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RESEARCH ARTICLE

Phylogeny, historical biogeography and climate niche differentiation in extant species of Ceratophryidae (Anura, Hyloidea) frogs in South America

Arjen A. W. van der Kamp[1,2], H. Mauricio Ortega-Andrade[1,3]

1 *Biogeography and Spatial Ecology Research Group, Life Sciences Faculty, Universidad Regional Amazónica Ikiam, Tena, Ecuador*
2 *Wildlife Ecology & Conservation Group, Wageningen University, Droevendaalsesteeg 3A, 6708 PB Wageningen, Netherlands*
3 *División de Herpetología, Instituto Nacional de Biodiversidad, Quito, Ecuador*

Corresponding author: H. Mauricio Ortega-Andrade (mauricio.ortega@ikiam.edu.ec)

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Abstract

Ceratophryidae is a family of frogs containing twelve extant species distributed in South America. Several studies have been carried out concerning the systematics, morphology, karyotypes, and behaviour within this monophyletic family. However, little is known about the historical biogeography and the divergence in time of this group. Here, we present an updated phylogeny, along with a calibrated chronogram, to analyse the historical biogeographical pattern and climate niche differentiation among extant species of Ceratophryidae (Anura: Hyloidea) frogs in South America. A phylogeny based on morphological and genetic data was obtained from 256 morphological characters, six mitochondrial and eight nuclear genes for up to a total of 8428 characters in the homological matrix. Our results indicate that the genus *Ceratophrys* is sister to the clade *Chacophrys* + *Lepidobatrachus*. The divergence of the crown group of Ceratophryidae is estimated to have occurred 19.2 Ma at the beginning of the Miocene, with recent cladogenetic events related to the late Miocene (10.18–13.70 Ma) and the Pliocene (~5.3–2.6 Ma). Moreover, the Cerrado region is estimated to be the ancestral area of the family, as well for the genera *Chacophrys* and *Lepidobatrachus*. In addition, temperature seasonality and annual precipitation play a major role in the niche differentiation of extant species within Ceratophryidae. In conclusion, our data suggest multiple dispersal and vicariance events originating from the Cerrado region in the early Miocene, and recognize the role of the environment in the differentiation of the ecological niches among extant species of this family in South America.

Highlights

- Ceratophryidae is hypothesized to have originated in the semi-arid Cerrado region in the early Miocene (~19.2 million years ago), from where species have diversified by independent dispersal and vicariance events across other South American regions.
- *Ceratophrys* frogs are phylogenetically recovered as the sister taxon to the clade comprising *Chacophrys* and *Lepidobatrachus*.
- Three lineages of *Lepidobatrachus* and four lineages from *Ceratophrys* diverged in the Pliocene, around 5.3 to 2.6 million years ago.
- Niche differentiation among extant species of Ceratophryidae appears to reflect roles for temperature seasonality and annual precipitation.

Keywords

ancestral area reconstruction, biogeography, *Ceratophrys*, Cerrado, *Chacophrys*, chronogram, divergence time estimation, *Lepidobatrachus*, precipitation, seasonality

Introduction

Ceratophryidae (Anura: Hyloidea) (von Tschudi 1838) is a family of frogs with three extant genera (*Ceratophrys*, *Chacophrys*, *Lepidobatrachus*) and twelve species that are distributed in South America, from the Caribbean lowlands in Colombia and Venezuela to the Pampean region in central Argentina (Faivovich et al. 2014). *Ceratophrys* is the biggest genus within the family, containing eight species. *Chacophrys pierottii* (Vellard 1948) is the only species so far known that is ascribed to the *Chacophrys* genus. *Lepidobatrachus* is a genus containing three extant species distributed within the Gran Chaco region (Brusquetti et al. 2018). Ceratophryidae can be found in tropical rainforests, humid grasslands and semi-arid environments (Fernández and Fernández 1921; Barrio 1968; Duellman and Lizana 1994). These frogs are mostly active during the rainy season. Species of *Lepidobatrachus* are primarily aquatic, whereas adults from *Ceratophrys* and *Chacophrys* are mostly terrestrial (Faivovich et al. 2014).

Herpetologists have for a long time been interested in the biology of Ceratophryidae, for ecological, morphological and behavioural reasons. For example, larva from Ceratophryidae have an unique mechanism for sound production underwater (Salgado Costa et al. 2014). The morphological traits of these frogs are distinctive from those of other frog families (Quinzio et al. 2006; Fabrezi and Quinzio 2008; Fabrezi et al. 2009), and the ontogeny of the postcranial skeleton, as well as the relatively large skull, are also unique compared to other frog species (Wild 1997). Most Ceratophryidae contain an enlarged distal prehallical element that provides support for a heavily keratinized metatarsal spade used for digging (Fabrezi 2001). All Ceratophryidae have a basic chromosome number of x = 13 (Faivovich et al. 2014). However, most *Ceratophrys* have a diploid (2n = 2x = 26) karyotype, but the species *C. ornata*, *C. joazeirensis* and *C. aurita* have an octoploid (2n = 8x = 104) karyotype (Vieira et al. 2006; Faivovich et al. 2014). Lastly, some species of Ceratophryidae are known to be able to dig themselves into humid soil and make a cocoon of dead skin in order to decrease water loss when the humidity decreases in their surroundings (McClanahan et al. 1976; Faivovich et al. 2014). It has been found that frogs in captivity can persist in this cocoon-state for 20 months (Pisanó and Paz 1954).

Lynch (1971) presented the first phylogeny of the *Ceratophrys* genus as it is mostly considered today, wherein he included *C. cornuta* Linnaeus, 1768, *C. aurita* (Raddi 1823), *C. ornata* (Bell et al. 1841), *C. stolzmanni* (Steindachner 1882), *C. calcarata* (Boulenger 1890), *C. testudo* (Andersson 1945) and *Ch. pierottii*. Later, *C. cranwelli* (Barrio 1980) and *C. joazeirensis* (Mercadal 1986) were described and included in the group. A taxonomic review by Reig and Cei (1963) and Barrio (1968) defined the composition of the genus *Lepidobatrachus*, including three species: *L. asper* (Budgett 1899), *L. laevis* (Budgett 1899) and *L. llanensis* (Reig and Cei 1963). The phylogeny of Ceratophryidae has been well studied (Reig and Cei 1963; Lynch 1982; Maxson and Ruibal 1988; Perí 1994; Wild 1997; Fabrezi 2006; Frost et al. 2006; Fabrezi and Quinzio 2008; Pyron and Wiens 2011; Faivovich et al. 2014; Frazao et al. 2015; Fabrezi et al. 2016; Feng et al. 2017; Brusquetti et al. 2018; Gómez and Turazzini 2021; Hime et al. 2021; Barcelos et al. 2022). The phylogeny of Ceratophryidae has been well studied (Lynch 1982; Maxson and Ruibal 1988; Perí 1994; Wild 1997; Fabrezi 2006; Frost et al. 2006; Fabrezi and Quinzio 2008; Pyron and Wiens 2011; Faivovich et al. 2014; Frazao et al. 2015; Fabrezi et al. 2016; Feng et al. 2017; Brusquetti et al. 2018; Gómez and Turazzini 2021; Hime et al. 2021; Barcelos et al. 2022). However, the majority of studies have only utilized a limited number of species representatives from the Ceratophryidae family. Furthermore, the phylogenetic relationships between the genera within Ceratophryidae remain unclear. For example, several studies have suggested *Ceratophrys* as the sister taxon to the clade comprising *Chacophrys* and *Lepidobatrachus* (Fabrezi 2006; Faivovich et al. 2014; Frazao et al. 2015; Fabrezi et al. 2016; Brusquetti et al. 2018; Hime et al. 2021). Conversely, others have hypothesized *Chacophrys* as the sister group to the clade consisting of *Lepidobatrachus* and *Ceratophrys*. Moreover, studies encompassing a broader spectrum of Ceratophryidae species exhibit varying phylogenetic placements within the *Ceratophrys* genus. For example, genetic analyses conducted by Faivovich et al. (2014) revealed a distinct evolutionary relationship between *C. cranwelli* and *C. ornata* in comparison to the morphological research conducted by Gómez and Turazzini (2021) and Barcelos et al. (2022).

The fossil records of Ceratophryidae have undergone extensive examination. Various studies have attempted to estimate the divergence times within Ceratophryidae. Notably, Gómez and Turazzini (2021) and Barcelos et al. (2022) integrated fossils as calibration points into phylogenetic analyses with extant species of the family. Despite these efforts, the crown age divergence of Ceratophryidae remains uncertain (Barcelos et al. 2022). Some studies propose a late Paleogene origin, ~66–23 Ma (Heinicke et al. 2009; Hutter et al. 2017), while others suggest a divergence in the Mid-Miocene. Feng et al. (2017) constructed a chronogram of the three major clades of Gondwanan frogs, incorporating *C. cornuta* and *Lepidobatrachus* as representatives of Ceratophryidae. Brusquetti et al. (2018) constructed a chronogram for the genus *Lepidobatrachus* and Gómez and Turazzini (2021) made a time-adjusted phylogeny of Ceratophryidae, including the fossil record of the whole family of Ceratophryidae.

Several studies have delved into evolutionary radiations through phylogenetic analysis at a biogeographical scale in amphibians (Crawford and Smith 2005; Wiens et al. 2006; Santos et al. 2009; Gonzalez-voyer et al. 2011; Castroviejo-fisher et al. 2014). However, to date, no ancestral area reconstructions have been conducted for the Ceratophryidae family. Such reconstructions hold the potential to elucidate the environmental niche differentiation of the species within their distributional areas in South America. A more comprehensive understanding of the evolutionary history and biogeography of species inhabiting these regions can be achieved through such reconstructions (Crawford and Smith 2005; van der Meijden et al. 2007; Castroviejo-fisher et al. 2014).

We aim to investigate the phylogeny, historical biogeographic pattern and climate niche differentiation in extant species of Ceratophryidae (Anura: Hyloidea) frogs in South America. We focus on the following research questions: (1) How are the three genera of Ceratophryidae phylogenetically related to each other based on morphological and genetic characters? (2) What are the divergence times of species within the Ceratophryidae family? (3) What is the biogeographical ancestral area of Ceratophryidae species in South America? (4) Which environmental variable(s) play a major role in niche differentiation among extant species of Ceratophryidae?

Materials and methods

Taxon and character sampling

Data from eleven of the twelve frog species of the family Ceratophryidae are included. *Ceratophrys testudo* was excluded, as this species has not been collected since the holotype (Andersson 1945). DNA sequences from several specimens per species were available for most species on GenBank. Based on the results from Feng et al. (2017), *Calyptocephallela gayi* (Calyptocephalellidae), *Gastrotheca weinlandii* (Hemiphractidae), *Pseudis paradoxa* (Hylidae) and *Rhinoderma darwinii* (Hemiphractidae) were selected as outgroup species where *Calyptocephallela gayi* was used to root the tree. The GenBank accession numbers can be found in Suppl. material 3: table S1.

The phylogenetic tree of Ceratophryidae was constructed by first analysing a concatenated matrix with 8172 bp DNA sequences from GenBank. The six mitochondrial gene sequences used include portions of cytochrome oxidase subunit I (COI) gene, cytochrome b (cytb) gene, 12S rRNA gene, the intermediate partition sequence tRNAVal gene, 16S rRNA gene and NADH dehydrogenase subunit 1 (ND1) gene. The eight nuclear gene sequences include the Seven In Absentia Homolog 1 (SIAH1) gene, Tyrosinase precursor (TYR) gene, Recombination activating protein 1 (RAG-1) gene, Ribosomal protein L3 (RPL3) gene, Proopiomelanocortin A (POMC) gene, Chemokine receptor 4 (CXCR4) gene, lactose dehydrogenase beta chain gene (lactose_dh) and Fibrinogen A alpha polypeptide gene (fib_A) (Suppl. material 3: table S1). In addition, 256 morphological characters from Gómez and Turazzini (2021) were added as additional characters in the mixed matrix to enrich the dataset (Suppl. material 3: table S1).

Phylogenetic analysis

Genes were selected based on the number of species with available sequences in GenBank (https://www.ncbi.nlm.nih.gov/genbank/; accessed in March 2023). Sequences for every gene were separately aligned with the MAFFT v.7.450 (Katoh and Toh 2010) alignment auto algorithm in Geneious Prime v.2022.1.1 (Kearse et al. 2012) using the default scoring matrix (200PAM /k = 2), gap open penalty (1.53) and offset value (0.123). The alignments were then modified and trimmed for a maximum coverage between the sequences. A character data matrix was generated using Mesquite software v.3.81 (Maddison and Maddison 2023). The specimen with the most complete alignment (i.e., most genes being aligned) was chosen as the specimen to represent each species for the character data matrix. If possible, the gaps in the character data matrix were filled by the gene alignments from another specimen of the same species (Suppl. material 3: table S1). The concatenated sequence matrix was partitioned by gene (Suppl. material 3: table S1).

IQ-TREE v.1.6.12 (Nguyen et al. 2015) was used to estimate the maximum likelihood (ML) phylogeny of the concatenated sequence matrix. An edge-linked partition model (Chernomor et al. 2016) was chosen based on the phylogenomic alignments. The partition model estimated an independent substitution model and evolutionary rate for the 38 partitions. Each partition has its own specific partition rate, while the model rescales all its branch lengths, including those for the different codon positions of all the coding genes. Branch support was assessed by applying both the Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) (Shimodaira and Hasegawa 1999) and the Ultrafast Bootstrap Approximation (UFB) (Hoang et al. 2018). For both methods, 1000 bootstrap replicates were used (Suppl. material 1: fig. S1). Another ML phylogeny with IQ-TREE v.1.6.12 was performed for the combined analysis by using both genetic and morphological data, with only the UFB test for branch support. Trees were visualized and edited with FigTree v.1.4.2 (Rambaut 2014).

Chronogram

We employed BEAST v. 2.7.4 (Bouckaert et al. 2014) to estimate the divergence time of Ceratophryidae based on the genetic matrix. The analysis ran for one hundred million generations utilizing a relaxed clock log-normal model, with log-data sampled every 10,000th iteration. A calibrated Yule Model with a 25% burn-in and a randomly generated starting tree were utilized. We estimated a TIM2+F+I+G4 substitution model for the genetic matrix in IQ-TREE v.1.6.12. Subsequently, a GTR model was set in BEAUti 2, incorporating rate parameters estimated as follows: A-C: 5.1986, A-G: 15.2835, A-T: 5.1986, C-G: 1.0000, C-T: 34.0549, G-T: 1.0000 (see Suppl. material 7: appendix S1). For the BEAST analysis, we employed the fossils *Ceratophrys ameghinorum* (Fernicola 2001) and *Lepidobatrachus australis* (Moreno 1889), along with the nodes 37, 41, 51, 52, and 53 from the time-calibrated phylogenetic tree of Feng et al. (2017), as calibration points. *C. ameghinorum* was used to establish a minimum bound between 5.33 and 4 million years ago (Ma) for the divergence of the clade containing *C. aurita* and *C. joazeirensis* from its sister group (Tomassini and Montalvo 2013). Similarly, *L. australis* was used to establish a minimum bound between 5.33 and 4 Ma, for the divergence of *Lepidobatrachus* from its sister group (Tomassini and Montalvo 2013; Gómez and Turazzini 2021).

Two independent runs were made, and sampled values were joined and analysed for convergence. Tracer v.1.7.2 (Rambaut et al. 2018) was used to assess the best run based on the likelihood effective sample size (ESS). All parameters in the model had an ESS higher than 900 (Suppl. material 4: table S2). TreeAnnotator (Bouckaert et al. 2014) was used to obtain a maximum clade credibility tree with divergence times from the best run. Chronogram tree visualization and editing was done with FigTree v.1.4.2.

Ancestral area reconstruction for Ceratophryidae

The divergence time reconstruction inferred for the phylogenies is the key input for biogeographical analysis (Canitz et al. 2022). We utilized the BioGeoBears package (Matske 2013) in RASP v.4.4 (Yu et al. 2020) to compare biogeographical models based on AICc and AICw-weighted statistics. RASP v.4.4 includes a likelihood version of the dispersal-vicariance analysis (DIVALIKE), the dispersal-extinction-cladogenesis model (DEC), and the Bayesian analysis of biogeography (BAYAREALIKE). Furthermore, it includes the free parameter "j" ("jump dispersal") to simulate founder-event speciation (Matske 2013), additional to the dispersal (d) and extinction (e) rates. Every species in the analysis was coded according to the biogeographic regions within South America outlined by Hutter et al. (2017): (A) Cerrado, (B) Temperate, (C) Atlantic Forest, (D) Amazonia and the Guiana Lowlands province, and (E) Choco. As no species from the Ceratophryidae are distributed in the Tropical Andes and Guiana Shield, we excluded those regions from the analyses. It was decided to include the Guiana Lowlands province within the Amazonia region to simplify the analysis, as some occurrences of *C. cornuta* were found in this region. We verified the species-specific geographical data acquired from the biodiversity information system GBIF (https://www.gbif.org/; accessed in May 2023) and cross-referenced them with the distribution polygons per species obtained from the IUCN Red List website (https://www.iucnredlist.org/). A total of 6744 occurrences for all the extant Ceratophryidae species (except for *C. testudo*) and the four outgroup species were gained from the GBIF. Errors, doubtful, and duplicated occurrences were removed, resulting in a final dataset of 2860 records: 1383 for the outgroup and 1477 for Ceratophryidae species (Fig. 1, Suppl. material 5: table S3).

The maximum number of areas in ancestral ranges was restricted to three, in accordance with the current distributional range codified for each species across the designated regions. We estimated the probabilities of ancestral areas for each node, drawing from a subset comprising 1000 randomly selected trees in RASP v.4.4.,

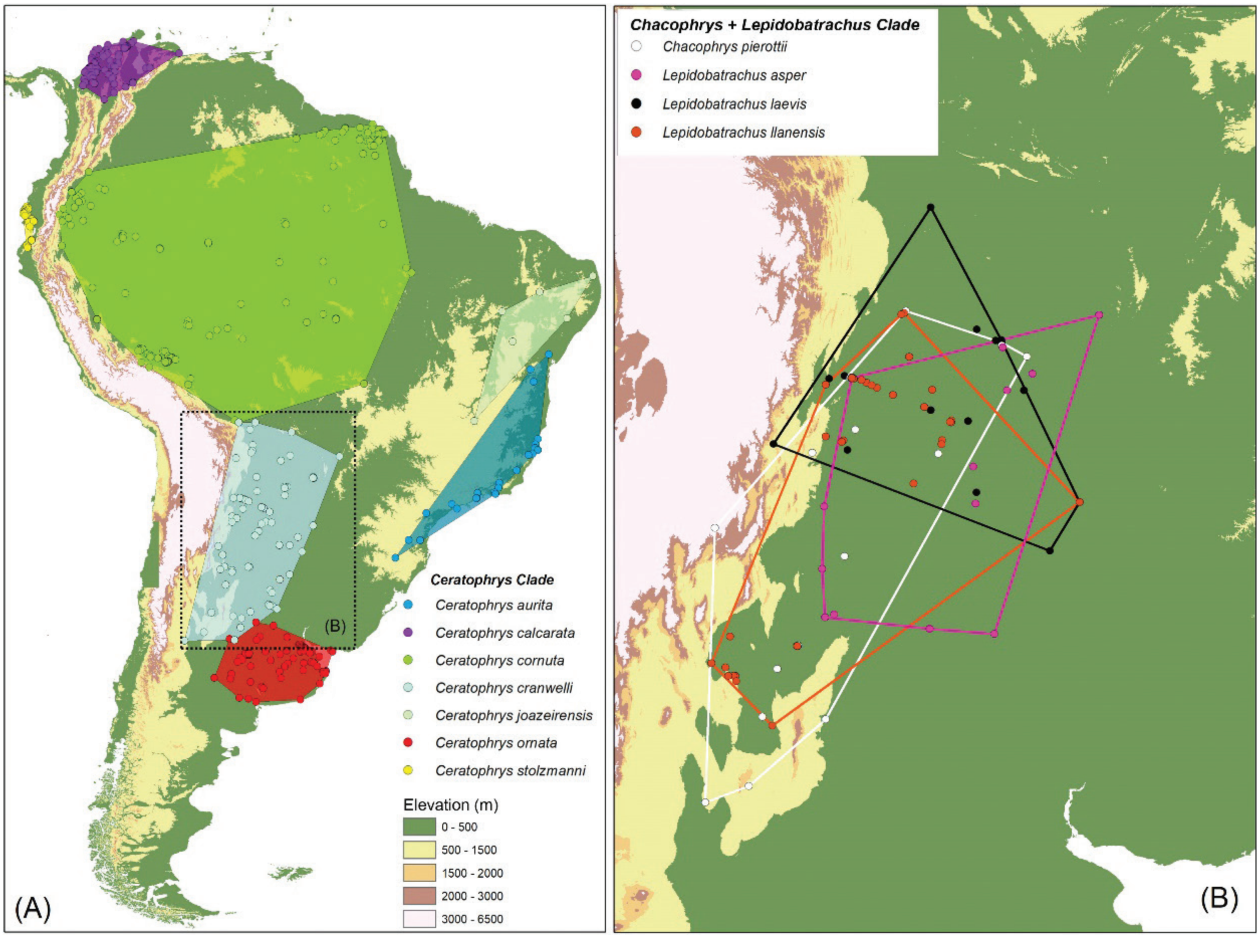

Figure 1. Biogeographic map for species in the Ceratophryidae family. (**A**) Map of South America with convex hull polygons representing the occurrences for every species of *Ceratophrys*. (**B**) The Gran Chaco region with convex hull polygons that represent the occurrences for species of *Lepidobatrachus* and *Chacophrys*.

utilizing the chronogram and trees derived from BEAST2 software as input files (Yu et al. 2015).

Phylogenetic environmental niche differentiation

The nineteen bioclimatic variables were downloaded from the WorldClim2 database (http://www.worldclim.com/version2; accessed in December 2023), with a 30 seconds (~1 km^2) spatial resolution (Hijmans et al. 2005). We generated a database of environmental data extracted for each location point per species using ARCMAP 10.5 (ESRI 2015). We performed three methods to analyse the relatedness of environmental variables to phylogenetic groups in Ceratophryidae: (a) We used the Jackknife method (Sinharay 2010) to estimate the relative contributions of environmental variables for each species, ran in Maxent v.3.4.3 software (Phillips et al. 2017); (b) A principal components analysis (PCA) was performed using PAST v.4.03 (Hammer et al. 2001) to determine the environmental variables with the highest loading per phylogenetic groups; and (c) a phylogenetic Principal Components Analysis (pPCA) was performed using the mean environmental data from 19 variables and the chronogram tree to analyse the evolutionarily covariance of phylogenetic species related to environmental niche differentiation by region. We utilized the phyl.pca function to run the pPCA, and the phylomorphospace function to project the phylogeny and the biogeographic regions onto the first two environmental principal components. Both functions were applied with the phytools package (Revell 2012; Revell and Harmon 2022) in R software (R-Core-Team 2023).

Results

Phylogenetic analysis

The genetic and the combined analysis (genetic + morphology) show a similar phylogeny, where the monophyly of *Ceratophrys*, *Chacophrys* and *Lepidobatrachus* is well supported for Ceratophryidae (Fig. 2 and Suppl. material 1: fig. S1). The phylogeny yielded *Ceratophrys* to be the sister clade of *Chacophrys* + *Lepidobatrachus* with a 100% Ultra Bootstrap Approximation (UFB). *Ceratophrys* is divided into major two clades, where the first clade contains *C. calcarata* and *C. cornuta* (UFB = 100%), and the second clade includes *C. stolzmanni* as sister (UFB = 82%) of the clade containing *C. aurita*, *C. joazeirensis*, *C. cranwelli* and *C. ornata* (UFB = 100%).

Time of divergence of the Ceratophryidae clade

The divergence times are shown in Table 2 and the chronogram is provided in Suppl. material 2: fig. S2 and Suppl. material 8: appendix S2. The chronogram estimated with BEAST suggests that the crown group of Ceratophryidae originated 19.2 Ma in the early Miocene (95% Highest Posterior Density [HPD] 14.7–24.1 Ma, node 5). Within the Ceratophryidae family, the crown group of *Ceratophrys* is estimated at 17.2 Ma (95% HPD 12.5–21.7 Ma, node 9) in the Miocene; the crown group of *Chacophrys* diverged from *Lepidobatrachus* near to 13.7 Ma (95% HPD 8.2–19.1 Ma, node 6); and the crown group of *Lepidobatrachus* originated 5.0 Ma in the Pliocene (95% HPD 4.0–6.5 Ma, node 7). All divergence times within Ceratophryidae are estimated to fall in the Neogene (23.0–2.6 Ma; Fig. 3, Suppl. material 2: fig. S2).

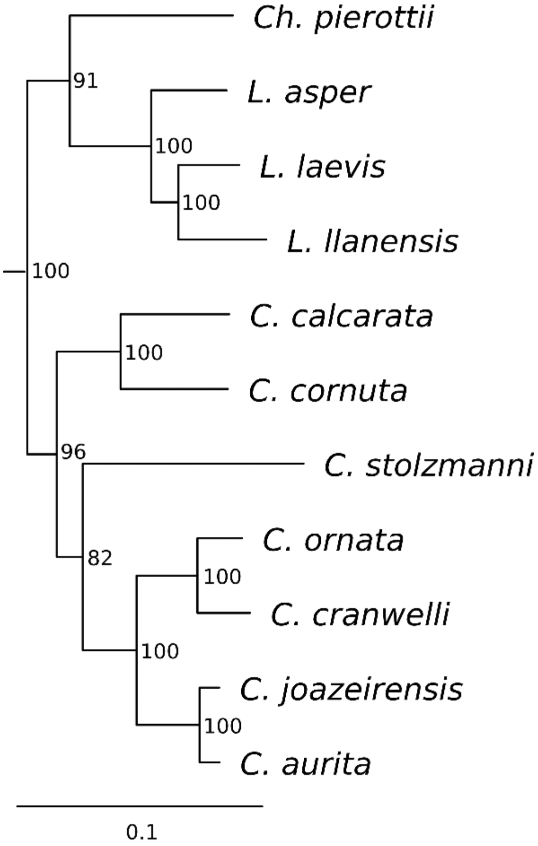

Figure 2. Phylogenetic relationships of Ceratophryidae based on genetic (Faivovich et al. 2014) and morphological data (Gómez and Turazzini 2021) with Ultrafast Bootstrap Approximation percentages. Outgroup relationships can be found in Suppl. material 3: table S1.

Ancestral area reconstruction for Ceratophryidae

The Dispersal-extinction-cladogenesis model (DEC = –30.38 lnL; AICc = 65.76; AICc_wt = 39%) was the best among other biogeographical models (Table 3). We identified five dispersals followed by five vicariance events in the biogeographical history of the Ceratophryidae clade to explain distribution of extant species in South American regions (Fig. 3; Suppl. material 9: appendix S3). The ancestral area reconstruction suggests an origin around 19.8 million years ago (95% HPD 14.72–24.05 Ma, node 5) in the Cerrado region for Ceratophryidae (Fig. 3, Table 2). Speciation of *Chacophrys pierottii* (node 6, 13.70 Ma [95% HPD 8.15–19.50 Ma]) and the crown

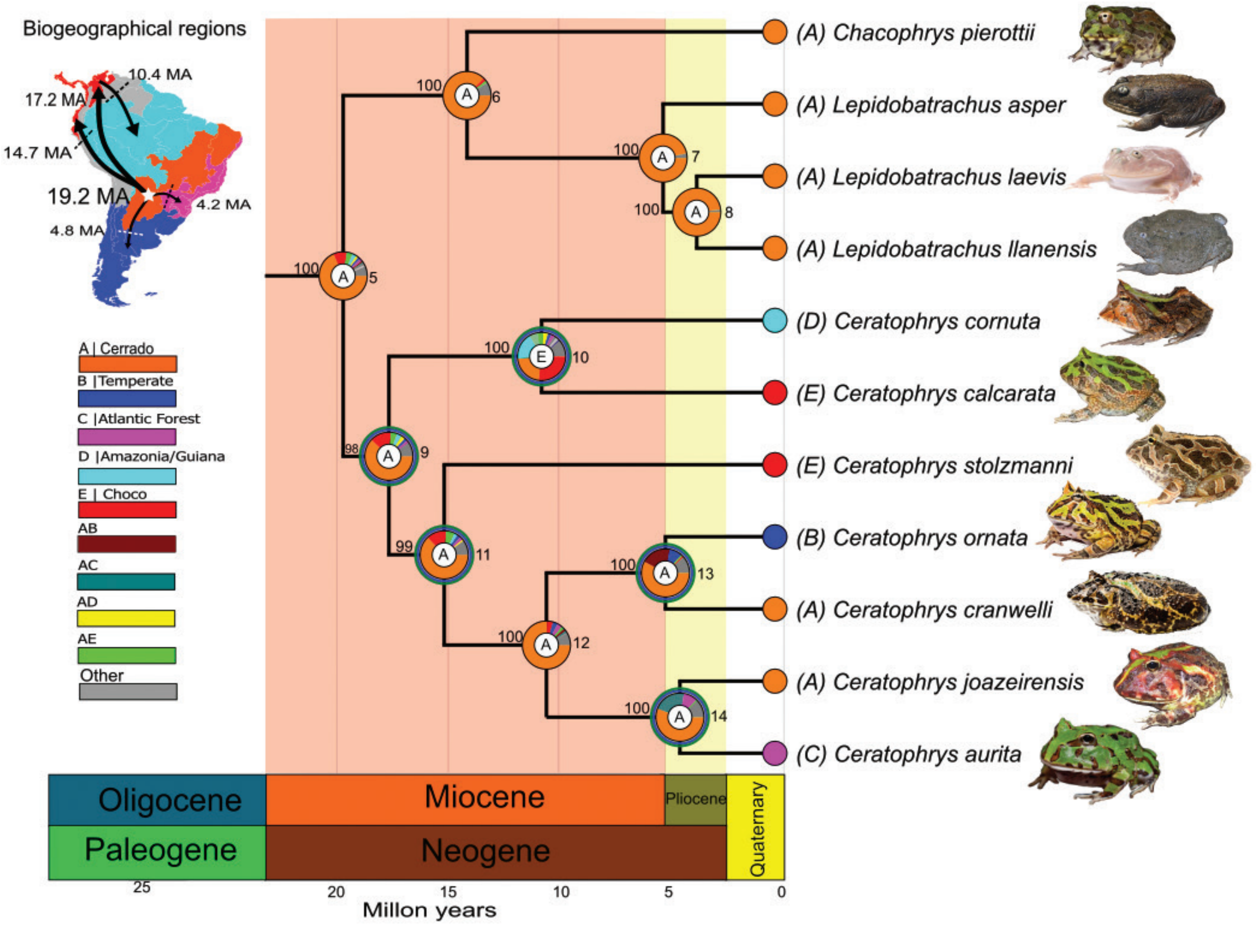

Figure 3. Ancestral Area Reconstruction for extant species of Ceratophryidae in South America. A dispersal-extinction-cladogenesis model (DEC) for Ceratophryidae reconstructed with RASP v.4.4. software. The condensed tree is a chronogram based on 10,001 trees derived from genetic data reconstructed by Bayesian inference analysis in BEAST2 for Ceratophryidae. Biogeographical region categories are based on Hutter et al. (2017). The colored circles on internal nodes visualize the marginal probabilities of ancestral area for the corresponding clade of that node. Letters inside each node represents the most probable ancestral area, whereas support values and node number are located on left and right of each node, respectively. Biogeographic events are highlighted for dispersal event (blue) and vicariance events (green) surrounding each node. The geographical distribution of taxa across regions is represented using color coding, with each extant taxon depicted at the tip nodes. Symbology in the map of South America: Estimated time event of speciation are detailed in million years ago (Ma), arrows denote dispersal events, dashed lines indicate vicariance events, a star symbol marks the estimated origin region for Ceratophryidae. Biogeographic information on speciation events per node can be found in Table 2 and Suppl. material 8: appendix S2. Photograph credits are detailed in Suppl. material 10: appendix S4.

group *Lepidobatrachus* (node 7, 4.95 Ma [4.00–6.47 Ma]) also has its origin in the Cerrado region around 13.70 Ma to 3.46 Ma (Fig. 3). Independent dispersion and vicariance events occurred between approximately 17 Ma to 10 Ma, leading to the migration from the Cerrado region to both the Choco and Amazonia. This migration marked the diversification of *Ceratophrys cornuta*, *C. calcarata*, and *C. stolzmanni* (Fig. 3, nodes 9–11). Consequently, *C. cornuta* inhabited the Amazonia region (area D), while *C. calcarata* colonized the Choco region (area E), in a vicariance event estimated around 10.4 Ma (95% HPD, 5.1–15.8 Ma, see Fig. 3). The distribution of *C. stolzmanni* (14.71 Ma, Cerrado → Choco|Cerrado) and *C. calcarata* (17.2 Ma, Cerrado → Choco; 10.3 Ma, Choco → Amazonia|Choco), suggests an independent origin due to sequential dispersal and vicariance events from Cerrado to colonize the Choco region (area E, see Fig. 3). The most recent speciation event within Ceratophryidae are estimated to have occurred in the Pliocene, producing the split of *C. ornata* and *C. cranwelli* (4.8 Ma [95% HPD 2.1–8.2 Ma]; node 13), as well as *C. joazeirensis* and *C. aurita* (4.2 Ma [95% HPD 4.0–4.7 Ma]; node 14). These splits reflect, respectively dispersal and vicariance events from the Cerrado towards the Temperate and the Atlantic Forest (Fig. 3).

Phylogenetic environmental niche differentiation

The Jackknife resampling test shows the environmental importance of Temperature Seasonality (BIO4) for several species of Ceratophryidae (*C. aurita*, *C. cranwelli*, *C. ornata*, *Ch. pierottii*, *L. asper*, *L. laevis* and *L. llanensis*). Mean Temperature of Coldest Quarter (BIO11) is environmentally important for *C. calcarata*, Precipitation of Driest month

Table 1. Estimates of relative contributions of the environmental variables by Jackknife test for extant Ceratophryidae species in South America. Blue colors correspond to high percentage of environmental importance of variables, green are medium values and white are lower values.

Bioclimatic variables	*Ceratophrys aurita*	*Ceratophrys calcarata*	*Ceratophrys cornuta*	*Ceratophrys cranwelli*	*Ceratophrys joazeirensis*	*Ceratophrys ornata*	*Ceratophrys stolzmanni*	*Chacophrys pierottii*	*Lepidobatrachus asper*	*Lepidobatrachus laevis*	*Lepidobatrachus llanensis*
BIO1 = Annual Mean Temperature	5.2	2.7	1.1	0.2	6.6	4.3	0	0	0	0	0.4
BIO2 = Mean Diurnal Range	8.6	0.2	1.4	0.1	0	0.7	4.8	0	0.3	0	0.1
BIO3 = Isothermality (BIO2/BIO7) (×100)	6.9	2.9	15.3	0.8	0	11.1	0	0.4	0	0	0.8
BIO4 = Temperature Seasonality (standard deviation ×100)	45.7	0.3	3.7	55.2	0.8	54.9	1.2	50.6	46.6	47.4	47.3
BIO5 = Max Temperature of Warmest Month	0.1	2.8	0.7	5.7	0	0.1	0.1	11.1	16.4	31.7	12.4
BIO6 = Min Temperature of Coldest Month	0	3.9	1.1	0.2	0	0.1	1.1	0.3	0	0	0
BIO7 = Temperature Annual Range (BIO5-BIO6)	0	0.6	0.2	0	0	0.2	30.2	0.2	0.4	0	0.7
BIO8 = Mean Temperature of Wettest Quarter	0	1.1	0.6	12.1	0	3.3	0.1	22.2	24.6	0.4	17.8
BIO9 = Mean Temperature of Driest Quarter	0	2.4	4.8	0.1	7.9	0.1	0	0.1	0.6	0	0
BIO10 = Mean Temperature of Warmest Quarter	0	0.5	2.8	2.2	0	3.3	0	0.2	0.2	0	0.5
BIO11 = Mean Temperature of Coldest Quarter	0	64.7	2	0	13.2	0.1	0.2	0	0	0	0
BIO12 = Annual Precipitation	0	0.5	2.8	0	37.8	0.6	2.1	0.3	1.8	0.7	0.2
BIO13 = Precipitation of Wettest Month	0	0.2	2.7	0.3	0	0.1	0.5	0	1.8	0.6	0
BIO14 = Precipitation of Driest Month	29	0.2	31	0.2	0	14.1	0.8	7.7	0	4.6	0.1
BIO15 = Precipitation Seasonality (Coefficient of Variation)	1.7	1.1	0.4	11.6	0	0.6	17.7	2.3	1.2	0.7	8.5
BIO16 = Precipitation of Wettest Quarter	0	7.4	21.7	0.1	0	0	0	0.2	0	0	0
BIO17 = Precipitation of Driest Quarter	0	5.5	0.1	0.3	32.1	0	0	0	0.9	0.2	0
BIO18 = Precipitation of Warmest Quarter	0.1	0.1	4.6	10.3	0	6.2	8.2	0.2	4.5	13	4.1
BIO19 = Precipitation of Coldest Quarter	2.7	2.8	2.9	0.6	1.8	0.2	32.9	4.2	0.6	0.6	7

Table 2. Time of divergence in Ceratophryidae. Results by node numbers (shown in Fig. 3), Clade = Major clades, UFB = Ultrafast Bootstrap Approximation, PP = Posterior Probability, Height = Time associated in million years (Ma) 95% HPD = 95% Highest Posterior Density intervals.

Node number	Clade	UFB	Height	95% HPD
1	Outgroup	N/A	131.79	-
2	Outgroup	N/A	71.67	66.36–77.01
3	Outgroup	N/A	64.08	59.26–68.83
4	Outgroup	N/A	54.78	39.84–66.50
5	Ceratophryidae	100	19.18	14.72–24.05
6	*Chacophrys* + *Lepidobatrachus*	91	13.70	8.15–19.50
7	*Lepidobatrachus*	100	4.95	4.00–6.47
8	*Lepidobatrachus*	100	3.46	2.03–5.01
9	*Ceratophrys*	96	17.16	12.53–21.74
10	*Ceratophrys*	100	10.38	5.12–15–78
11	*Ceratophrys*	82	14.71	10.41–19.20
12	*Ceratophrys*	100	10.18	6.67–13.90
13	*Ceratophrys*	100	4.84	2.08–8.16
14	*Ceratophrys*	100	4.24	4.00–4.72

Table 3. Comparative statistics of biogeographic models. Abbreviations: -lnL = log-likelihood; d = dispersal rate; e = extinction rate; j = founder-event speciation; AICc = corrected Akaike Information Criterion; AICc_wt = weighted AICc percentage values; DEC = Dispersal Extinction Cladogenesis model; DIVALIKE = BioGeoBEARS implementation of DIVA model; BAYAREALIKE = BioGeoBEARS implementation of BayArea model.

Model	-lnL	d	e	j	AICc	AICc_wt
DEC	-30.38	0.021	0.44	0	65.76	39%
DEC+J	-30.35	0.024	0.66	0.00001	68.88	8%
DIVALIKE	-30.69	0.015	0.21	0	66.38	29%
DIVALIKE+J	-30.36	0.013	0.28	0.025	68.9	8%
BAYAREALIKE	-31.93	0.012	0.16	0	68.85	8%
BAYAREALIKE+J	-30.51	0.011	0.27	0.041	69.2	7%

(BIO14) for *C. cornuta*, Annual Precipitation (BIO12) for *C. joazeirensis* and Precipitation of Coldest Quarter (BIO19) for *C. stolzmanni* (Table 1).

The environmental PCA analysis for *Chacophrys* and *Lepidobatrachus* displays overlap between the four species (Fig. 4A). Annual Precipitation (BIO12) accumulates 89.7% of the total variance on Principal Component 1 (PC1), while Temperature Seasonality (BIO4) and Precipitation of Warmest Quarter (BIO18) accumulates 7.4% of the variance in PC2. Both components include 97.1% of variance. The PCA analysis for the *Ceratophrys* group shows that Temperature Seasonality (BIO4) and Annual Precipitation (BIO12) together accumulate 89.9% of the total variance in PC1. This analysis reveals a separation between *C. stolzmanni* from *C. cornuta* and *C. aurita* in the environmental space (Fig. 5). Precipitation of Coldest Quarter (BIO19) and Precipitation of the Warmest Quarter (BIO18) accumulates 5.4% of the variation in the PC2 (Fig. 4B, Suppl. material 6: table S4).

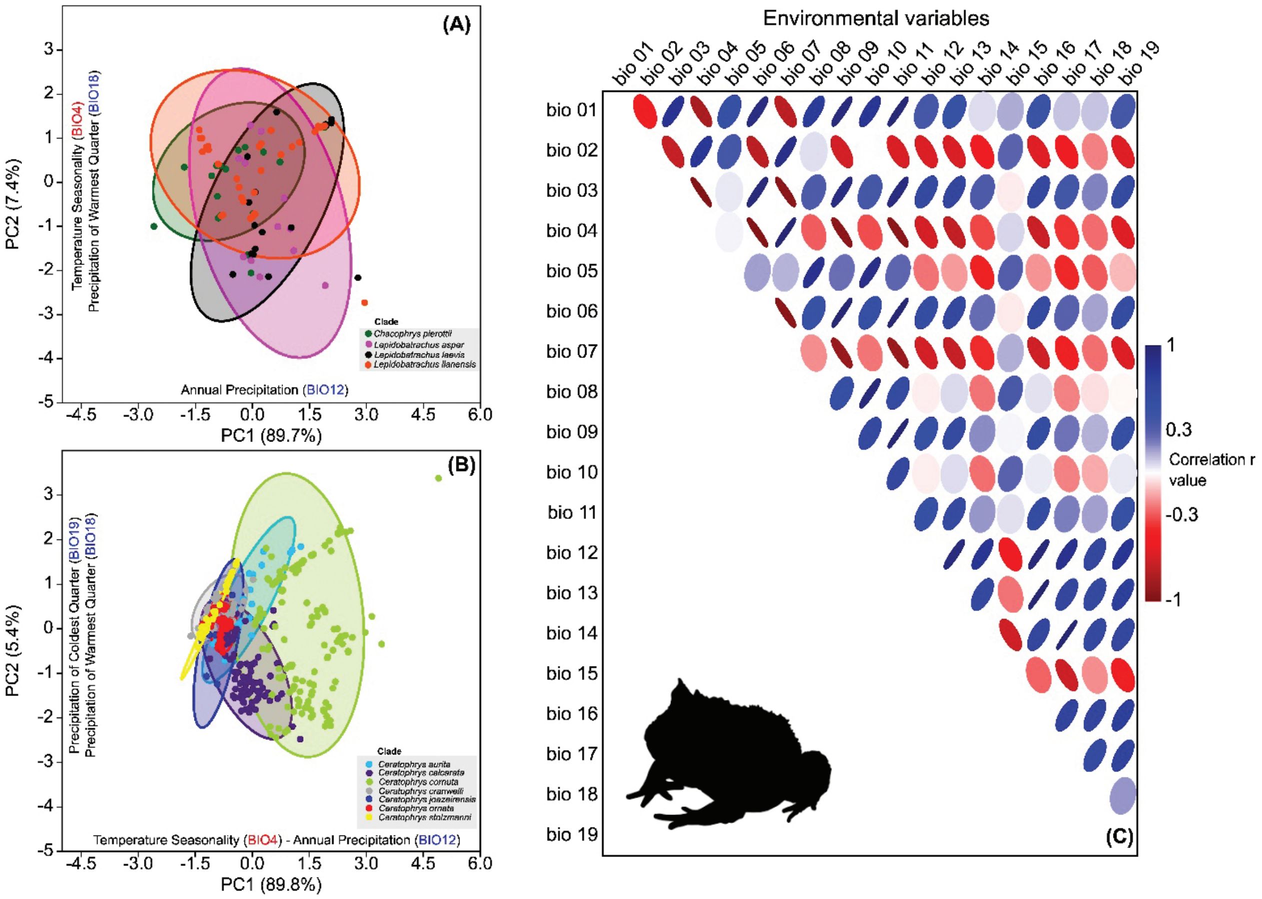

Figure 4. Scatter plot of the Principal Components Analysis (PCA) and correlation matrix for the nineteen environmental variables from WorldClim2 for Ceratophryidae. PCA for extant (**A**) species of *Chacophrys*, *Lepidobatrachus*, and (**B**) *Ceratophrys* in South America**.** Values in the x-axis are the percentage of variance explained mostly by the principal component 1 (PC1) and on the y-axis principal component 2 (PC2). The colored dots represent individual occurrences per species in the PCA and are surrounded by 95% ellipses. (**C**) Correlation plot with positive (blue) or negative (red) direction that show the collinearity between environmental variables. Variables names are detailed in Table 1.

The pPCA shows that the first principal component has the greatest multivariate load from Precipitation Seasonality (BIO15, positive load) and overall precipitation (BIO12; negative load). The second PC is mostly accumulated by Temperature Seasonality (BIO4; negative load) and Isothermality (BIO3; positive load) and. *C. cornuta* is highly related with precipitation variables and Temperature seasonality, located far from all other members of this family in the environmental space (Fig. 5). There is a group which include all species of *Lepidobatrachus*, *Ch. pierottii* and *C. ornata* highly related to Precipitation seasonality (BIO15) and Isothermality (BIO3). *Ceratophrys stolzmanni* and *Ceratophrys joazeirensis* are mostly related to Precipitation seasonality (BIO15). Vicariance events are associated with variation in niche differentiation of extant *Ceratophrys* species in the environmental space by regions (Fig. 5). Species from *Chacophrys* and *Lepidobatrachus* have evolved and originated within the Cerrado region (Fig. 3) where they inhabit similar environmental niches characterized by arid ecosystems. This stands in contrast to the diverse environmental niches observed within the *Ceratophrys* clade (Fig. 4 and Fig. 5).

Discussion

We present for the first time a phylogeny based on both genetic and morphological characters, as well as a calibrated chronogram, to explore the historical biogeographic patterns and the evolutionary role of environmental variables in the diversification of Ceratophryidae in South America.

Phylogeny

The phylogenetic tree (Fig. 2) supports previous studies about the phylogeny of the family Ceratophryidae. Nonetheless, there are still differences. The comparison between the combined (DNA + morphology) and the DNA based data set resulted in the same topology. However, the combined dataset gave a higher node support in the topology compared with the DNA based data set (Fig. 2, Suppl. material 1: fig. S1). Morphological characters could exhibit phylogenetic signal that complements genetic data, which tend to increase node support, especially in cases where genetic variation is low, when molecular markers fail to resolve deep phylogenetic relationships (Wahlberg et al. 2005) or

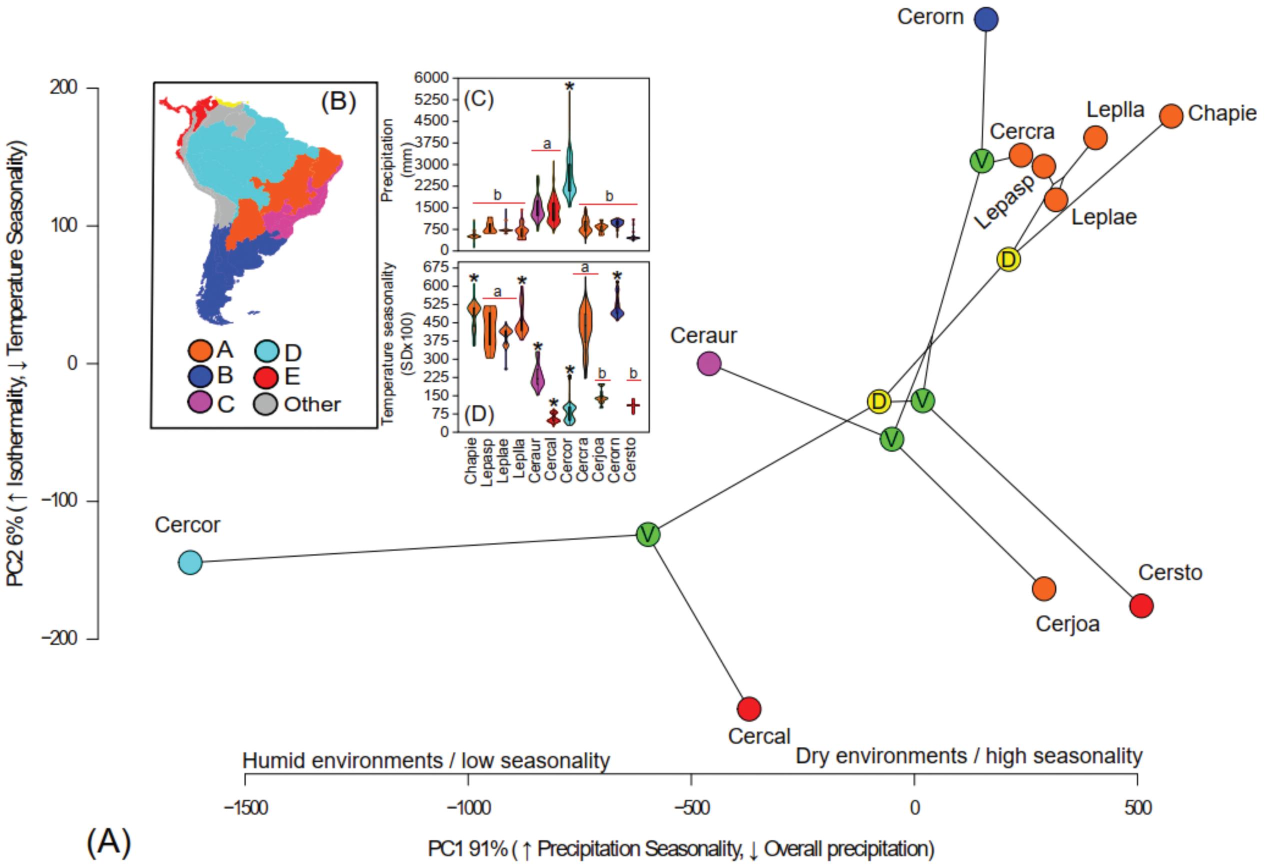

Figure 5. Phylomorphospace of Phylogenetic Principal Components for extant *Ceratophrys* species in South America. (**A**) Phylogenetic PCA for Ceratophryidae species to represent the environmental niche differentiation data based on the phylogenetic chronogram. Tip colors indicate (**B**) Biogeographical region categories based on Hutter et al. (2017): **A:** Cerrado, **B:** Temperate, **C:** Atlantic Forest, **D:** Amazonia and the Guiana Lowlands province, **E:** Choco; because none of the species from Ceratophryidae are distributed in the Tropical Andes and Guiana Shield, we excluded those regions from the analyses (Other). (**C**) Precipitation annual mean (mm) data from BIO12 variable. (**D**) Temperature seasonality (Standard Deviation × 100) data from BIO4 variable. Species codes: Ceraur = *Ceratophrys aurita*; Cercal = *Ceratophrys calcarata*; Cercor = *Ceratophrys cornuta*; Cercra = *Ceratophrys cranwelli*; Cerjoa = *Ceratophrys joazeirensis*; Cerorn = *Ceratophrys ornata*; Cersto = *Ceratophrys stolzmanni*; Chapie = *Chacophrys pierottii*; Lepasp = *Lepidobatrachus asper*; Leplae = *Lepidobatrachus laevis*; Leplla = *Lepidobatrachus llanensis*. Biogeographic events are highlighted with letters (**D**, yellow) for dispersal event and (V, green) for vicariance events based on the DEC developed in RASP software. Statistic groups assessed by Tukey test in (**C**) and (**D**) are represented by letters, whereas species that have significative differences ($p < 0.05$) among all other species are represented by asterisk (*).

when distantly related taxa share similar genetic sequences due to convergent evolution (Edwards et al. 2012). *Chacophrys* was expected to be the sister group of *Ceratophrys* + *Lepidobatrachus* based on the 93% Jackknife support from Barcelos et al. (2022). However, this is inconsistent with our phylogeny, which exhibits a high node support for the clade which includes *Ceratophrys* as sister group to *Chacophrys* + *Lepidobatrachus* (Fig. 2). On the other hand, other studies have hypothesized a similar phylogenetic relationship (Fabrezi 2006; Faivovich et al. 2014; Frazao et al. 2015; Brusquetti et al. 2018; Hime et al. 2021), despite Faivovich et al. (2014) recovering low support for this group (<50%).

All nodes within *Lepidobatrachus* in our phylogeny (Fig. 2) are well supported and agree with the results from previous studies about this genus (Faivovich et al. 2014; Brusquetti et al. 2018; Gómez and Turazzini 2021; Barcelos et al. 2022). In the *Ceratophrys* clade, we found *C. stolzmanni* to be phylogenetically linked to two clades formed by *C. aurita* + *C. joazeirensis* and *C. cranwelli* + *C. ornata*, with relative high support values (82% UFB, 100% UFB in the inner clades). This contrasts with previous studies (Faivovich et al. 2014; Barcelos et al. 2022), where *C. stolzmanni* was found to belong to a clade also including *C. cornuta* + *C. calcarata*, but with lower support values (<60% parsimony jackknife frequencies) compared to our study. It was hypothesized that *C. cranwelli* and *C. ornata* were sister taxa, as detailed by Faivovich et al. (2014). However, in the phylogeny presented by Barcelos et al. (2022), the Jackknife frequency node support was <50%. Our phylogeny has a 100% node support on this clade confirming that *C. cranwelli* and *C. ornata* are sister taxa (Fig. 2). These different results can be explained by the fact that Barcelos et al. (2022) utilized morphological data, whereas the analysis of Faivovich et al. (2014) relied exclusively on genetic data. Our study supplements these analyses by incorporating both genetic and morphological characters (Fig. 2).

Time of divergence of Ceratophryidae

Our chronogram mostly agreed with previous studies in estimating divergence times in Anura, despite the inclusion of only a few representatives of Ceratophryidae in those analyses (Fabrezi et al. 2009; Faivovich et al. 2014; Brusquetti et al. 2018). We present the first genetically calibrated chronogram of the Ceratophryidae family (excluding *C. testudo*). The divergence of the crown group of Ceratophryidae is estimated to have occurred ~19.2 Ma (95% HPD 14.7–24.1 Ma) at the beginning of the Neogene (Fig. 3, Suppl. material 2: fig. S2). The divergence time estimation of the MRCA of *Chacophrys* and *Lepidobatrachus* is congruent with the ML tree of Roelants et al. (2007), at around 18.8 Ma (95% HPD 12.7–25.1 Ma). These results agree with the divergence time estimations within *Lepidobatrachus* (Ruane et al. 2011; Frazao et al. 2015; Brusquetti et al. 2018; Hime et al. 2021).

Ancestral area reconstruction for Ceratophryidae

The ancestral area for Ceratophryidae reveals that the Cerrado (area A; Fig. 3) is the core area estimated to be the biogeographic origin for this group. The high diversity and closely related fossil species of this clade documented in the Cerrado region, such as *Baurubatrachus pricei* and *Beelzebufo ampinga* (Barcelos et al. 2022), support the hypothesis of the origin of this clade in this region in South America (Fig. 3). This agrees with the estimated ancestral area for *Chacophrys* and *Lepidobatrachus* frogs, which is also suggested to be in the Cerrado region (Figs 3–5). Faivovich et al. (2014) predicted that most diversification in Ceratophryidae occurred in semi-arid environments with three independent transitions to different humid environments in the Choco, Amazonia and Atlantic Forest. We found that temperature and precipitation seasonality are important factors for extant species located in the Cerrado region (i.e. *Ch. pierottii*, *Lepidobatrachus* species, *C. cranwelli* and *C. joazeirensis*), whereas main precipitation is related to humid tropical inhabitants like *C. cornuta* and *C. calcarata* (Fig. 5).

The node related with extant *Ceratophrys* species (node 9) has a high marginal probability to assign the Cerrado region as the ancestral area (A = 61.89%), but low probability to support the RASP route in the speciation events (10.5%; Table 2; Suppl. material 8: appendix S2). The most probable scenario entails independent dispersions originating from the Cerrado to other regions, succeeded by vicariance events in the Choco, Amazonia, Atlantic Forest, and Temperate Forest (Fig. 3, Suppl. material 8: appendix S2). Extant species of this group show limited dispersal capabilities and a strong dependence of their habitat (Zeisset and Beebee 2008). Inside this clade, a vicariance event caused the divergence of *C. cornuta* and *C. calcarata* ~10.4 Ma, where they inhabited the Amazonia and Choco regions, respectively (Fig. 3). The Eastern Andes Cordillera experienced surface uplifts during the middle Miocene period (Leier et al. 2013; Carrapa et al. 2014), and Gregory-Wodzicki (2000) suggested that not more than half of the modern elevation of the North-eastern Cordillera was present by ~10 Ma (Hoorn et al. 2010) . This uplift may explain the vicariance event causing the divergence of *C. calcarata* isolated in the Choco region and *C. cornuta* in the Amazonian Basin. It this case, the MRCA of both species has a hypothetical wider distribution in the Choco and the Amazonia regions, which were isolated by the rise of the young Eastern Andes Cordillera after the Pebas system (23 to 10 Ma) transitioned to the Acre system (10–7 Ma) in the late Miocene (Fig. 3). On the other hand, the divergence time estimation data indicates a vicariance event in the distribution of *C. stolzmanni*, occurring ~14.4 Ma (95% HPD 10.5–18.5; node 11; Fig. 3), which was caused by the formation of the Andes in southern Ecuador (Evenstar et al. 2015). The divergence of *C. aurita* from *C. joazeirensis* and *C. cranwelli* + *C. ornata* (node 12) is probably caused by the epeirogenic uplift of the Brazilian Shield during Middle- Late Tertiary elevations (Ab’sáber 2000; Klöcking et al. 2020).

Cocoon production is observed in all species of Ceratophryidae, but is unknown for the species *C. testudo*, *C. calcarata* and *C. cornuta* (Faivovich et al. 2014). In agreement with these findings, cocoon formation originated in the MRCA of the clade, estimated to have occurred 19.2 Ma in the early Miocene (Faivovich et al. 2014). The formation of a cocoon, which acts as protection against water loss in dry environments, could have been lost in *C. cornuta* and *C. calcarata*, both inhabitants of the wet tropical forest in the Amazonia and the Choco regions, around 10.4 Ma (node 10, Fig. 3).

Phylogenetic environmental relatedness

Three species of *Ceratophrys* (*C. cornuta*, *C. calcarata* and *C. aurita*) inhabit areas with a high average precipitation. *C. cornuta* is distributed in tropical humid environments, separated from all other species in the environmental space (Fig. 5). *C. aurita* is an inhabitant of the Atlantic Forest, characterized by a higher temperature seasonality compared with the former two species (Fig. 5). All other species from *Ceratophrys*, *Lepidobatrachus* and *Chacophrys* are associated with more semi-arid environments characterized by higher and variable temperature seasonality (Fig. 5, Table 1). Biogeographically, environmental niche differentiation is shown even in phylogenetic sister species (e.g., *C. aurita*–*C. joazeirensis*; *C. ornata*–*C. cranwelli*; Figs 3–5).

Faivovich et al. (2014) hypothesised that most diversification in Ceratophryidae occurred in semi-arid environments, with three independent transitions to humid environments by *C. cornuta*, *C. aurita* and *C. ornata*. Our results suggest two independent environmental niche differentiation shifts to wet environments, one associated with *C. cornuta* and *C. calcarata*, and the other associated with *C. aurita* (Figs 3–5). Nevertheless, Faivovich et al. (2014) also concluded an independent transition from *C. ornata* to a more humid environment of the Pampean region in central Argentina. However, our pPCA and PCA tests showed a relationship of this species with highly variable Temperature seasonality and precipitation of the driest month (Figs 4, 5, Table 1). The niche differentiation observed between sister species *C. joazeirensis* and *C. aurita* can be attributed to variances in average precipitation (Figs 4, 5). *C. joazeirensis* primarily inhabits the Cerrado Brazilian forest (area A, Fig. 3), characterized by lower precipitation levels in contrast to the Atlantic Forest (area C), where *C. aurita* is found.

Conclusion

We present the first calibrated chronogram hypothesis, providing divergence time estimations for Ceratophryidae. Within this family, *Ceratophrys* emerges as the sister taxon to the clade comprising *Chacophrys* and *Lepidobatrachus*. Our chronogram revealed a pronounced surge in species radiation within this clade during the Miocene, around 19.2 million years ago (Ma). Notably, three lineages of *Lepidobatrachus* and four lineages from *Ceratophrys* diverged in the Pliocene, around 5.3 to 2.6 Ma. Ceratophryidae is hypothesized to have originated in the semi-arid Cerrado region, from where species have diversified by dispersal and vicariance events across other South American regions, exhibiting distinct environmental niches shaped by variation in temperature seasonality and annual precipitation in extant species. The biogeography, evolution, and niche adaptation of extant Ceratophryidae species are linked with their likely origin in semi-arid ecosystems in the Cerrado region, exhibiting marked temperature seasonality across South America (Brusquetti et al. 2018). This origin is characterized by environmental niche differentiation, particularly evident in three species of *Ceratophrys*, as they adapted to more humid environments (Faivovich et al. 2014).

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Author contributions

Conceptualization, methodology, formal analysis, investigation, validation, formal analysis, data curation, visualization, supervision, HMOA, AvdK; writing original draft preparation, AvdK, HMOA; writing—review and editing, AvdK, HMOA; project administration, funding acquisition, HMOA. Both authors have read and agreed to the published version of the manuscript and declared not conflict of interest.

Data accessibility statement

Supplemental material is available in ZENODO repository.

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Supplementary materials

Supplementary material 1

fig. S1: Phylogeny base only in genetic data (.png)
Link: https://doi.org/10.21425/fob.17.132672.suppl1

Supplementary material 2

fig. S2: Chronogram for Ceratophryidae (.png)
Link: https://doi.org/10.21425/fob.17.132672.suppl2

Supplementary material 3

table S1: GenBank accession numbers (.xlsx)
Link: https://doi.org/10.21425/fob.17.132672.suppl3

Supplementary material 4

table S2: Tracer v.1.7.2 output (.xlsx)
Link: https://doi.org/10.21425/fob.17.132672.suppl4

Supplementary material 5

table S3: Environmental data for Principal components analysis (.xlsx)
Link: https://doi.org/10.21425/fob.17.132672.suppl5

Supplementary material 6

table S4: Phylogenetic Principal Components Analysis (.xlsx)
Link: https://doi.org/10.21425/fob.17.132672.suppl6

Supplementary material 7

appendix S1: BEAUti (.xml) file with the chronogram model parameters (.xml)
Link: https://doi.org/10.21425/fob.17.132672.suppl7

Supplementary material 8

appendix S2: RASP v.4.3 output of the ancestral area reconstruction (.docx)
Link: https://doi.org/10.21425/fob.17.132672.suppl8

Supplementary material 9

appendix S3: Tree file of the calibrated chronogram by BEAST v. 2.7.4 (.tree)
Link: https://doi.org/10.21425/fob.17.132672.suppl9

Supplementary material 10

appendix S4: Photograph credits (.pdf)
Link: https://doi.org/10.21425/fob.17.132672.suppl10