morphological traits

The first two components of the morphometric-based PCAs explain 31 and 41% of the variation in males and 36 and 53% in females based on the raw data, and 34–61% in males and 35 and 67% in females using the size-corrected data (Supplemental Fig. S3). The resulting morphometric space suggests an almost complete overlap among major mtDNA clades (Fig. 4A). Among them, the *R. proboscidea* clade overlaps with the other major clades but is partly differentiated both using raw data (driven by its smaller SVL; Supplemental Table S7) and log-shaped ratios accounting for size differences (Fig. 4A).

In turn, when focusing on closely related species (i.e., within the major mtDNA clades), we found evidence of morphometric differentiation in only a few cases (a selection of these results is represented in Fig. 4; complete results available in Supplemental Fig. S3). Among them, *R. exostosica* marginally overlaps with *R.* sp. Purus and *R.* sp. Aripuana, considering both males and females and raw and size-corrected data. Some differentiation is also noticed when comparing *R. castaneotica* with the other species of the *R. proboscidea* clade, albeit for females only. All the other candidate species overlap extensively in morphometric space, indicating little differentiation in size and shape. However, such broad morphometric overlap involved many taxa diagnosable on the basis of qualitative morphological data, supporting that candidate species have diverged in phenotypic axes other than morphometric attributes (see below).

Qualitative morphological traits

In contrast with morphometric data, qualitative morphological characters provide diagnostic features (i.e., are fixed within species or unique in combination), especially the shape of snout (1), cranial crests in males and females (2–7), neural spines (10), protrusion at the mouth angles (11), and the dorsolateral row of tubercles (12), allowing many of the delimited MOTUs to be distinguished (Supplemental Table S8; more details in Supplemental Appendix 2). The shape of the snout and the development of its apical ridge reliably distinguish closely related members of the *R. proboscidea* clade: *R. proboscidea* has a truncate snout in dorsal view with an apical ridge forming a proboscis and two species (*R. teotoniensis* and *R.* cf. *proboscidea* 10) have a pointed snout in dorsal view (Supplemental Fig. S1), while the remaining species in their clade have a rounded snout in dorsal view. *Rhinella* cf. *proboscidea* 2 (eastern Guiana Shield) can be differentiated from the remaining species in its clade by the possession of an acute snout in lateral view (protruding in the other species). Finally, the apical ridge is developed in *R.* cf. *proboscidea* 8 (Mato Grosso State), but it is low in *R. castaneotica* and *R.* cf. *proboscidea* 5 (Acre State) (Supplemental Fig. S1). These last

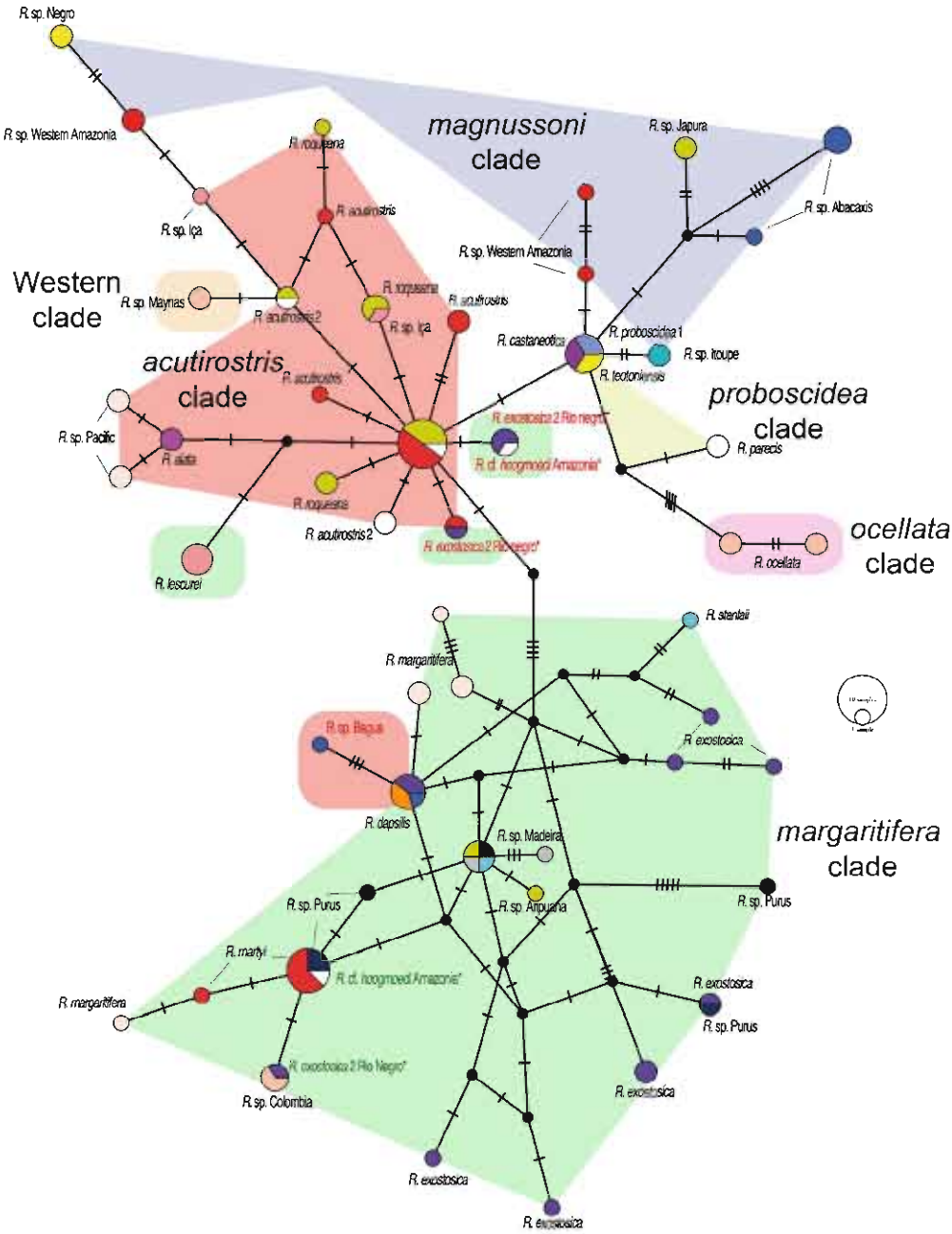

Fig. 3. Median-joining network based on 110 POMC nuDNA phased alleles from 55 specimens of *Rhinella* gr. *margaritifera*. The colours and labels of alleles correspond to the delimited MOTUs (Fig. 1). Polygons correspond to major mtDNA clades (Fig. 1). Red labels and asterisks indicate putatively introgressed samples (i.e., samples clustering incongruently with mtDNA-based results) and green labels indicate the alleles from the same individuals clustering congruently with mtDNA.

two candidate species are also differentiated by the presence of a preocular crest and the more developed lateral row of tubercles in *R.* cf. *proboscidea* 5.

Three cranial crests characters (canthal, supratympanic, and postocular) vary in development in both males and females (Fig. 5). Those variations are however only noted in clades whose members present developed cranial crests (i.e., *R. margaritifera* and *R. acutirostris* clades). Other characters are fixed among many MOTUs of these two clades such as snout shape

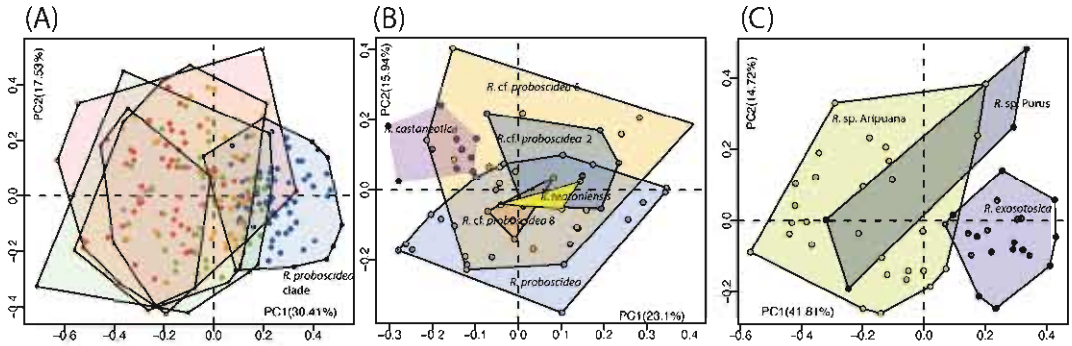

Fig. 4. Selected Principal Component Analyses (PCAs) based on 17 log-shaped morphometric ratios in *Rhinella* gr. *margaritifera* (1197 specimens total, 773 males and 418 females), using (A) all the measured female specimens, and (B) considering only the *R. proboscidea*, and (C) *R. exostosica* mtDNA major clades (Fig. 1). Colours are following the mtDNA-based species delimitation (Fig. 1). All the PCAs are available in Supplemental Fig. S3.

(dorsally truncated, rounded, pointed, acute, or mucronate) and dorsolateral row of tubercles (spinose, tubereulated, and overlapping parotoids or not).

Within the *R. acutirostris* clade, *R. roqueana* is distinct from the remaining species by possessing hypertrophied supratympanic crests and hypertrophied neural spines, whereas *R. acutirostris* and *R. acutirostris* 2 differ from each other mainly by snout shape in lateral view (acuminate in *R. acutirostris* and acute in *R. acutirostris* 2) and by the presence of low neural spines in *R. acutirostris* (Supplemental Fig. S1) and more developed supratympanic and postocular cranial crests in *R. acutirostris* 2 (Fig. 5).

Within the *R. margaritifera* clade, *R. exostosica* and *R.* sp. Aripuana are the only MOTUs with hypertrophied bony protrusions at the mouth angles (Fig. 5; Supplemental Fig. S1), being barely distinguished from one another by the development of preocular and supraorbital crests. It is noteworthy that a population from Santa Isabel do Rio Negro (Amazonas, Brazil) genetically assigned to *R. exostosica* is morphologically similar to *R. acutirostris* (Supplemental Fig. S1). *Rhinella margaritifera*, the most taxonomically challenging species of the group, also has qualitative morphological diagnostic characters. Although there is genetic similarity between *R. margaritifera* and *R. dapsilis*, the latter is distinct in lacking developed cranial crests in both sexes (well-developed in *R. margaritifera*, especially the supratympanic crest; Fig. 5), and by its fleshy proboscis (absent in *R. margaritifera*). *Rhinella margaritifera* is also distinguished from its putative synonym *R. martyi* by having lower protrusion at the mouth angles (developed in *R. martyi*) and more developed neural spines. Populations assigned to *R. scitula* and *R. paraguayensis* have no differences in qualitative morphological characters from *R. stanlaii* (Supplemental Fig. S1).

Bioacoustics

The two first components of the PCA based on all 104 recordings accounted for 62.4% of the total variation in acoustic traits (Fig. 6). In this analysis, the clusters representing *R. parecis*, *R. castaneotica*, and *R. lescurei* are well segregated from the remaining species, which overlap in three different clusters largely corresponding to three mtDNA major clades (*R. margaritifera*, *R. acutirostris*, and *R. proboscidea* clades). However, a few incongruencies between call traits and mtDNA phylogenetic relationships are noteworthy: (1) a recording assigned to *R. exostosica* from the Negro River clusters with the calls from species of the *R. acutirostris* clade, instead of the *R. margaritifera* clade; and (2) recordings of *R. ocellata* and *R.* sp. Itoupé, which are genetically distant to any other recorded species, overlap with the calls from species of the *R. margaritifera* clade. Nevertheless, their calls are clearly distinct in the sonograms (Supplemental Table S9, Supplemental Fig. S4), and, therefore, it appears that the call variables considered here do not fully capture call differences between these candidate species.

Each of the three acoustic clusters largely corresponds to major mtDNA clades, further supporting genetic evidence of species differentiation. In the PCA focusing on the *R. acutirostris* cluster (and *R. exostosica* from the Negro River), the two first principal components accounted for 56.6% of the total acoustic variation, segregating *R.* sp. Iça, *R. acutirostris* 1 and *R. exostosica* (from the Negro River region) from *R. acutirostris* 2 and *R. roqueana* (Fig. 6B). These last two species partially overlap in acoustic space but have clearly distinct calls, with *R. acutirostris* 2 emitting a long series of short calls (often more than 40 consecutive calls) and *R. roqueana* emitting short series (4–11). In the PCA focusing on the *R. margaritifera* cluster, the two first

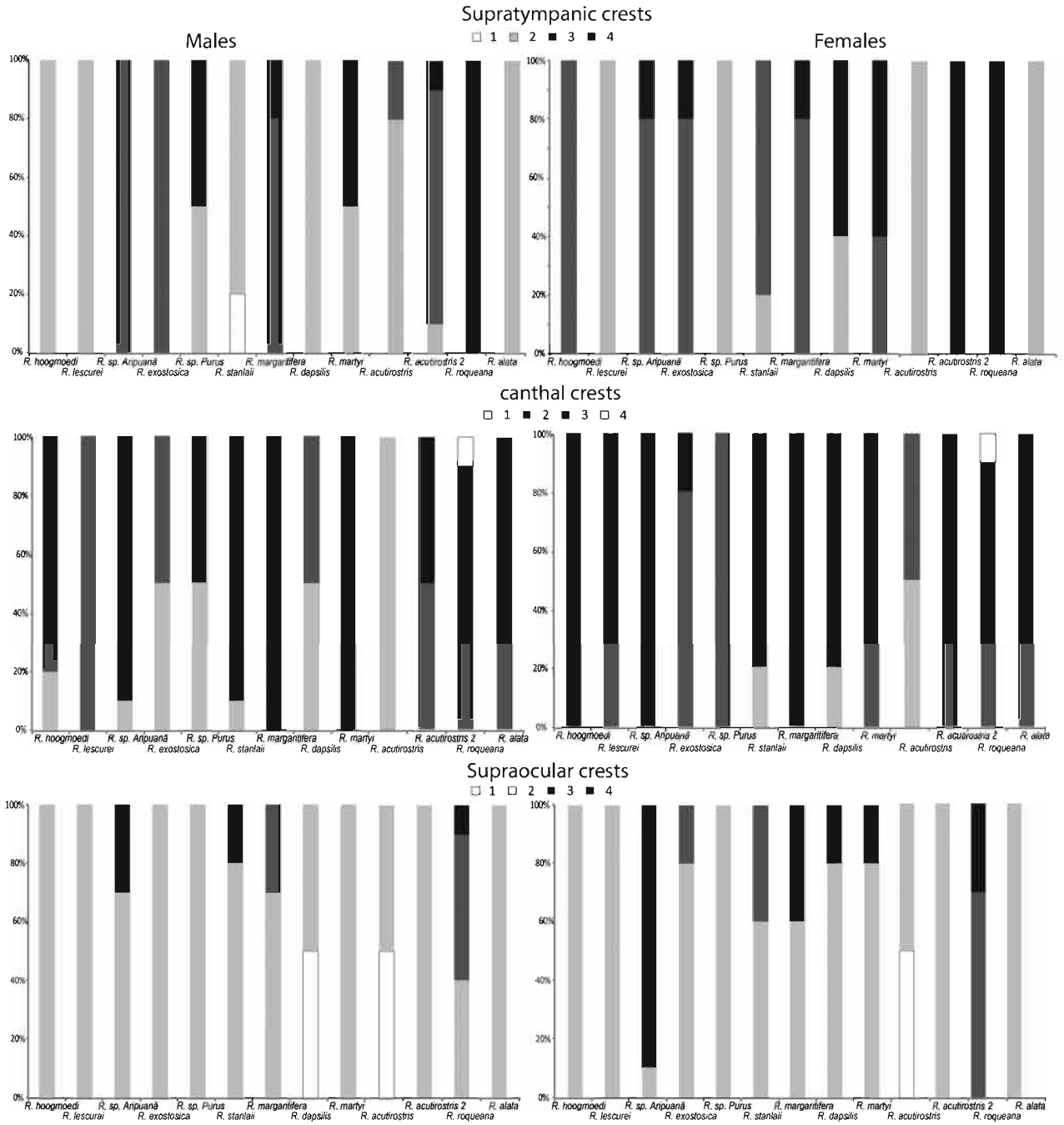

Fig. 5. Proportions of states (1: absent; 2: low; 3: high; 4: hypertrophied) of three qualitative morphology characters (canthal; supraocular; supratympanic crests) observed in 10 males and 10 females of the *R. margaritifera* and the *R. acutirostris* clades (populations from the Negro River were excluded).

components accounted for 46.6% of the total variation, segregating *R. dapsilis*, *R. stanlaii*, and *R. exostosica* from the remaining species (Fig 6D). However, *R. margaritifera*, *R. martyi*, and *R.* sp. Aripuana largely overlap in acoustic space, even when analyzed separately; to illustrate the similarity between *R. sebbeni* and *R. gildae*, they were also considered independently from *R. margaritifera* (Fig. 6; Supplemental Table S8).

In the PCA focusing on the *R. proboscidea* cluster, the two first components accounted for 44.7% of the

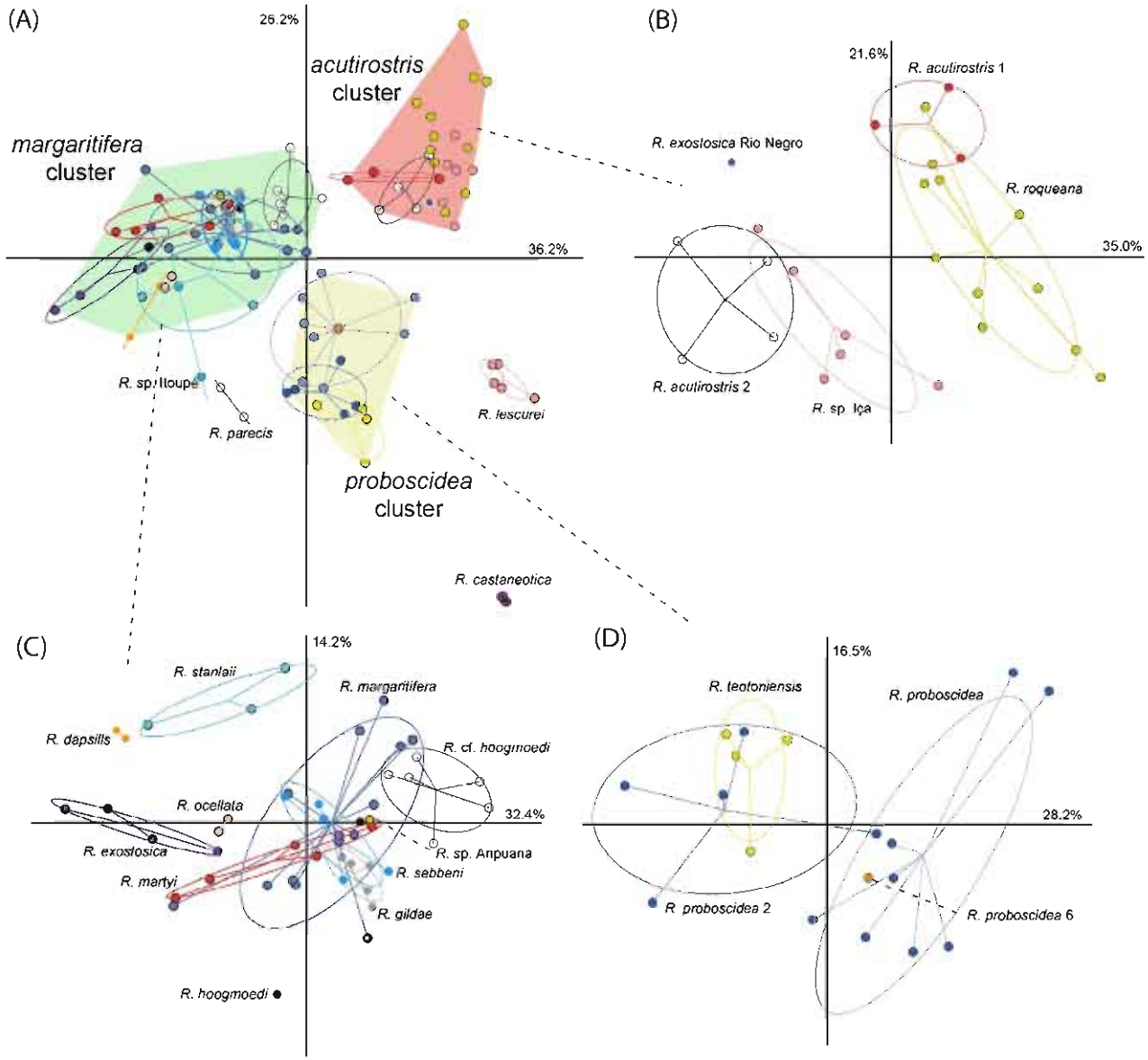

Fig. 6. Principal Component Analyses (PCAs) based on 12 acoustic variables from 104 recorded males of *Rhinella* gr. *margaritifera*. Plots consider the entire group (A) or focus on the *R. acutirostris* (B), *R. margaritifera* (C), and *R. proboscidea* (D) clusters to facilitate visualization of overlap and segregation among candidate species. Ellipses are graphical representations of the 67% dispersal from the centroids.

total variation, segregating two subgroups: *R. proboscidea* + *R. proboscidea* 6, and *R. teotoniensis* + *R. proboscidea* 2. Each MOTU within each pair largely overlap in acoustic space. Nevertheless, *R. teotoniensis* and *R. proboscidea* 2 are spatially very distant and distinguished by the number of notes per call and length of silences among notes (Supplemental Table S9, Supplemental Fig. S4).

Integrative support for candidate species

Based on the integration of mtDNA, nuDNA, morphometrics, qualitative morphology, and bioacoustic lines of evidence, we consider 25 MOTUs as Confirmed Candidate Species (CCS, including one false negative, i.e., a phenotypically divergent species that was not identified by mtDNA delimitation). Moreover, 30 MOTUs are considered Unconfirmed Candidate Species

(UCS) because of missing information for particular data types. In some cases, assigning these MOTUs to currently recognized taxa was difficult (Supplemental Appendix 2).

Briefly, two lineages occur near the imprecise type locality of *R. proboscidea*. Therefore, we tentatively assigned one of them to this taxon, a decision supported by morphological examinations, notably of the holotype, but which might require further analysis. The many unnamed lineages related to *R. proboscidea* are left for future dedicated analyses of this complex.

As for *R. margaritifera*, we also examined the type material and considered that the type locality is most likely in south-eastern Amazonia (Supplemental Appendix 2). Therefore, given molecular, morphological, and acoustic similarity among *R. sebbeni*, *R. gildae*, and *R. margaritifera*, we suggest that they should be considered synonyms of the latter. In contrast, morphological differences between *R. martyi* and *R. margaritifera* exist, so the former should probably be removed from the synonymy of the latter. Nevertheless, given that uncertainty remains around the type locality of *R. margaritifera*, we consider that formal taxonomic decisions will require further investigation (Supplemental Appendix 2).

Finally, *R. dapsilis* is embedded within *R. margaritifera* according to molecular data, even though they are morphologically and acoustically distinct. We thus considered that this case represents a false negative and that these two taxa should remain separate (Supplemental Appendix 2). In turn, we argue that *R. scitula* (and thus *R. paraguayensis*) should be considered a synonym of *R. stanlaii*. In total, we found that 19 of the 25 CCS correspond to nominal species (Fig. 2; Supplemental Table S1), while six species remain unnamed and undescribed.

Molecular dating and biogeography

The resulting mitogenomic phylogenetic relationships are highly supported, with posterior probabilities above 0.99 for most of the tree nodes (exceptions correspond to the recent relationships between a few tree terminal branches). The tree topology corroborated the existence of six major clades in *R. gr. margaritifera* (Fig. 7, Supplemental Fig. S5). The geographic distribution patterns across and within these major clades are strikingly different. For instance, early diverging clades such as the *R.* 'western' clade are circumscribed to westernmost Amazonia, while species in the *R. magnussoni* clade display a striking pattern of small allopatric ranges scattered throughout Amazonia. The other three major clades in the group are widely distributed, but the *R. proboscidea* clade does not seem to occur in westernmost Amazonia, while the *R. acutirostris* clade is conversely absent from eastern Amazonia. In turn, the *R. margaritifera* clade is widespread throughout Amazonia, but its constituent species display little spatial overlap; only *R. lescurei* overlaps with *R. martyi* and *R. margaritifera* in the Guiana Shield. It is also noteworthy that, within the *R. margaritifera* clade, the subclade of species related to *R. margaritifera* (i.e., the *R. margaritifera-dapsilis* complex) is almost non-overlapping geographically with the subclade of species related to *R. exostosica* (*R. exostosica* complex).

Historical biogeographic inference using BioGeoBEARS suggests that *R.* gr. *margaritifera* originated from Andean ancestors and started to diversify ca. 9.2 Ma (8.1–10.2), most likely in western Amazonia (Figs 7 and 8; Supplemental Fig. S6; Supplemental Table S10). The complementary analyses using different sets of calibration points provided very similar time estimates. The crown age of *R.* gr. *margaritifera* was found to be slightly younger when constraining only the *Rhinella* crown age (mean 8.4 Ma; 6.7–10.1), and larger HPD were recovered when using fossil calibrations (mean 10.1 Ma, 8.2–12.5) (Supplemental Fig. S7).

The *R.* 'western' clade is the first to have diverged, followed by the *R. magnussoni* clade, which also seems to have started to diversify in western Amazonia and dispersed once into the Guiana Shield (according to models including the j parameter and DEC) ca. 6 Ma, and once more recently (2.5–3.0 Ma) into the Brazilian Shield. The *R. ocellata* clade was then the third major clade to have diverged and has seemingly secondarily dispersed into the Dry Diagonal between 7.5 and 4.5 Ma.

The clade formed by the remaining three, and younger, clades (*R. margaritifera*, *R. acutirostris,* and *R. proboscidea* clades) also probably originated in western Amazonia (according to models including the j parameter and DEC). The *R. acutirostris* clade occurs almost exclusively in this region and dispersed into the west of the Andes ca. 2.0–3.5 Ma. The ancestor of the clade grouping the *R. margaritifera* and *R. proboscidea* clades probably also dispersed toward eastern Amazonia ca. 6 Ma, where it seems to have mainly diversify (also according to model DEC + j). Nevertheless, a more precise centre of origin for this clade remains ambiguous, considering the low support of its internal relationships and the wide distribution of some of its MOTUs. Some lineages of the *R. margaritifera* clade seem to have only recently dispersed back into western Amazonia, notably within the *R. margaritifera-dapsilis* and *R. exostosica* complexes (Fig. 8). A last recent noteworthy dispersal is that of *R. hoogmoedi* from the Guiana Shield into the

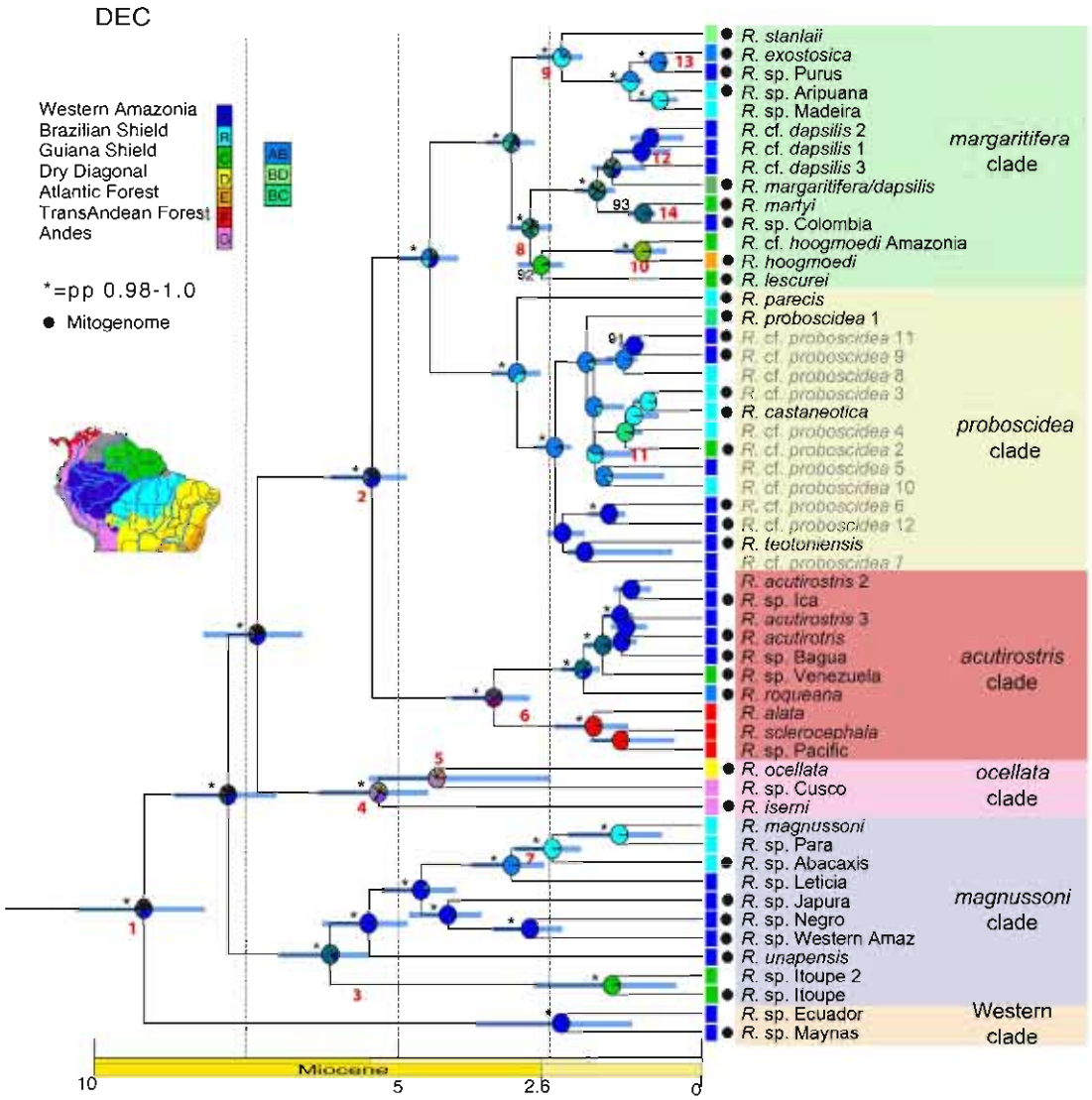

Fig. 7. Results of BioGeoBEARS (DEC) analysis of the mitogenomic subtree obtained from BEAST 2.5. The full tree is presented in Supplemental Fig. S5 and results of the DEC + J in Supplemental Fig. S6.

Atlantic Forest ca. 1 Ma. Most of the diversification events in the clade, including within the *R. acutirostris*, *R. margaritifera*, and *R. proboscidea* clades, are relatively recent, i.e., happening during the last 2.6 Ma (i.e., during the Pleistocene). By contrast, divergenees within the *R. magnussoni* clade are mostly older than 2.6 Ma. False-positives in our species delimitation step might have influenced these eladogenetie patterns. However, most of the tree terminals correspond to species supported as distinct based on both phenotype and genotype, making it unlikely that this diversification scenario merely results from species overestimation.

Discussion

This study provides (1) a re-evaluation of thc species richness and distributions within the *Rhinella* gr. *margaritifera* by combining multiple lines of evidence, and (2) an analysis of the spatio-temporal process of diversification of the group. These toads are a prominent eomponent of Amazonian terrestrial anuran communities owing to their typically high abundanees and relatively large sizes. However, field herpetologists have long regarded this clade as challenging because of high levels of polymorphism within and among populations and

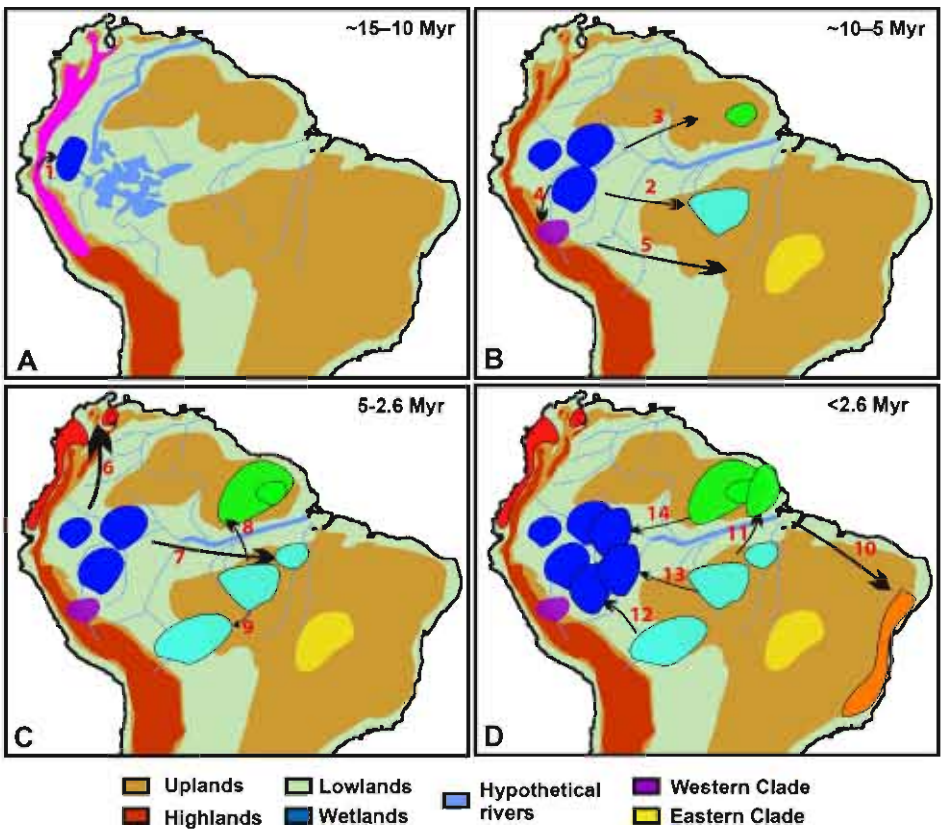

Fig. 8. Hypothesized scenario of diversification and dispersal of *Rhinella* gr. *margaritifera* throughout the Neotropics. (A) Divergence between the ancestors of the Andean *R. festae* and lowlands *R. margaritifera* groups during the Pebas system; (B) early diversification in western Amazonia and subsequent eastward dispersal after the demise of the Acre system; (C) Trans-Andean, trans-Amazon dispersals; (D) late westward dispersals and toward the Atlantic Forest. Fourteen putative dispersals are numbered in red and corresponding branches are also numbered in Fig. 7.

unclear taxon diagnoses. Our analyses confirm this view, supporting high overlap among MOTUs in morphometric space. This overlap stems from large morphological variation within DNA-based candidate species, attributable to both ontogenetic and geographic variation, and subtle morphological distinctiveness across candidate species. We found several instances of incongruence across DNA-based delimitation methods, as well as across mtDNA and a taxonomic scheme supported by clear morphological differences. Our combined analyses also uncovered possible instances of genetic introgression, which may further explain why delimiting species boundaries in *R.* gr. *margaritifera* has long been a challenging task. This study illustrates well the limitations inherent to using a single mtDNA locus for species delimitation. Therefore, we derived a final species scheme by refining MOTU boundaries to better reflect patterns of both genetic and morphological coherence and distinctiveness. This combined approach provides a more consistent and likely evolutionarily more meaningful delimitation, particularly for challenging complexes such as *R. margaritifera* and *R. proboscidea* (Supplemental Appendix 2).

In the face of this complexity, we do not pretend to provide a definitive picture of species limits in *R.* gr. *margaritifera*. Nevertheless, our analyses bring clarity into several taxonomic issues while allowing an examination of the group's diversification history. For instance, candidate species within the *R. magnussoni* clade were overall consistent across approaches and datasets. Likewise, our analyses provide an improved assessment of species richness and distributions within other subclades, including some of the most challenging ones, such as the *R. margaritifera* subclade. Despite persistent instances of unclear species boundaries within particular subclades, our comprehensive sampling of major clades and terminal lineages provides new insights into the group's diversification history. In particular, we inferred diversification events to be concentrated remarkably recently (ca. 9 Ma) relative to other anuran lineages with similar distribution and species richness (see below). This intense period of diversification is linked to

multiple dispersal events throughout Amazonia and its neighbouring regions. We discuss these major findings in detail below.

Species diversity and distributions

We delimited 55 DNA-based candidate species among which 25 are morphologically and/or acoustically diagnosable from their close relatives (Fig. 2; Supplemental Table S8–S9) and thus considered them CCS. Despite our extensive effort, the remaining candidate species (30) lacked sufficient data for an evaluation of their morphological and acoustic diagnosability and are thus considered UCS. Supposing that the deeply divergent lineages within the *R.* 'western' clade and the unsampled *R. angeli* represent additional CCS, the total number of confirmed species in this group may reach at least 27. Given that 20 taxa are currently recognized within *R.* gr. *margaritifera*, these estimates strengthen the perception that the group's diversity remains underestimated. Specifically, the number of recognized species might increase one- to three-fold depending on whether only CCS or the total number of MOTUs are considered. In agreement with these findings, almost every investigation of a focal neotropical amphibian clade has uncovered similarly high numbers of candidate species (in anurans broadly, Vacher et al., 2020; in *Allobates*, Réjaud et al., 2020; *Boana* gr. *albopunctata*, Fouquet et al., 2021; *Osteocephalus*, Ortiz et al., 2022; *Amazophrynella*, Moraes et al., 2022). However, considering the recent 9 Myr old crown age, *R.* gr. *margaritifera* seems to have undergone faster speciation than any co-distributed anuran lineage of similar age. Coincidentally, Hutter et al. (2017) detected an increase in diversification rate within *Rhinella*. Even though this signal was associated with taxa from the Atlantic Forest, their analysis was taxonomically incomplete for Amazonia. It might be interesting to determine whether this increase in diversification extends to a larger proportion of *Rhinella* under improved sampling.

On the other hand, we suspect that some of the delimited MOTUs represent Deep Conspecific Lineages (DCL) as opposed to separate species (i.e., false positives). In particular, we might expect misestimation of species boundaries owing to limitations inherent to mtDNA data, including shared alleles resulting from introgressive hybridization and mitochondrial capture among some of the MOTUs (e.g., Hillis, 2019). Hybridization seems to be frequent in bufonids, sometimes even between distantly related species (in *Rhinella*: Sequeira et al., 2011; Guerra et al., 2011; Thomé et al., 2012; Correa et al., 2012; Pereyra et al., 2021; Rivera et al., 2022), though to our knowledge has not yet been documented in *R.* gr. *margaritifera*. Herein, we identified incongruences between mtDNA and nuDNA trees for populations along the Negro River (*R. exostosica* and *R.* cf. *hoogmoedi*) and in northern Peru (*R.* sp. Bagua), which are partly corroborated by acoustic and morphological data (e.g., *R. exostosica*). These discrepancies might result from historical or current introgression events; notably, both cases correspond to geographic regions where major clades have apparently come into secondary contact (see below). Additionally, these areas are also where narrowly distributed mtDNA lineages occur (e.g., *R.* cf. *hoogmoedi*, *R.* sp. Colombia, *R.* sp. Bagua, *R.* cf. *dapsilis* 1–3, *R. acutirosris* 3). These mtDNA lineages may represent captured lineages (introgressing mtDNA material), as suggested for the *R. marina* complex (Pereyra et al., 2021). The Branco-Negro interfluvium has been previously characterized as a zone of secondary contact and hybridization in birds (Naka et al., 2012), possibly following past shifts in habitat distributions. Notably, this region also corresponds to a hybrid zone between species of *Loxopholis* lizards (Brunes et al., 2019). In the case of *R. exostosica*, hybridization is likely contemporaneous or recent because this species shares alleles with the co-occurring *R. acutirostris* (Fig. 4). Specimens harbouring these distinct mtDNA lineages are indistinguishable based on morphology and emit highly similar calls (Fig. 6). In turn, a potential instance of introgression involving *R.* cf. *hoogmoedi* might be more ancient, because its calls are only slightly distinct from those of *R. margaritifera* and somewhat intermediary relative to species in the *R. acutirostris* clade (Fig. 6). Moreover, *R.* cf. *hoogmoedi* shares a single allele with *R. exostosica* (Fig. 3). Coincidentally, the position of *R.* cf. *hoogmoedi*, an Amazonian lineage, as closely related to *R. hoogmoedi* from the Atlantic Forest in the mtDNA tree, is intriguing because it suggests recent dispersal over long distances (see below). Therefore, species boundaries and historical processes in these two lineages deserve further investigation. Similar caution needs to be taken in the case of *R. dapsilis* and *R.* sp. Bagua. *Rhinella dapsilis* is morphologically and acoustically distinct from the other species of its clade, but almost identical in mtDNA to *R. margaritifera*, although a distribution hiatus in central Amazonia exists between them. The co-occurrence and allele sharing between *R. dapsilis* and *R.* sp. Bagua, for which we do not have phenotypic data, call for further investigation but suggest possible mtDNA introgression over remarkably long distances, mirroring the case of *R.* cf. *hoogmoedi* from the Negro River and *R. hoogmoedi* from the Atlantic Forest.

Interestingly, the four major clades of this group (*R. margaritifera*, *R. proboscidea*, *R. magnussoni*, and *R. acutirostris* clades) are widely distributed in Amazonia and thus geographically overlap with each other, but species within each of these clades are generally allopatric. The few instances of co-occurrence among species from the same major clade are *R. lescurei* with *R. margaritifera* + *R. martyi* (*R. margaritifera* clade), and *R. acutirostris* 2 with *R. roqueana* (*R. acutirostris* clade). As a result, alpha diversity patterns typically involve 3–4 species per locality in Amazonian lowlands. This scenario of multiple closely related species in sympatry might involve niche partitioning (Bar-Massada, 2015; Wiens, 2011), notably in breeding habitat. In turn, broad species ranges might result from possibly high dispersal capacities in these toads. Even though each major clade is broadly distributed in Amazonia, the *R. margaritifera* and *R. proboscidea* clades are centred on eastern Amazonia and are only marginally distributed in western Amazonia (*R. dapsilis*, *R. exostosica*, *R. teotoniensis*, *R. proboscidea*) as well as in the Dry Diagonal (*R. margaritifera*, *R. stanlaii*), and in the Atlantic Forest (*R. hoogmoedi*). Conversely, the *R. acutirostris* clade is only marginally distributed in the Brazilian Shield (*R. roqueana*) and trans-Andean forests (*R. alata*, *R. sclerocephala*, *R.* sp. Pacific). Many MOTUs within these groups have large distributions, notably *R. margaritifera*, *R. exostosica*, *R. roqueana*, and *R. acutirostris*. These distributions encompass several anuran bioregions delimited by Godinho and Da Silva (2018) or Vacher et al. (2020), suggesting great dispersal capacities along rivers and ecological gradients. Those species are large-bodied and breed along seasonally flooded habitats (*igapós* and *várzea*) or puddles along riverbeds. These characteristics might partly explain their wide ranges. This hypothesis is also reinforced by the contrasting distribution patterns of species in the *R. magnussoni* clade, where species exhibit small ranges within single Amazonian bioregions. Notably, *R. magnussoni* (Lima et al., 2007; Moraes et al., 2014) and *R.* sp. Itoupe (A.F. pers. obs.) breed in phytotelmata and are associated with unflooded (*terra firme*) habitat, which might prevent their dispersal across major hydrographic features. A parallel situation can be hypothesized for *R. castaneotica*, which also breeds in phytotelmata and is geographically circumscribed to a small range in eastern Amazonia, contrasting with the wide range of the river-breeder *R. proboscidea*. Similarly, in treefrogs such as *Osteocephalus*, breeding ecology differs among co-occurring species as well as their range size (Ortiz et al., 2022). We note, however, that the breeding biology of species in *Rhinella* is not fully known and deserves further investigation.

Historical biogeography

Major divergences between *Rhinella* gr. *margaritifera* and closely related clades, as well as divergences within the group, are concomitant with major historical shifts in Neotropical climate and landscape inferred from geological records. For instance, the divergence between *R.* gr. *margaritifera* and its sister group *R.* gr. *festae* was estimated to date back to ca. 17 Ma, which coincides with the Miocene Climatic Optimum and the maximum extent of the vast lacustrine environment known as Pebas system (Hoorn et al., 2022). This lacustrine system has been assumed to have isolated populations of terrestrial organisms in the Andean foothills and neighbouring regions from eastern Amazonia in many groups (e.g. Fouquet et al., 2022). The period that followed was characterized by gradual global cooling and coincided with dispersals of primarily Andean anuran lineages toward Amazonian lowlands, notably dendrobatids such as *Ameerega* and *Ranitomeya* (Santos et al., 2009), centrolenids (Castroviejo-Fisher et al., 2014), and possibly some lineages within the bufonid genus *Atelopus* (Lötters et al., 2011) and the strabomantid *Pristimantis* (Fouquet et al., 2022). These climatic changes might have promoted divergence between cold-adapted species and others forced to follow milder conditions in the lowlands (Santos et al., 2009).

Within *R.* gr. *margaritifera*, there is strong evidence that diversification started in western Amazonia ca. 9 Ma, a period coinciding with one of the major landscape changes in Amazonia: the demise of the Pebas system and the setup of a transcontinental Amazon drainage system (Hoorn et al., 2017). Early diversification in western Amazonia may have been favoured by the opening of dispersal pathways (at least between western Amazonia and the Guiana Shield) and the increasing availability of new and diverse rainforest environments along Andean slopes and nearby lowlands following the retraction of western mega wetlands (Hoorn et al., 2010). Nevertheless, a fluvio-lacustrine environment known as the Acre system persisted across the Amazonia sedimentary basin until ca. 6 Ma, thus possibly keeping western Amazonia and the Brazilian Shield isolated. Many Amazonian anuran lineages seem to have originated in western Amazonia previous to 10 Ma, during the apex of the Pebas system, which would have kept these lineages isolated from populations in most other lowland forest regions, followed by dispersal throughout Amazonia ca. 10–5 Ma onward. This diversification scenario is observed in other anuran clades, such as *Ameerega* (Guillory et al., 2020; Santos et al., 2009), *Ranitomeya* (Muell et al., 2022), *Osteocephalus* (Ortiz et al., 2022), *Boana* gr. *albopunctata* (Fouquet et al., 2021), and possibly *Atelopus*

(Lötters et al., 2010) and *Leptodactylus* gr. *melanonotus* (Carvalho et al., 2022). Similarly, *R.* gr. *margaritifera* displays three initial independent dispersals ca. 6 Ma from the west toward the Dry Diagonal (*R. ocellata*), the Guiana Shield (*R.* sp. Itoupé), and the Brazilian Shield (*R. margaritifera* and *R. proboscidea* clades), all coinciding with the demise of the Acre system (Albert et al., 2018). These patterns support our hypothesis that western Amazonia corresponded to the geographical core of *R.* gr. *margaritifera*'s diversification, and that the Miocene wetlands represented an early biogeographic barrier to this group. We also found evidence that the extant diversity of *R.* gr. *margaritifera* was mainly a product of continuous dispersal events followed by diversification, rather than primarily generated by *in situ* speciation. This contrasts with the evolutionary history of the small-bodied Amazonian bufonid genus *Amazophrynella* (Moraes et al., 2022), whose diversification appears to have involved little dispersal throughout the Neogene.

Subsequent dispersals of *R.* gr. *margaritifera* from western Amazonia toward the Brazilian Shield have apparently occurred in the *R. magnussoni* clade ca. 3 Ma and, more recently, within the *R. acutirostris* clade. Conversely, dispersals from the Brazilian Shield toward western Amazonia occurred very recently in the *R. margaritifera* clade (*R. exostosica* and *R. dapsilis*). Such dispersals also happened in the *R. proboscidea* clade (<3 Ma); however, the number of events and the timing is more difficult to assess because phylogenetic relationships within this clade remain ambiguous. These reciprocal dispersals might have led to secondary contact between the *R. margaritifera* and *R. acutirostris* clades, notably along the Negro River and in western Amazonia, where introgressions appear to have occurred. These dispersals might have been favoured by multiple river captures (Albert et al., 2018, 2021; Pupim et al., 2019; Ruokolainen et al., 2019) and the highly dynamic nature of Amazonian floodplain river courses in general, allowing populations to shift from one bank to the other via recurrent cutting-off of meanders (Jackson & Austin, 2013).

The largest riverine barrier in Amazonia is represented by the lower course of the Amazon River itself, separating the Brazilian Shield from the Guiana Shield. Transamazon dispersals younger than 5 Ma are relatively rare in terrestrial anurans associated with *terra firme*, ranging from none in *Synapturanus* (Fouquet et al., 2021) and *Amazophrynella* (Moraes et al., 2022), to single oddities in *Pristimantis* gr. *conspicillatus* (in *P. koehleri*; Fouquet et al., 2022) and *Allobates* (in *A. femoralis*; Réjaud et al., 2020), and twice in *Adenomera* (in *A. andreae* and *A. hylaedactyla*; Fouquet et al., 2014). The frequency of these events seems to increase in groups associated with riverbeds and open habitats: three times in *Osteocephalus* (Ortiz et al., 2022) and four times in *R.* gr. *margaritifera*. Although the exact timing has not been estimated per se, 35 recent (probably Pleistocene) trans-Amazon dispersals involving anuran lineages can be hypothesized based on molecular candidate species with cross-Amazon River ranges in Vacher et al. (2020) (Table S10). The majority (18) of these species are open habitat or aquatic species, for which the lower Amazon does not seem to have represented a barrier during the last 3 Ma. However, 15 of these are forest species associated with riverbeds. These species have not dispersed across the lower Amazon River until 3 Ma, after which dispersal was possibly favoured by passive dispersal of adults and/or larvae during floods.

In *R.* gr. *margaritifera*, recent dispersal from the Guiana Shield toward the Atlantic Forest (*R. hoogmoedi*) – two currently disjunct areas – is estimated to have occurred ca. 1 Ma. Similarly, recent dispersals between these areas have been found in the viperid snake *Bothrops bilineatus* (Dal Vechio et al., 2018) and, more broadly between Eastern Amazonia and the Atlantic Forest, in many squamates, mammals, and birds (reviewed by Ledo & Colli, 2017), as well as other forestial anurans such as *Lithobates palmipes* (Coelho et al., 2022) and *Boana semilineata* (Fouquet et al., 2016). Palynological and speleothem studies (Auler et al., 2004; De Oliveira et al., 1999; Wang et al., 2004), as well as niche modelling (Ledo & Colli, 2017), support the expansion of cold-adapted species along a northern route connecting eastern Amazonia and the Atlantic Forest during the Last Glacial Maximum.

In conclusion, multiple multidirectional dispersals have led to a complex diversification history and species distribution patterns in *R.* gr. *margaritifera*. The combination of (1) a relatively young clade crown age, (2) introgression events, (3) ontogenic morphological variation, and (4) ancient species names tied to unclear type localities largely explains why the taxonomy and systematics of the group is so challenging. The diversification history of this species group illustrates the great capacity to disperse across major geographical barriers such as Amazonian rivers, the Dry Diagonal, and Andean mountains compared with other terrestrial Neotropical anurans. Such great dispersal capacity might explain a particularly intense diversification (>27 species in less than 9 Ma) compared with other Amazonian terrestrial anurans. In turn, this pattern seems more similar to what has been inferred in more dispersive vertebrates in this region, such as birds and mammals (e.g., Machado et al., 2019; Smith et al., 2014), highlighting

the potential role of inherent organismal attributes in shaping broad-scale biodiversity patterns.

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Supplemental material

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