



Historical biogeography highlights the role of Miocene landscape changes on the diversification of a clade of Amazonian tree frogs

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Abstract

The diversification processes underlying why Amazonia hosts the most species-rich vertebrate fauna on earth remain poorly understood. We studied the spatio-temporal diversification of a tree frog clade distributed throughout Amazonia (Anura: Hylidae: *Osteocephalus*, *Tepuihyla*, and *Dryaderces*) and tested the hypothesis that Miocene mega wetlands located in western and central Amazonia impacted connectivity among major biogeographic areas during extensive periods. We assessed the group's diversity through DNA-based (16S rRNA) species delimitation to identify Operational Taxonomic Units (OTUs) from 557 individuals. We then selected one terminal for each OTU ($n=50$) and assembled a mitogenomic matrix (~14,100 bp; complete for 17 terminals) to reconstruct a Bayesian, time-calibrated phylogeny encompassing nearly all described species. Ancestral area reconstruction indicates that each genus was restricted to one of the major Amazonian biogeographic areas (western Amazonia, Guiana Shield and Brazilian Shield, respectively) between ~10 and 20 Mya, suggesting that they diverged and diversified in isolation during this period around the Pebas mega wetland. After 10 Mya and the transition to the modern configuration of the Amazon River watershed, most speciation within each genus continued to occur within each area. In *Osteocephalus*, only three species expanded widely across Amazonia (< 6 Mya), and all were pond-breeders. Species with other breeding modes remained mostly restricted to narrow ranges. The spectacular radiation of *Osteocephalus* was probably driven by climatic stability, habitat diversity and the acquisition of new reproductive modes along the Andean foothills and western Amazonia. Our findings add evidence to the importance of major hydrological changes during the Miocene on biotic diversification in Amazonia.

Keywords Amazonia · *Dryaderces* · Hylidae · Neotropics · *Osteocephalus* · *Tepuihyla*

Introduction

The Neotropics harbour the world's highest species diversity for many animal and plant groups (Raven et al., 2020). The origin and distribution of this astonishing diversity have intrigued biologists for almost two centuries (e.g. Wallace,

1854), notably in Amazonia, which encompass > 6.5 million km² of mostly rainforest vegetation. Our understanding of the diversification processes and assembling of biotas in the Neotropics has accelerated over the last decades, with Amazonia acting as the major biodiversity source for the entire continent (Antonelli et al., 2018). Nevertheless, the historical diversification of major lineages in the Neotropics has been mostly tackled at the continental scale, whereas diversification processes within Amazonia remain unresolved (Cracraft et al., 2020). This is at least partly because including sufficiently dense and taxonomically complete sampling across this broad region, together with rigorous temporal estimations of lineage diversification throughout Neogene (2.6–23 Mya), remains challenging.

Several diversification hypotheses have been formulated to explain the origin of Amazonian biodiversity. Hypotheses involving major landscape changes that lead to allopatric

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speciation have received the most attention (for a review, see Leite & Rogers, 2013). For example, the riverine barrier hypothesis (Wallace, 1854) postulates that populations of terrestrial organisms were isolated following the formation of the largest Amazonian rivers. Paleogeographic data suggests that this hypothesis should be largely limited to speciation since ~10 Mya, following the establishment of the modern Amazon River watershed (Albert et al., 2018; Hoorn et al., 2010). Another model of allopatric speciation is the marine incursions hypothesis (Webb, 1995), which postulates that during the middle Miocene, an extensive mega wetland with marine influence occupied western-central Amazonia (termed the ‘Pebas system’) and thus formed a barrier for rainforest organisms between ~10 and 23 Mya (Albert et al., 2018; Bicudo et al., 2019; Hoorn et al., 2010). However, it is important to keep in mind that large uncertainties surround Amazonia’s hydro-geological history, as illustrated by different models in Bicudo et al. (2019). The end of the Pebas system (draining north to the Caribbean Sea) was followed by the onset of the transcontinental Amazon River (draining east to the Atlantic Ocean) at 9–10 Mya (Hoorn et al., 2010, 2017). Nevertheless, the mega wetlands might have persisted in south-western Amazonia as the Acre system between 7 and 10 Mya (Hoorn et al., 2010). Therefore, over the last 10 Mya, western Amazonia underwent profound and progressive transformations, transitioning from a mega wetlands environment towards a fluvial and *terra firme*-dominated landscape (Albert et al., 2018).

Most comprehensive studies on speciation and biogeography of tetrapods in Amazonia have been conducted on birds (Ribas & Aleixo, 2019) and primates (e.g. Boubli et al., 2015; Byrne et al., 2018), primarily because their diversity and taxonomy are reasonably well known. A recent review suggests that most speciation in birds and primates within Amazonia occurred relatively recently (Pliocene and Pleistocene; 0.01–5.3 Mya) (Cracraft et al., 2020), and often implied successive dispersals across rivers from western to eastern Amazonia (Silva et al., 2019; Smith et al., 2014). In contrast, the diversity and biogeography of other groups, including Amazonian amphibians, are less well resolved. Frogs are a particularly valuable group to study due to their exceptional diversity in Amazonia and limited dispersal capabilities (Zeisset & Beebe, 2008).

Some frog groups originated out of Amazonia, notably in the Andes, and secondarily dispersed into Amazonia during the Miocene (5.3–23 Mya) (e.g. Centrolenidae, Castroviejo-Fisher et al., 2014; Dendrobatidae, Santos et al., 2009). Other groups are only found in the Amazonian lowlands and their diversification occurred in situ during the Miocene (e.g. *Adenomera*, Fouquet et al., 2014; *Allobates*, Réjaud et al., 2020; *Amazophrynella*, Moraes et al., 2022; Physelaphryninae, Fouquet et al., 2012; *Synapturanus*, Fouquet et al., 2021a; *Pipa*, Fouquet et al., 2022). In general, the diversification

events in these ground-dwelling and aquatic frogs appear to reflect the presence of ancient mega wetlands (e.g. Pebas system) with separation between western and eastern Amazonian clades (> 10 Mya) and then establishment of the trans-continental Amazon River (~10 Mya) separating northern and southern Amazonian clades. However, there have been few biogeographic studies of the exceptionally diverse arboreal frog fauna in Amazonia, for which biogeographic patterns are expected to differ due to ecological differences with terrestrial species (Fouquet et al., 2015). For example, Fouquet et al. (2021b) found that diversification of tree frogs of the *Boana albopunctata* group is relatively recent (Pliocene and Pleistocene) and included dispersals widely across Amazonia. This diversification was thus similar to patterns observed in birds and primates but in contrast to those seen in ground and aquatic frogs.

The tree frog clade consisting of *Osteocephalus* (29 species), *Tepuihyla* (9 species) and *Dryaderces* (2 species) (Hylidae: Lophiohyliinae; Frost, 2022; Ortiz & Ron, 2018) is endemic to Amazonia and the Pantepui region (Duellman et al., 2016; Jungfer et al., 2013). This clade is diverse and widespread and thus offers the potential for valuable insights into Amazonian biogeography. However, this clade also exemplifies the gap existing in our understanding of the extant Amazonian diversity because new species are frequently described (e.g. Chasiluisa et al., 2020; Duellman, 2019; Melo-Sampaio et al., 2021); many known populations are suspected to belong to undescribed species (Jungfer et al., 2013; Vacher et al., 2020), and some relationships among species and species groups remain unresolved (Blotto et al., 2020). Moreover, the spatio-temporal aspects of their diversification and biogeography remain virtually unexplored. Duellman et al. (2016) estimated the divergences among the three genera between ~25 and 28 Mya, the crown age of *Osteocephalus* ~18.2 Mya (15.5–20.9), and Salerno et al. (2012) estimated the crown age of *Tepuihyla* (including *Tepuihyla exophthalma* Smith & Noonan, 2001 as ‘*Osteocephalus exophthalmus*’ therein) to be 14.7–23.6 My old. However, these estimates are suspected to be overestimated based on recent genomic studies (Feng et al., 2017; Hime et al., 2021).

The spatial distribution of the three genera in this clade covers Amazonia. *Dryaderces* is endemic to the Brazilian Shield, *Tepuihyla* is restricted to the Pantepui region (Guiana Shield) and adjacent north-western Amazonia, and *Osteocephalus* has diversified extensively throughout Amazonia but is particularly diverse along the Andean foothills and western Amazonia (Duellman, 2019; Jungfer et al., 2013; Ron et al., 2016). Within *Osteocephalus*, five species groups are currently recognised based on their monophyly and reproductive modes (Jungfer et al., 2013). The *O. leprieurii* and *O. taurinus* groups are almost entirely pond-breeders, and the *O. buckleyi* group mostly breeds in streams. These

three groups are widespread across Amazonia. The *O. albo-guttatus* and *O. planiceps* groups are restricted to western Amazonia and reproduce in phytotelmata. The evolution of breeding ecology in *Osteocephalus* is of particular interest because it may have contributed to its extensive diversification, including enabling co-occurrence of species (Jungfer et al., 2013).

Based on the fact that the crown age of the most recent common ancestor between *Osteocephalus* and *Trachycephalus* (another genus in Lophiohylinae) has been estimated from genomics to be about 23 My old (Feng et al., 2017), the diversification of our focal clade must have taken place through the Neogene. Therefore, the origin of the three major clades (genera) distributed in the Amazonian areas described above (i.e. western Amazonia, Guiana Shield and Brazilian Shield) either predates 10 Mya and hence overlaps with the Pebas system as a potential barrier, or is more recent and occurred across major Amazonian rivers. Given the higher diversity of *Osteocephalus* in western Amazonia, we hypothesize that this region acted as a centre of diversification and that the different species groups dispersed throughout the Amazon River watershed after the demise of the Pebas and Acre systems.

To test these assumptions, we used spatially dense sampling of mitochondrial sequences (16S rRNA) from 557 individuals of the Amazonia-wide tree frog clade formed by *Osteocephalus*, *Tepuihyla* and *Dryaderces* to delimit Operational Taxonomic Units (OTUs). We subsequently built a mitogenomic matrix (complete for 17 OTUs) to reconstruct a time-calibrated phylogeny and estimate their ancestral geographic distribution, in order to imply diversification processes.

Materials and methods

DNA-based species delimitation

We analysed a 16S rRNA fragment to identify major mitochondrial DNA (mtDNA) lineages that may represent species (i.e. a general lineage species concept; de Queiroz, 1999, 2007). Thus, this study does not represent an integrative species delimitation. The 16S locus has been widely used in amphibian systematics (Vences et al., 2005) and, for our focal clade, sequences were available from previous studies encompassing most taxa and regions (e.g. Jungfer et al., 2013; Vacher et al., 2020). We generated new 16S sequences from recent field collections ($n = 52$) (laboratory protocols are in Electronic Supplement 1), which were aligned with available GenBank sequences ($n = 505$), thus resulting in 16S sequences from 557 individuals (*Osteocephalus*: $n = 508$; *Tepuihyla*: $n = 34$; and *Dryaderces*: $n = 15$). This dataset included sequences of 34 out of 40 described species (Frost, 2022; Ortiz & Ron, 2018) and encompassed

the entire geographic range for the three genera. GenBank accession numbers and associated information to all analysed sequences, including sources, are available in Electronic Supplement 2. The entire dataset was aligned using the E-INS-i strategy in MAFFT 7 (Katoh & Standley, 2013). The alignment was manually reviewed and its extremes were trimmed in Aliview 1.26 (Larsson, 2014) to keep the majority of homologous nucleotides (583 bp) among individuals.

The species delimitation was undertaken using three single-locus methods (ABGD, mPTP and GMYC). The Automatic Barcode Gap Discovery (ABGD) is a distance-based method which infers a ‘gap’ putatively existing between intra- and interspecific genetic distances to partition the dataset (Puillandre et al., 2012). The analysis was conducted using a prior of intraspecific divergence (K80) of 0.1–10% ($P = 0.001–0.1$), X (relative gap width) = 1, and number of steps = 30 (Réjaud et al., 2020). The analysis was performed on the online web server (available at <https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>). We selected the 11th recursive partition ($P = 0.0049$) in the ranking of distances, which corresponds to the end of a plateau for a number of delimited groups ‘species’ and thus, it was considered stable (Puillandre et al., 2012). Partitions with ‘ P ’ values higher than this resulted in very few groups and the merging of many recognised species.

The multi-rate Poisson Tree Processes (mPTP) is a tree-based method that incorporates different levels of intraspecific divergence without input thresholds (Kapli et al., 2017; Zhang et al., 2013a). We first reconstructed a maximum likelihood tree in RAxML 8.2 (Stamatakis, 2014) using one partition, 1000 iterations, and the GTRCAT approximation. The outgroup was defined in the GMYC analysis (see below). After removing the outgroup, we performed mPTP using 50 million iterations of Markov chain Monte Carlo (MCMC), sampling every 100,000 iterations, and discarding an initial 10% as burn-in.

The General Mixed Yule Coalescent (GMYC) is also a tree-based method that classifies the branches as interspecific (using a Yule model of constant speciation rate and no extinction) or intraspecific (modelled using a neutral coalescent process) (Monaghan et al., 2009; Pons et al., 2006). We first reconstructed an ultrametric phylogeny in BEAST 2.6 (Bouckaert et al., 2019) to estimate the timing of diversification events using a birth–death model and the uncorrelated relaxed clock lognormal of rate variation among branches (Drummond et al., 2006). The substitution model for 16S (one partition) was defined using the Bayesian Information Criterion (BIC) as implemented in PartitionFinder 2.1 (Lanfear et al., 2016). We used *Trachycephalus coriaceus* Peters, 1867 as an outgroup to constrain the root age of the tree with a normal prior distribution. This secondary calibration corresponds to the time to the crown age of the most recent common ancestor (TMRCA) between *Osteocephalus* and *Trachycephalus*

(23.52 Mya; 15.1–32.5), an estimation based on 95 nuclear genes (~88,000 bp) and relevant fossil calibration (Feng et al., 2017). We ran four independent chains on the CIPRES online cluster with 250 million MCMC steps, 10,000 generations of thinning, and discarding an initial 10% as burn-in. The sampling adequacy (ESS > 200) and convergence of parameters among independent runs were verified in Tracer 1.7 (Rambaut et al., 2018). We combined and re-sample the independent tree files (excluding the initial 10% as burn-in) in LogCombiner 2.6 (Bouckaert et al., 2019) resulting in a file with 9000 trees. From this file, we extracted the Maximum Clade Credibility (MCC) tree in TreeAnnotator 2.6 (Bouckaert et al., 2019) excluding an additional initial 10% as burn-in. After removing the outgroup, we performed GMYC on the ultrametric tree using ‘splits 1.0’ R-package (Ezard et al., 2009) within R 4.0.2 (R Core Team, 2020), an interval of 1–10 Mya, and the single-threshold method (Fujisawa & Barraclough, 2013).

Operational Taxonomic Units (OTUs) were defined by applying a majority rule consensus to the results of the three delimitation methods (i.e. a cluster of sequences corresponding to an independent OTU when recovered as such in at least two of the three methods). We then assigned the OTUs to known taxa based on a priori sequence identification, type locality and known geographic range (Frost, 2022). In addition, we calculated uncorrected p-distances (mean and range) for 16S among OTUs in MEGA X (Kumar et al., 2018).

Dated mitogenomic phylogeny

We selected 50 terminals to be represented in a phylogenetic reconstruction based on mitogenomic data. Forty-two of these terminals correspond to the defined OTUs from the 16S species delimitation (see Results). However, four of these OTUs clustered sequences of 2–4 recognised species each. Because these species display diagnosable external morphology and other evidence lines supporting their distinctiveness, we followed the current taxonomy and added six corresponding terminals to the mitogenomic matrix, totalling 48 terminals. Finally, terminals for two recognised taxa without 16S sequences and thus not included in the delimitation (*Osteocephalus sangay* Chasiluisa et al., 2020 and *Tepuihyla shushupe* Ron et al., 2016) were also added to the mitogenomic matrix (see justification on inclusion of additional terminals in Appendix). Our mitogenomic matrix included 36 out of 40 currently recognised species (90%), with the absence of *Osteocephalus duellmani* Jungfer, 2011; *Osteocephalus germani* Ron et al., 2012; *Osteocephalus melanops* Melo-Sampaio et al., 2021; and *Tepuihyla luteolabris* Ayarzagüena et al., 1993a.

We performed complete mitogenome sequencing for 16 of the 50 terminals, with these 16 terminals evenly distributed

across the phylogeny. We used 200 ng of genomic DNA to generate high-quality mitogenomic assemblies through low-coverage shotgun sequencing. For library preparation, we used the Illumina TruSeq Nano DNA Sample Prep kit with supplier instructions at the Génopole facility (Toulouse, France). Genomic DNA was fragmented by sonication, then fragments were selected by size (50–400 bp), adenylated and ligated to indexed sequencing adapters. Eight cycles of polymerase chain reaction (PCR) were performed to amplify the libraries. We quantified and validated the libraries, which were then multiplexed and sequenced on one lane of an Illumina HiSeq3000 flow cell (Illumina Inc.). Finally, assembly of reads was conducted in ORGanelle ASSEMBler (Boyer et al., 2016), following assembling and annotation steps described in Réjaud et al. (2020) and using as mitogenome reference *Osteocephalus taurinus* Steindachner, 1862 (GenBank accession: JX564881; Zhang et al., 2013b). For the remaining 34 terminals without complete mitogenomes, we gathered available GenBank accessions across multiple mitochondrial genes (12S, 16S, ND1, ND2, COI and CytB; see Electronic Supplement 2). Finally, we generated a complete mitogenome for the outgroup *Trachycephalus coriaceus*.

We extracted the non-coding (12S and 16S rRNAs) and all coding (CDS) regions considering the reading frame (therefore removing D-Loop and tRNAs) using Geneious v5.4 (<https://www.geneious.com>). We aligned each gene independently in MAFFT 7 using the strategies: E-INS-i for rRNA genes (multiple conserved domain and long gaps); and G-INS-i for protein-coding genes (global homology) (Kato et al., 2019). Finally, we concatenated the independent alignments in Geneious v5.4. Our final alignment consisted of 51 terminals and 14,098 characters (50% of missing data): 17 terminals including the outgroup had complete mitogenome (~14,000 bp), and 34 terminals had on average 3555 bp (1176–5155). We predefined four partitions: one for combined 12S and 16S rRNAs, and one for each codon position of concatenated protein-coding genes. We estimated the best-fit substitution model for each partition using Bayesian Information Criteria (BIC) as implemented in PartitionFinder 2.1.

We reconstructed a time-calibrated phylogeny using BEAST 2.6. Initially, we used a similar tree model setting as described for GMYC delimitation method (see above); however, some inconsistencies were detected on the parameters’ posterior, probably caused because of the large CI of our calibration prior. Therefore, we used the Calibrated Yule tree model instead as this allows a proper sampling when a single calibration point is used (Heled & Drummond, 2012), as in our case. In absence of relevant fossils for calibration of our focal clade, we constrained the tree root prior to using the secondary calibration node estimated by Feng et al. (2017): TMRCA *Osteocephalus-Trachycephalus* (mean = 23.52 Mya, SD = 3.6 Mya, and normal distribution). Each partition was parametrized using the substitution model informed by PartitionFinder 2.1, but excluding

the parameter of invariant sites proportion (Jia et al., 2014; Stamatakis, 2016). We used a linked, uncorrelated relaxed clock lognormal of rate variation among branches for all partitions (Drummond et al., 2006; Duchêne et al., 2020). MCMC parametrization in BEAST, runs assessment, and obtention of the MCC tree was similar as described for GMYC method (see above), except that each chain ran 100 million steps.

In addition, using the same file with 9000 trees (mitogenomic analysis) resulting from LogCombiner 2.6, we constructed a lineage through time (LTT) plot in ‘ape 5.5’ R-package (Paradis & Schliep, 2019) to explore changes in lineage accumulation over time. Finally, we conducted a maximum likelihood analysis on the mitogenomic matrix in RAxML 8.2 (Stamatakis, 2014), using 1000 iterations, and the GTRCAT approximation for the four partitions combined in a single tree.

Ancestral area reconstruction

We estimated the ancestral geographic range for the genera based on the mitogenomic phylogeny using ‘BioGeoBEARS 1.1.2’ R-package (Matzke, 2013). We coded the current distribution of our ingroup terminals using three areas: western Amazonia, the Guiana Shield and Brazilian Shield. These areas differ in geological history (Hoorn & Wesselingh, 2010), and are well-recognised biogeographically (Godinho & da Silva, 2018; Oliveira et al., 2017). The areas are approximately delimited by the rivers: Negro, Madeira and the lower course of the Amazon (see Electronic Supplement 3). The analysis compares the likelihood of several models of ancestral state reconstruction and their fit to the data, weighted by Akaike’s information criterion (AICc; Akaike, 1973). Six models were evaluated: Dispersal-Extinction Cladogenesis (DEC) model, a likelihood version of the Dispersal-Vicariance Analyses (DIVALIKE), and a Bayesian range-evolution model (BAYAREALIKE). The other three models correspond to modifications of the former ones by adding a parameter of founder-event speciation (+J). We included these latter three models in the model selection in face of recent debate about using this parameter (Klaus & Matzke, 2020; Ree & Sanmartín, 2018).

Results

Species delimitation (16S)

There were considerable differences in the estimated number of groups ‘species’ found among the three methods: mPTP=16, ABGD=42 and GMYC=159. The mPTP result (16 groups) was the most conservative, but 2–7 recognised, morphologically diagnosable species clustered together in some of these groups. This meant that the mPTP result failed to detect 19 described species. The ABGD result (42 groups) was more in agreement with current taxonomy, with 25 groups

correctly assigned each to a described species, four groups contained 2–4 recognised species (thus failing to detect five described species and one paraphyletic lineage of ‘*Osteocephalus cabrerai*’ Cochran & Goin, 1970 from the Guiana Shield), and 13 groups corresponded to non-described species. Therefore, the number of recognised species that failed into being detected was approximately four times higher in mPTP than in ABGD. The GMYC method was deemed to over-split the dataset (159 groups). Nineteen described species were identified as single groups in GMYC result, but 15 species were formed by two or more groups. Therefore, GMYC method overall identified almost every clade in the tree as a different group. The majority rule consensus over the three methods was consistent with the intermediate result provided by ABGD, delimiting 42 OTUs (Fig. 1a). Results of each method and consensus are in Electronic Supplements 2 and 4.

A taxonomic account describing the correspondence between the 42 OTUs and known taxa is in the Appendix, including a detailed rationale for the addition of eight terminals to the mitogenomic matrix, thus leading to 50 putative species. Out of these, 14 OTUs could not be associated to any known taxon and were labelled as ‘aff.’ relative to their closest taxon in the mitogenomic phylogeny. The geographic distribution of all 50 putative species is illustrated in Electronic Supplement 3. The mean uncorrected p-distances for 16S among the 50 putative species, calculated within eight groups (the genera *Dryaderces* and *Tepuihyla*, and six *Osteocephalus* species groups; Electronic Supplement 1), was >3% for the majority of the comparisons (57%). The rest of comparisons had distances between 1 and 3% (38%), or <1% (5%).

Dated mitogenomic phylogeny

Thirty-nine out of 50 nodes in the Bayesian phylogeny were highly supported (~80%), displaying posterior probabilities (PP) ≥ 0.95 (Fig. 1a). Nodes with PP < 0.9 were mostly bracketed by short branches and occurred between clades/terminals with and without complete mitogenome. The topology and bootstrap support (BS) of the maximum likelihood (ML) phylogeny was overall consistent with the Bayesian phylogeny, except for four low-supported nodes whose positions varied between analyses (Fig. 1a). One of these corresponds to the ‘*O. mimeticus* group’, which is the sister group of the *O. buckleyi* group (in Bayesian), but sister to the *O. leprieurii* group (in ML). A similar discrepancy in the position of this lineage (i.e. *Osteocephalus mimeticus* Melin, 1941) was reported by Blotto et al. (2020). We have considered the clade containing *Osteocephalus mimeticus* and *O. aff. mimeticus* as a new species group (‘*O. mimeticus* group’), and not as part of the *O. buckleyi* group as suggested by Jungfer et al. (2013), because it displays age of divergence similar to the other five traditional species groups (mean ≥ 10 Mya).

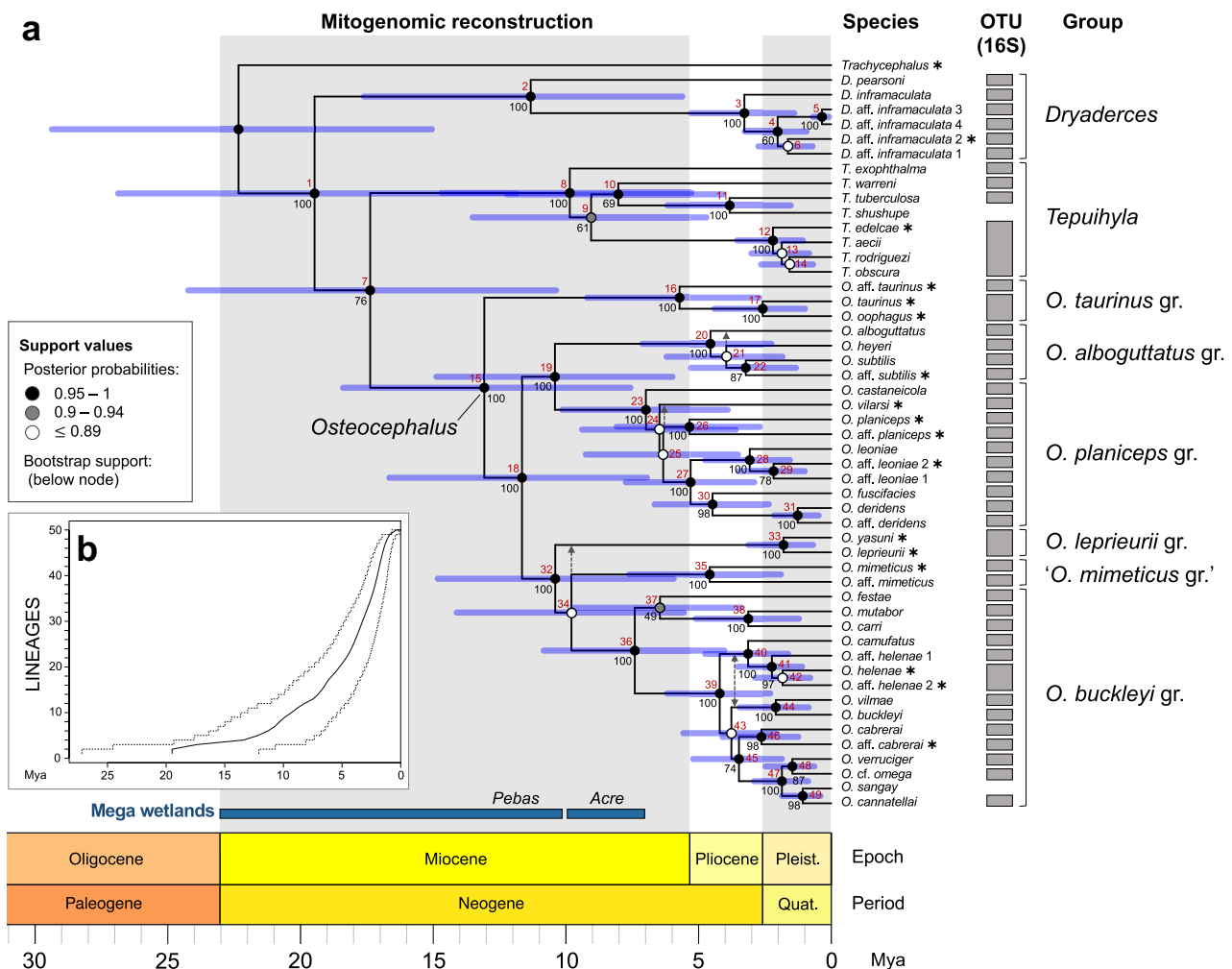


Fig. 1 Panel **a**, time-calibrated phylogeny of *Osteocephalus*, *Tepuihyla* and *Dryaderces* in Amazonia, resulting from a mitogenomic analysis in BEAST and displaying the mean and 95% CI (blue bars) of divergences. Nodes are numbered in red (age details are in Electronic Supplement 1), and support values are depicted for Bayesian (following node symbol colours) and maximum likelihood analyses (following numbers below the nodes). Topological discrepancies between analyses for four low-supported nodes (21, 25, 34 and 43)

are illustrated with grey dashed arrows. Terminals with complete mitogenome (~14,000 bp) are represented with asterisks, including the outgroup *Trachycephalus coriaceus*. Majority rule consensus results of 16S species delimitation are next to the terminals (each box corresponds to an Operational Taxonomic Unit 'OTU'). Terminals are grouped according to genera or species groups (gr.) within *Osteocephalus*. Panel **b**, lineage through time plot for the ingroup depicting mean accumulation of lineages and 95% CI

Our phylogeny (Fig. 1a) is generally consistent with previously proposed topologies (Blotto et al., 2020; Duellman et al., 2016; Jungfer et al., 2013; Kok et al., 2015; Ron et al., 2016; Salerno et al., 2012). However, it differs on three major aspects that could be explained by the inclusion of more mtDNA data: (1) *Osteocephalus* is sister to *Tepuihyla* with high support (PP=0.95; BS=76), instead of *Dryaderces* according to previous studies; (2) most internal nodes within *Tepuihyla* are highly supported, and show that *T. exophthalma* diverged first in the genus and that *Tepuihyla warreni* Duellman & Hoogmoed, 1992 is sister to *Tepuihyla tuberculosa* Boulenger, 1882 + *T. shushupe* (similar to the ML topology of Blotto et al. (2020),

although this inference was considered secondary therein and this relationship was not supported); and (3) internal nodes among *Osteocephalus* species groups are highly supported (except for the '*O. mimeticus* group' position).

Dryaderces is estimated to have diverged from *Tepuihyla* + *Osteocephalus* 19.5 Mya (11.8–26.9); and *Tepuihyla* from *Osteocephalus* 17.4 Mya (10.4–24.2). Crown ages of the three genera are as follows: *Osteocephalus* (13.1 Mya (7.6–18.4)), *Dryaderces* (11.3 Mya (5.6–17.6)) and *Tepuihyla* (9.8 Mya (5.3–14.7)). Age information for all nodes depicted in Fig. 1a is in Electronic Supplement 1. Lineage through time plot shows a constant lineage accumulation (Fig. 1b).

Ancestral area reconstruction

Consistent results of ancestral areas were uncovered across models, with the few exceptions being around recent nodes (see Electronic Supplement 5). The best-fit model was DEC (Fig. 2) (model selection is in Electronic Supplement 1). However, other models supporting alternative states at particular nodes are mentioned when relevant. Without the inclusion of other Lophiohylineae genera, the ancestral area for the entire focal clade remains ambiguous within Amazonia (Fig. 2a).

The ancestors of each genus diverged during the early-middle Miocene, each originating in a distinct Amazonian area (*Osteocephalus*: western Amazonia, *Tepuihyla*: the Guiana Shield and *Dryaderces*: Brazilian Shield; Fig. 2a, b). From these initial splits until ~10 Mya the diversification of each genus took place within each area, except for *Tepuihyla* which also included western Amazonia (Fig. 2a, c). However, other models indicate that the ancestral area of *Tepuihyla* crown age was predominantly the Guiana Shield (Electronic Supplement 5). In contrast, the inferred area for the ancestor of *T. warreni* (*T. tuberculosa* + *T. shushupe*) included consistently western Amazonia and the Guiana Shield in all models (Fig. 2a; Electronic Supplement 5), indicating that this node corresponds to the minimum age of colonization of *Tepuihyla* from the Guiana Shield to western Amazonia (mean 8 Mya).

From ~10 Mya to the present, the diversification of each genus continued mostly within their respective ancestral areas. In *Osteocephalus*, the inferred areas for all six species groups during ~5–10 Mya indicate that they co-occurred in western Amazonia (Fig. 2a, d–e). Posteriorly, the diversification for three out of the six species groups continued essentially within western Amazonia (*O. alboguttatus*, *O. planiceps* and ‘*O. mimeticus*’ groups). In contrast, the *O. buckleyi*, *O. leprieurii* and *O. taurinus* groups dispersed out of western Amazonia relatively recently (last ~6 My), and diversified in the rest of Amazonia (Fig. 2a). Each of these latter three groups currently contain species restricted to one of the three Amazonian areas, and a widespread, pond-breeding species occurring in all areas (Fig. 2a; Electronic Supplement 3). These dispersals out of western Amazonia are seemingly not synchronous among the three groups (Fig. 2a). The nodes associated to all dispersals display some ambiguity on the inferred ancestral areas in DEC model (Fig. 2a) and across models (see comments in Electronic Supplement 1). Combined, the models suggest that the dispersion sequence of *Osteocephalus* was as follows: *O. taurinus* group first, from western Amazonia to the Guiana Shield and subsequently to Brazilian Shield; *O. buckleyi* group second, from western Amazonia to Brazilian Shield and subsequently to Guiana Shield; and the terminal of *Osteocephalus leprieurii* Duméril & Bibron, 1841 third, from western Amazonia to the Guiana Shield or Brazilian Shield equally likely.

Discussion

Species diversity

Our final delimitation ($n = 50$ putative species), together with four missing taxa in our sampling (*O. duellmani*, *O. germani*, *O. melanops* and *T. luteolabris*), indicates that the three genera may harbour 54 species. The 14 unidentified OTUs (candidate species) represent a 35% increase in the current known diversity for the three genera combined (40 currently valid nominal species; Frost, 2022; Ortiz & Ron, 2018). The ratio of potential undocumented diversity in our focal clade (35%) is relatively low compared to what has been found in other widespread Neotropical frog groups using 16S (e.g. Fouquet et al., 2014, 2021a; Gehara et al., 2014; Vacher et al., 2020). The main reason for this lower ratio probably comes from better taxonomic knowledge because this clade has attracted more attention in recent years (Blotto et al., 2020; Chasiluisa et al., 2020; Jungfer et al., 2013; Moura et al., 2021; Ron et al., 2012, 2016).

Although our DNA-based delimitation is mostly based on a single and short fragment of 16S mtDNA, and thus is potentially prone to fail to indicate the existence or absence of species (e.g. Hickerson et al., 2006), most of the initially found 42 OTUs were readily confirmed by their correspondence with current taxonomy (Appendix). However, our results suggest that four of these OTUs included more than one nominal species since they were differentiated by their morphology and advertisement calls. This lack of accuracy is due to the combination of the small size of our locus and the recent divergence among some species (Fig. 1). Our results also corroborate that phenotypically recognised species with low 16S distances (< 3%) are frequent in this group (Jungfer et al., 2013).

Further investigations using more variable markers, including nuclear DNA (nDNA), and phenotypic data are needed to clarify the status of the 14 unidentified OTUs and that of widespread ‘species’ that may represent species complexes (i.e. *Osteocephalus helena* Ruthven, 1919, *O. leprieurii* and *O. taurinus*; see Appendix; Jungfer et al. 2013). Finally, vast under-sampled regions remain in Amazonia and Pantepui regions, notably in Colombia and Venezuela (see Electronic Supplement 3), which may harbour undocumented species and extend the distribution of the known species. Therefore, although this study does not represent a thoroughly systematic review, it provides an exploratory assessment of diversity in this clade that will contribute to more detailed, integrative studies.

Diversification around the Pebas mega wetland

Our results suggest that the three genera diverged, and were isolated from each other, for the period of ~10–20 Mya

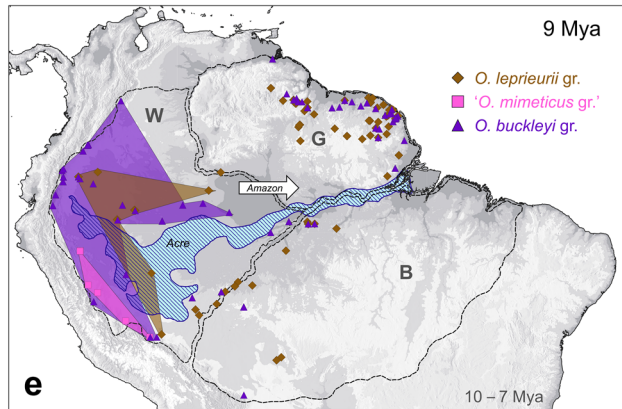
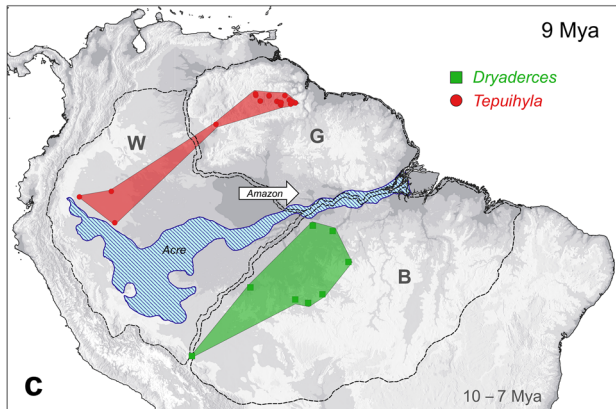
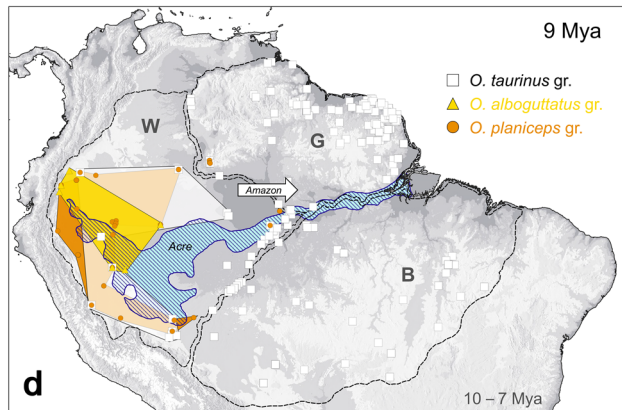
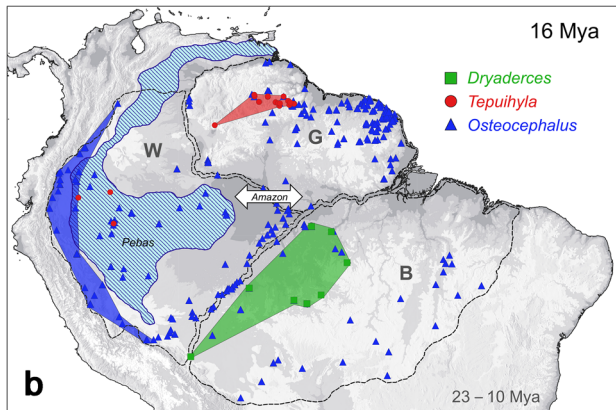
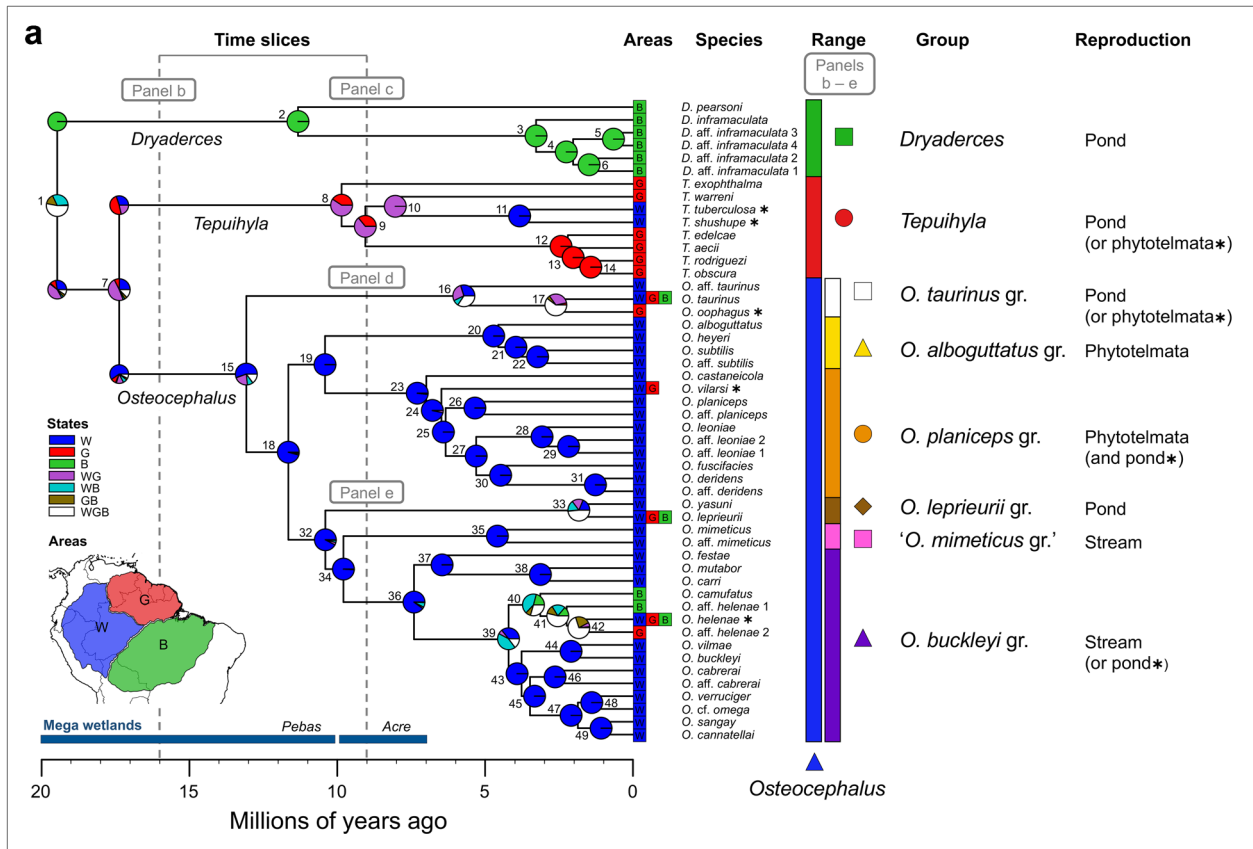


Fig. 2 Panel **a**, ancestral area reconstruction for the diversification of *Osteocephalus*, *Tepuihyla* and *Dryaderces* in Amazonia, resulting from the best-fit model (DEC) in BioGeoBEARS and based on the mitogenomic phylogeny (Fig. 1a) and three areas (western Amazonia 'W', Guiana Shield 'G' and Brazilian Shield 'B'). Species are grouped according to the three genera and six *Osteocephalus* species groups (gr.), with their generalised reproductive mode. Species displaying a reproductive mode being the exception within their groups are depicted with asterisks (*O. vilarsi* apparently displays both modes within its group; Ferrão et al., 2019). Two time slices show the hypothetical, ancestral distribution of the lineages (coloured polygons) at 16 Mya (panel **b**) and 9 Mya (panels **c–e**) according to DEC model and constrained by the extent of the Pebas and Acre mega wetlands. In panels **b–e**, the current distribution of the lineages is also plotted from individual 16S records; the species are combined in genera or species groups (see the distribution of each species in Electronic Supplement 2 and 3). An arrow indicates the flow direction of the Amazon River, and at the right bottom of the continent is the time span for that particular landscape configuration following Hoorn et al. (2010)

(early–middle Miocene). This period coincides with the existence of the Pebas mega wetland system, and the ancestral area of each genus (as well as the bulk of their subsequent diversification) matches the putative distribution of suitable rainforest areas during this period (Fig. 2a, b). Therefore, this major hydrological barrier coincides with the deep spatio-temporal pattern of divergence in our clade. Additional support for the hypothesis that Miocene mega wetlands represented a major barrier preventing dispersals and diversification in western Amazonia comes from the fact that the three genera started to diversify during the last stages of the Pebas system (the period that preceded the onset of the transcontinental Amazon River, triggered by intensified Andean uplift). These early diversifications within genera (~10–13 Mya; Fig. 1a) remained mostly in situ, except for *Tepuihyla* which may have dispersed westwards around this period following the disappearance of the barrier once formed by the northward-drained Pebas (Fig. 2b, c). This timeframe was also relevant for the highest diversification period of rocket frogs (*Allobates*) in western Amazonia (10–14 Mya; Réjaud et al., 2020), suggesting shared patterns of isolation and diversification.

In *Osteocephalus*, the divergence of the ancestors of all six species groups (gr.) was probably driven by the availability of new and diverse rainforest environments along Andean slopes and nearby lowlands following the retraction of mega wetlands in western Amazonia (Hoorn et al., 2010). Also, the concomitant evolution of new reproductive modes (phytotelmata- and stream-breeding) from a probable pond-breeding ancestor (Jungfer et al., 2013), may at least partly explain their rapid diversification and current co-occurrence in western Amazonia (Fig. 2d–e). According to our phylogeny, the evolution of phytotelmata-breeding from pond-breeding happened in at least two instances in *Osteocephalus* (in the ancestor of *O. alboguttatus* gr. + *O. planiceps* gr. 10.4 Mya, and more recently in *O. oophagus* within the *O. taurinus* gr. <2.6 Mya), and once in *Tepuihyla* (in the ancestor of *T. tuberculosa* + *T. shushupe* 3.8 Mya) (Fig. 2a). On the other hand, the evolution

of stream-breeding from pond-breeding appeared once in *Osteocephalus*, in the ancestor of *O. buckleyi* gr. + '*O. mimeticus* gr.' 9.8 Mya (Fig. 2a).

Other frog groups have similarly experienced transitions in reproductive mode during the Miocene in western Amazonia and Andes. *Chiasmocleis antenori* Walker, 1973 (Microhylidae), and presumably its closest relatives, transitioned from the generalised pond-breeding mode of *Chiasmocleis* to phytotelmata-breeding in bromeliads (de Sá et al., 2019; Peloso et al., 2014). The common ancestor of Dendrobatinae (*Ranitomeya* and relatives) transitioned from the generalised stream-breeding mode of dendrobatids to phytotelmata-breeding in bromeliads (Grant et al., 2006; Santos et al., 2009). Other Andean-derivate groups which diversified in the Miocene are strict stream-breeders (with some exceptions within Dendrobatidae) suggesting that their diversification was associated to Andean uplift and consequential availability of streams (e.g. *Atelopus* in Bufonidae (Lötters et al., 2011; Ramírez et al., 2020); Centrolenidae (Castroviejo-Fisher et al., 2014); Dendrobatidae (Santos et al., 2009); *Hyloscirtus* in Hylidae (Coloma et al., 2012; Duellman et al., 2016)). Therefore, we hypothesize that intensified Andean uplift around 10 Mya (and subsequently) might have provided new reproductive habitats that led to ecological divergence in *Osteocephalus* along the Andean slopes and western Amazonia, to utilize varied stream habitats and water-filled cavities of bromeliads (which radiated extensively in the northern Andes; Givnish et al., 2014; Zizka et al., 2020).

Diversification and dispersion throughout the modern Amazon River drainage

The lineage accumulation for the three genera appears to have been constant (Fig. 1b), and most speciation events have occurred in situ with only a few trans-Amazonian expansions. The onset of the modern Amazon River resulted in the Pebas mega wetland being first reduced in north-western Amazonia, compared to the south-western region that remained vastly flooded as the Acre system (7–10 Mya; Hoorn et al., 2010). This large area probably remained unsuitable since it seems to have also prevented dispersals (see below). This suggests that the first connection for non-flying, rainforest organisms between the three major areas would have been established between western Amazonia and the Guiana Shield, while the Brazilian Shield remained more isolated from the rest. This is supported by *Dryaderces* that did not disperse out of the Brazilian Shield. Also, despite *Dryaderces* was the first genus to diverge, most of its speciation occurred relatively recently in the lowlands (last ~3 My), with the exception of the deep divergence of *Dryaderces pearsoni* Gaige, 1929 (~11.3 Mya) which is the only *Dryaderces* species known to occur close to the Andes (Electronic Supplement 3). The relatively low and recent diversification of *Dryaderces* in the Brazilian Shield may

be related to the significant climate instability of the eastern Amazonia during the Pleistocene (e.g. Cheng et al., 2013; Silva et al., 2019; Wang et al., 2017).

Assessing diversification within *Osteocephalus* is particularly biogeographically interesting, given the diversity, distributional patterns and variation in breeding modes in this genus. Our results suggest that the six *Osteocephalus* species groups formed ≥ 10 Mya in western Amazonia. Three of these dispersed and diversified eastwards relatively recently (since ~ 6 Mya; Fig. 2a). These dispersions occurred in one lineage in each of these groups and do not seem to have been synchronous (Fig. 2a). The *O. taurinus* gr. probably dispersed first towards the Guiana Shield, supporting the relatively early connection between western Amazonia and the Guiana Shield (as discussed above for *Tepuihyla*). On the other hand, expansion of *terra firme* rainforest after the Acre system disappeared (Albert et al., 2018) may explain the more recent dispersal of the *O. buckleyi* gr. from western Amazonia towards the Brazilian Shield (compared to that of the *O. taurinus* gr.). Nevertheless, the dispersion and diversification of the *O. buckleyi* gr. were not extensive in the latter area (Electronic Supplement 3).

The *Osteocephalus* groups that contain few and widespread species in the three major Amazonian areas display the ‘ancestral’ pond-breeding mode (*O. lepreurii* within *O. lepreurii* gr., *O. taurinus* within *O. taurinus* gr., and *O. helenae* within the *O. buckleyi* gr.; Fig. 2a) (*O. helenae* may represent a recent transition back to the pond-breeding state because it reproduces in ponds associated to black-water flooded forest (*igapó*) on the margin of rivers). In contrast, the groups that diversified in western Amazonia and the Andean foothills (*O. alboguttatus* gr., *O. planiceps* gr., ‘*O. mimeticus* gr.’ and most of *O. buckleyi* gr.) have high species diversity, species with relatively small distributions (Electronic Supplement 3), and ‘derived’ reproductive modes (i.e. phytotelmata- or stream-breeding; Fig. 2a). This pattern of distribution and reproductive mode suggests that the potential large availability of ponds across Amazonian lowlands might have facilitated range expansions in pond-breeding species, whereas phytotelmata- and stream-breeding habitat may have been relatively more restricted.

Resolving the details of dispersal and speciation events during the Pliocene and Pleistocene (last 5.3 My) is complicated because of spatio-temporal uncertainties over the formation of the large tributaries of the Amazon River (Albert et al., 2018), whether or not these acted as dispersal barriers for these frogs, and the possibility of river captures (Pupim et al., 2019; Ruokolainen et al., 2019). Nevertheless, the high diversification within *Osteocephalus* suggests that climatic stability in western Amazonia and steep environmental gradients along the Andean foothills (including their effects on stream habitats and bromeliad diversity and abundance) probably played a significant role in its diversification (Cheng et al., 2013; Ron et al., 2012).

Conclusions

We assessed the diversity and historical biogeography of a diverse, Amazonia-wide clade of tree frogs. The dense sampling of 16S sequences obtained from this and previous studies, combined with our mitogenomic reconstruction, led to a comprehensive phylogenetic and biogeographic assessment of *Osteocephalus*, *Tepuihyla* and *Dryaderces*. Our results suggest that the genera diverged and diversified in isolation around Miocene mega wetlands through most of the Neogene and Quaternary. Only *Osteocephalus* experienced a spectacular radiation along Andean foothills and western Amazonia including the appearance of new reproductive modes, as well as few relatively recent, trans-Amazonian dispersals in pond-breeding species. Nevertheless, the diversification within genera < 10 Mya needs to be further studied incorporating genomic and phenotypic data. These approaches will be particularly relevant to unveil cryptic diversity in widespread ‘species’. Overall, our study provides a spatio-temporal framework for future research on the evolution and systematics of these tree frogs, and adds evidence to the biogeographic understanding of arguably the world’s most biodiverse region.

Appendix

Taxonomic account

Our mitogenomic phylogeny included 50 putative species as ingroup (Fig. 1a), and a distribution map based on our analysed sequences is illustrated in Electronic Supplement 3. This phylogeny included 36 out of 40 species currently recognised (Frost, 2022; Ortiz & Ron, 2018), with the absence of *Osteocephalus duellmani* Jungfer, 2011; *Osteocephalus germani* Ron et al., 2012 (see comments below on ‘*Osteocephalus helenae*’ species complex); *Osteocephalus melanops* Melo-Sampaio et al., 2021; and *Tepuihyla luteolabris* Ayarzagüena et al., 1993a. From the 40 recognised species, two of them (*O. germani* and *Osteocephalus vilmae* Ron et al., 2012) were considered synonyms of *Osteocephalus helenae* Ruthven, 1919 and *Osteocephalus buckleyi* Boulenger, 1882, respectively, by Jungfer et al. (2013) and currently are not listed in Frost (2022). However, we recognise these binomials as valid following discussion in this account (under *O. buckleyi* group) and justification in Ortiz and Ron (2018). Therefore, thirty-six out of the 50 putative species were associated to an available taxon name, while the remaining 14 (ten in *Osteocephalus* and four in *Dryaderces*) could not be associated to any described species and thus correspond to lineages (candidate species) pending of integrative study. They were labelled using ‘aff.’ relative to their closest related taxon in the mitogenomic phylogeny. Below

we discuss our taxonomic conclusions under each ‘species group’ including the correspondence between described species and the 42 OTUs resulting from the 16S-based species delimitation, and a rationale for the addition of eight terminals to the mitogenomic matrix (thus $n = 50$). The species groups are the genera *Dryaderces* and *Tepuihyla*, and each clade within *Osteocephalus* that have diverged approximately ≥ 10 million years ago ‘Mya’ to other clades (Fig. 1a).

***Dryaderces* Jungfer et al., 2013**

The majority rule consensus delimited six OTUs within *Dryaderces* (Fig. 1a). Two of them unambiguously correspond to described species: *Dryaderces inframaculata* Boulenger, 1882; and *D. pearsoni* Gaige, 1929. One out of two sequences of *D. inframaculata* corresponds to its first molecular record (Ortiz et al., 2020). Both sequences of *D. pearsoni* are from nearby their type locality. The four undescribed OTUs are phenotypically similar to *D. pearsoni*, but genetically closer to *D. inframaculata* (thus labelled as *D. aff. inframaculata* 1–4 (‘morph *pearsoni*’)). Three of them (*D. aff. inframaculata* 1, 3 and 4) were previously considered as a single undescribed species, labelled as ‘*D. pearsoni* [Ca1] CCS’ by Jungfer et al. (2013).

***Tepuihyla* Ayarzagüena et al., 1993b**

The majority rule consensus delimited four OTUs within *Tepuihyla* (Fig. 1a). Three of them unambiguously correspond to described species with sequences from their type locality or nearby sites, and/or from their known distribution: *Tepuihyla exophthalma* Smith & Noonan, 2001; *T. tuberculosa* Boulenger, 1882; and *T. warreni* Duellman & Hoogmoed, 1992. The fourth OTU is formed by sequences from specimens assigned to four recognised species (*T. aecii* Ayarzagüena et al., 1993a; *T. edelcae* Ayarzagüena et al., 1993a; *T. obscura* Kok et al., 2015; and *T. rodriguezi* Rivero, 1968). Given these species are phenotypically distinct (see ‘Definition and diagnosis’ section in Kok et al. (2015)), the low divergence among them in 16S led to not detect them as different in the majority rule consensus. Therefore, a terminal of each species was included in the mitogenomic matrix.

Finally, a terminal for *T. shushupe* Ron et al., 2016 (holotype) was also added to the mitogenomic matrix given it did not have 16S data and thus was not included in the delimitation analyses. Although *T. shushupe* morphologically resembles its sister species, *T. tuberculosa*, they differ in molecular data, iris colouration and advertisement call (Ron et al., 2016). *Tepuihyla luteolabris* Ayarzagüena et al., 1993a was absent in our mitogenomic phylogeny and no genetic sequences are known for this species to infer its relationships.

***Osteocephalus* Steindachner, 1862**

The delimitation will be presented following the traditional five *Osteocephalus* species groups of Jungfer et al. (2013), plus an additional species group here labelled as ‘*Osteocephalus mimeticus* group’. The species in this latter group were previously considered as part of the *O. buckleyi* group by Jungfer et al. (2013), but we recognise it as an additional group because it displays an age of divergence similar to the other five species groups (mean ≥ 10 Mya).

***Osteocephalus alboguttatus* group**

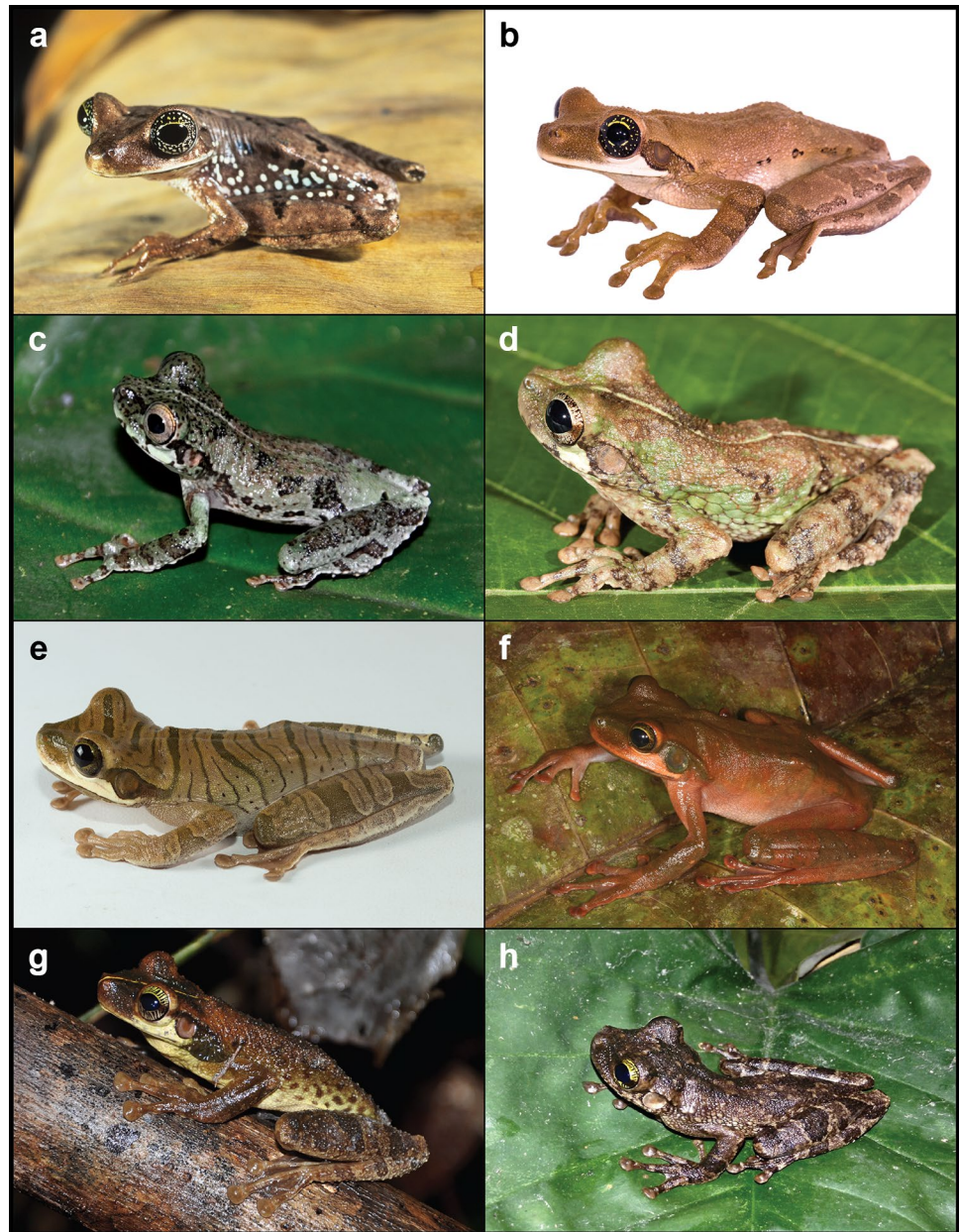
The majority rule consensus delimited four OTUs within the *Osteocephalus alboguttatus* group (Fig. 1a). Three of them unambiguously correspond to described species with sequences from their type locality: *Osteocephalus alboguttatus* Boulenger, 1882 (Fig. 3a in the Appendix); *O. heyeri* Lynch, 2002; and *O. subtilis* Martins & Cardoso, 1987. The sequences of the fourth OTU (*O. aff. subtilis*) are from Serra do Divisor (border between Brazil and Peru), and form a highly divergent sister group, geographically close, to *O. subtilis sensu stricto* (Fig. 1a; Electronic Supplement 3). *Osteocephalus melanops* Melo-Sampaio et al., 2021 was recently described and thus it could not be included in our analyses. However, it is phenotypically distinct from the other species in this group and displays a sister relationship with *O. alboguttatus* (Melo-Sampaio et al., 2021).

***Osteocephalus buckleyi* group**

The majority rule consensus delimited 13 OTUs within the *Osteocephalus buckleyi* group (Fig. 1a). Ten of them unambiguously correspond to described species, with sequences from their type locality or nearby sites, and/or from their known distribution: *Osteocephalus buckleyi* Boulenger, 1882 (Fig. 3c in the Appendix); *O. cabrerai* Cochran & Goin, 1970 (Fig. 4a in the Appendix); *O. camufatus* Jungfer et al., 2016 (holotype); *O. cannatellai* Ron et al., 2012; *O. carri* Cochran & Goin, 1970; *O. festae* Peracca, 1904; *O. helenae* Ruthven, 1919 (Fig. 4b–d in the Appendix); *O. mutabor* Jungfer & Hödl, 2002 (Fig. 3e–f in the Appendix); *O. verruciger* Werner, 1901; and *O. vilmae* Ron et al., 2012 (Fig. 3d in the Appendix). The remaining three OTUs (*O. aff. cabrerai*, *O. aff. helenae* 1 and *O. aff. verruciger*) are discussed below.

Osteocephalus vilmae was synonymised under *O. buckleyi* by Jungfer et al. (2013). However, we associate the OTU containing three ‘*O. buckleyi*’ samples to the name *O. vilmae* in this study because these samples cluster with high support with other *O. vilmae* specimens (including the holotype) in previous phylogenies that include both mitochondrial

Fig. 3 Dorsolateral views of *Osteocephalus*. **a** *O. alboguttatus* sensu stricto, QCAZ15972, Puyo-Canelos Road, Pastaza, Ecuador. **b** *O. mimeticus* sensu stricto, AF4442 (sequenced), San José, San Martín, Peru. **c** *O. buckleyi* sensu stricto, Canelos, Pastaza, Ecuador. **d** *O. vilmae*, QCAZ51205, Pompeya-Iro Road km 80, Orellana, Ecuador. **e** *O. mutabor*, QCAZ56066, Lorocachi, Pastaza, Ecuador. **f** *O. mutabor*, QCAZ39588, Río Pucayacu, Pastaza, Ecuador. **g** *O. taurinus* sensu stricto, Reserva Ducke, Amazonas, Brazil. **h** *O. oophagus* sensu stricto, Reserva Ducke, Amazonas, Brazil. Photographs: **a**, **d–f** by Santiago Ron (BIOWEB, Licence: CC BY-NC-ND 4.0); **b** by Antoine Fouquet; **c**, **g–h** by Diego Ortiz



(mtDNA) and nuclear (nDNA) data; and this clade is sister to *O. buckleyi* sensu stricto with high support (Chasiluisa et al., 2020; Jungfer et al., 2013). Further justification for the validity of *O. vilmae* is presented in Ortiz and Ron (2018).

The single sequence of *O. aff. cabrerai* (from Iça River, Amazonas, Brazil) is sister and geographically close to *O. cabrerai* sensu stricto (Fig. 1a; Electronic Supplement 3). The long branches separating them suggest that each represents a distinct species but morphological and bioacoustics data remain to be examined.

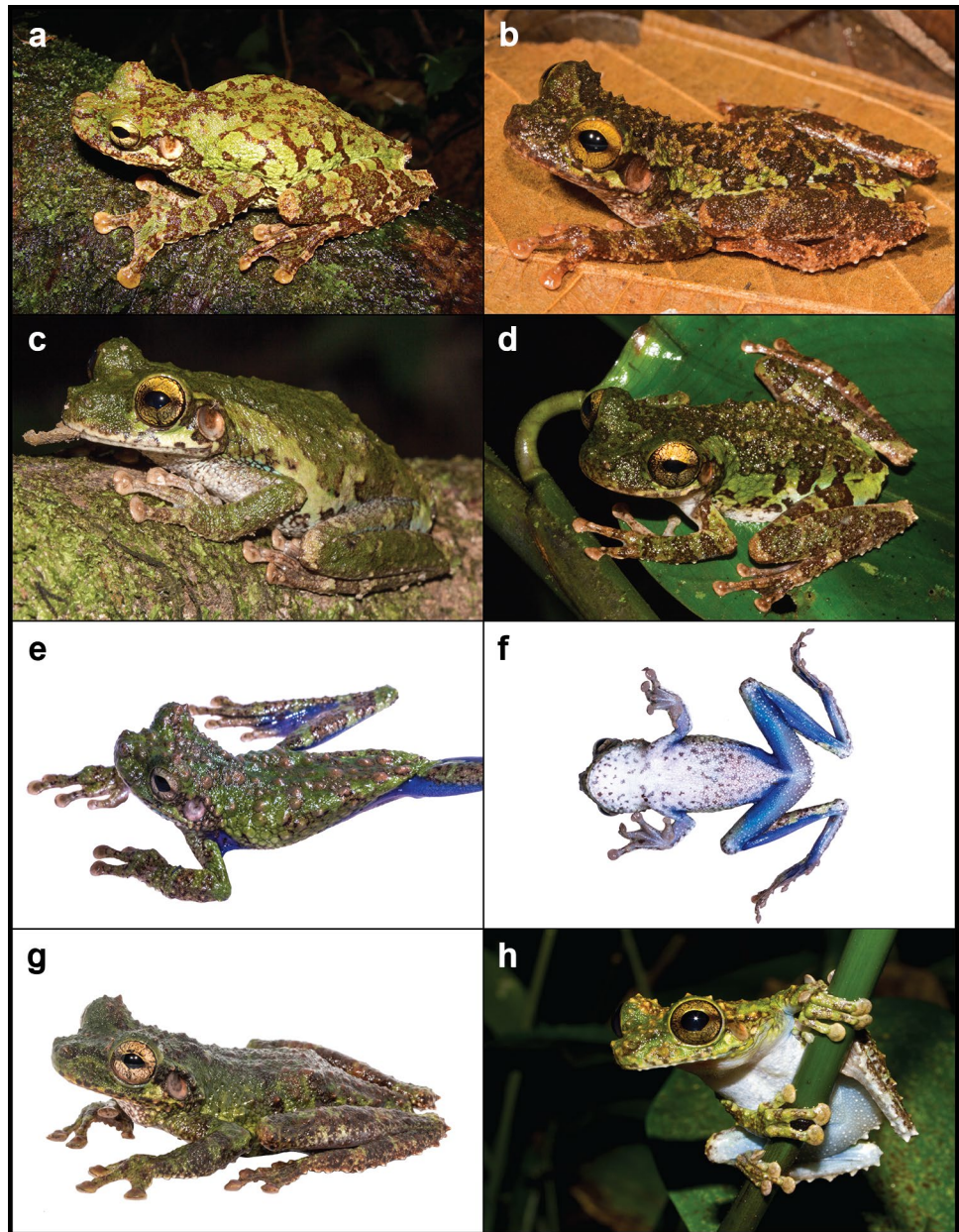
The single sequence of *O. aff. helenae* 1 is from Mataracú, Santa Cruz, Bolivia. It corresponds to the southernmost record of the *O. buckleyi* group (Electronic Supplement 3). *Osteocephalus aff. helenae* 1 is sister to *O. helenae* + *O.*

aff. helenae 2 (Fig. 1a; see comments below on ‘*O. helenae*’ species complex).

The sequences of *O. aff. verruciger* are from the Caquetá Department, Colombia, lying geographically close to the type locality of a recently described species, previously considered part of *O. verruciger* (i.e. *O. omega* Duellman, 2019). Therefore, we tentatively labelled this OTU as *O. cf. omega* (Fig. 1a). However, it is still necessary to determine whether they are conspecific.

Finally, a terminal for *O. sangay* Chasiluisa et al., 2020 (holotype) was added to the mitogenomic matrix because this species did not have 16S data and thus was not included in the delimitation analyses. *Osteocephalus sangay* is genetically and morphologically distinct from its sister species, *O.*

Fig. 4 Dorsolateral and ventral views of *Osteocephalus*. **a** *O. cabrerai*, CORBIDI120, Tarampoa, Sucumbíos, Ecuador. **b** *O. helenae*, AF2427 (sequenced), Nassau, Suriname. **c** *O. helenae*, Amapá, Brazil. **d** *O. helenae*, Voltaire, French Guiana. **e–f** *O. aff. helenae* 2 ‘morph *cabrerai*’, AF2357 (sequenced), Mapaou, French Guiana. **g** *O. aff. helenae* 2 ‘morph *cabrerai*’, AF3358 (sequenced), Bakhuis, Surinam. **h** *O. aff. helenae* 2 ‘morph *cabrerai*’, St. Georges, French Guiana. Photographs: **a** by Pablo Venegas (BIOWEB, Licence: CC BY-NC-ND 4.0); **b–h** by Antoine Fouquet



cannatellai (Chasiluisa et al., 2020). *Osteocephalus duellmani* Jungfer, 2011 was absent in our mitogenomic phylogeny and no DNA sequences are known for this species to infer its relationships. It has been tentatively allocated within the *O. buckleyi* group given its morphological affinities (Jungfer, 2011). Nevertheless, *O. duellmani* may be a junior synonym of *O. festae* Peracca, 1904 (Ortiz & Ron, 2020).

Comments on the ‘*Osteocephalus helenae*’ complex (within *O. buckleyi* group)

The delimited OTU corresponding to ‘*Osteocephalus helenae*’ Ruthven, 1919 sensu Jungfer et al. (2013) is widespread across Amazonia (Electronic Supplement 3) and represents

a species complex on its own. The sequences associated to the name ‘*O. helenae*’ consist of seven geographically structured clades which do not form a monophyletic group in our 16S tree given the positions of *O. aff. helenae* 1 and *O. aff. helenae* 2 (‘morph *cabrerai*’) (see below) (Electronic Supplement 4). *Osteocephalus* aff. *helenae* 1 was delimited as an independent OTU, whereas *O. aff. helenae* 2 (‘morph *cabrerai*’) clustered in the same OTU with ‘*O. helenae*’ in the majority rule consensus (Fig. 1a).

In the Guiana Shield, this latter OTU is formed by sequences that belong to two co-occurring distinct species, thus the low divergence among them in 16S led to not detecting them as different. One of those (sp. 1) morphologically resembles *O. buckleyi* Boulenger, 1882 and has been associated to the name

O. helenae Ruthven, 1919 even though the justification remains meagre (Jungfer et al., 2013). The holotype of ‘*Hyla helenae*’ (UMMZ52681) is a recently metamorphosed juvenile from Dunoon, Demerara River, Guyana. It apparently shows the typical characters of juveniles of most species of the *O. buckleyi* group according to Jungfer et al. (2013), who based on that, transferred the name ‘*Hyla helenae*’ to *Osteocephalus* under the *O. buckleyi* group. These authors assumed that there is only one species of the *O. buckleyi* group occurring in the Guiana Shield and therefore concluded to name all the samples within this clade under ‘*O. helenae*’, including previous reports of *O. buckleyi* from this region (e.g. Gorzula & Señaris, 1998; Kok & Kalamandeen, 2008) and other regions.

The other species from the Guiana Shield (sp. 2) was previously assigned to *O. cabrerai* Cochran & Goin, 1970 based on its morphological characteristics (Dewynter et al., 2016). However, the samples of ‘*O. cabrerai*’ from the Guiana Shield are not closely related to *O. cabrerai* sensu stricto from western Amazonia using mitochondrial DNA (mtDNA) (Electronic Supplement 4), thus representing another lineage closely related to ‘*O. helenae*’ instead. Therefore, we labelled sp. 2 as *O. aff. helenae* 2 (‘morph *cabrerai*’) and included an additional terminal for it in the mitogenomic matrix (Fig. 1a).

Both species, *O. helenae* sensu Jungfer et al. (2013) (Fig. 4b–d in the Appendix) and *O. aff. helenae* 2 ‘morph *cabrerai*’ (Fig. 4e–h in the Appendix) show a low-supported, sister relationship when analysing their complete mitogenomes (Fig. 1a), and differ in morphology and ecology. *Osteocephalus aff. helenae* 2 is larger than *O. helenae*; it has large and fleshy tubercles on all dorsal surfaces, including tarsus and lower jaw (tubercles smaller and more restricted to dorsum in *O. helenae*), and hidden surfaces are deep blue in *O. aff. helenae* 2 (tan or bluish grey in *O. helenae*) (Dewynter et al., 2016). In sympatric areas, both are allopatric species: *O. aff. helenae* 2 is found in rocky, fast, clear water streams, while *O. helenae* in ponds and flooded forests (*igapó*) around black water rivers; their calls also differ (A. Fouquet, pers. obs.). Finally, *O. aff. helenae* 2 may actually be circumscribed to the eastern Guiana Shield (Electronic Supplement 3) and its occurrence in northern Guyana still need to be confirmed but is unlikely. Moreover, the habitat found in Dunoon (flat, flooded riparian forest) matches the habitat of sp. 1 ‘*O. helenae*’ but not the one of sp. 2 ‘*O. aff. helenae* 2’ (rocky streams). Therefore, we consider that the assignation of Jungfer et al. (2013) for ‘*O. helenae*’ is correct. However, to completely solve this issue would require the sampling of adult specimens and the analysis of sequences from the type locality of *O. helenae*.

Finally, closely related samples from other Amazonian regions were also considered part of ‘*O. helenae*’ by Jungfer et al. (2013), including the synonymy of *O. germani* Ron et al., 2012. *Osteocephalus germani* was described from the Cusco Department, Andean slopes of Peru. Unfortunately,

there were no 16S sequences available for the type material of *O. germani* to include it in our species delimitation and is absent in our mitogenomic phylogeny. However, a recent, dense-sampled phylogeny with a focus on the *O. buckleyi* group (Chasiluisa et al., 2020) showed that ‘*O. germani*’ individuals clustered in a clade that contain samples of *O. aff. helenae* 2 ‘morph *cabrerai*’, suggesting that both species may be closely related (in mtDNA) within the ‘*O. helenae*’ complex. *Osteocephalus germani* differs from *O. aff. helenae* 2 in morphology (Dewynter et al., 2016; Ron et al., 2012), and it is distributed in an opposite extreme of Amazonia (Andean slopes in south-western Amazonia) compared to *O. helenae* and *O. aff. helenae* 2 (eastern Guiana Shield) (Electronic Supplement 3), thus making unlikely that these three species are conspecific. Because ‘*O. helenae*’ is a species complex and this issue was unknown by Jungfer et al. (2013), we consider that their synonymy of *O. germani* under *O. helenae* was premature (see Ortiz & Ron, 2018). A thorough review of the ‘*O. helenae*’ complex is opportune including genomic nDNA and bioacoustics.

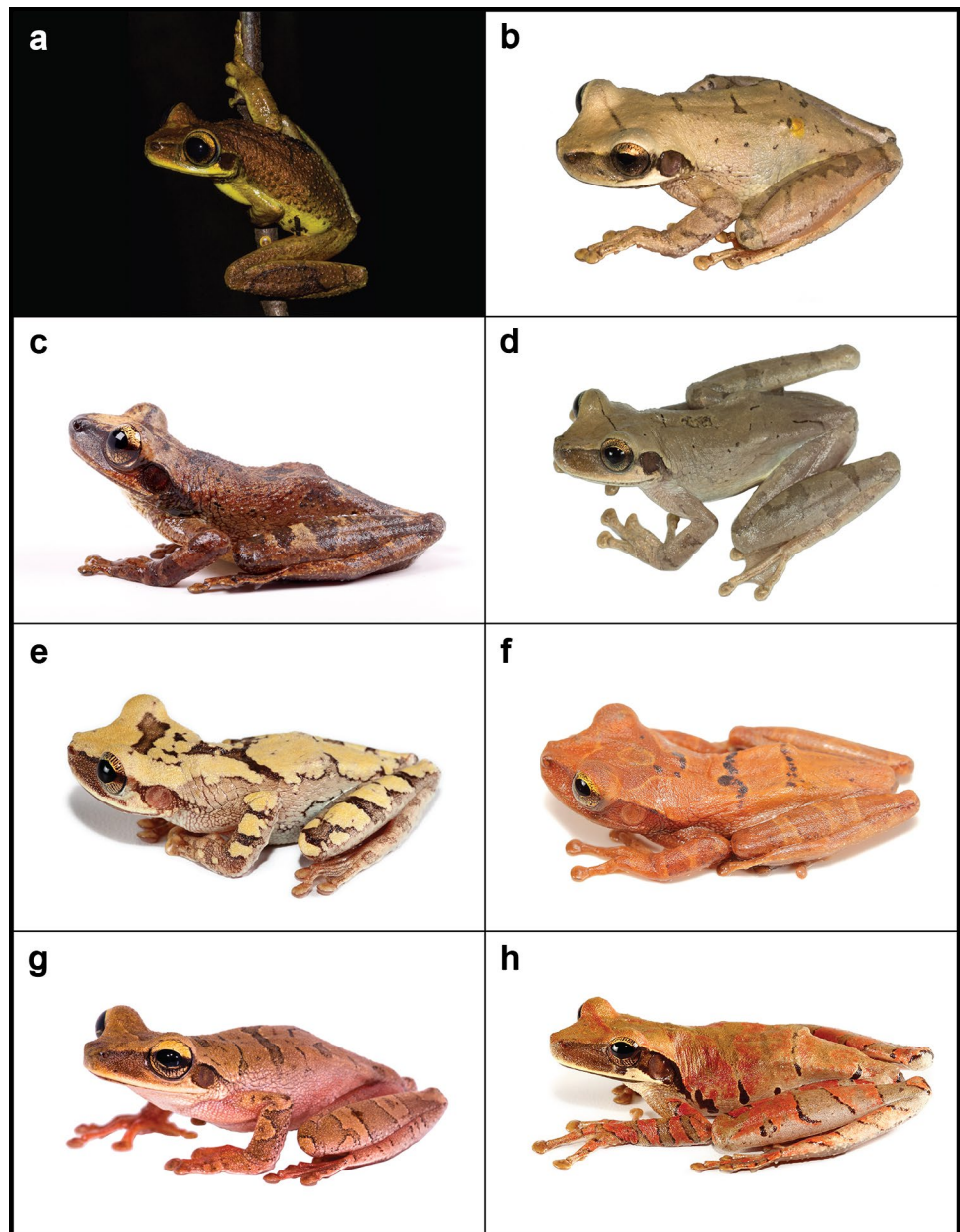
***Osteocephalus leprieurii* group**

The majority rule consensus delimited one OTU for the entire *Osteocephalus leprieurii* group (Fig. 1a). Two species have been formally described in this group: *Osteocephalus leprieurii* Duméril & Bibron, 1841 (Fig. 5a–b in the Appendix) from the Guiana Shield; and *O. yasuni* Ron & Pramuk, 1999 (Fig. 5c–d in the Appendix) from western Amazonia. Given these two species are phenotypically distinct (see ‘Diagnosis’ section in Ron and Pramuk (1999)), the low divergence in 16S led to not detecting them as different in the majority rule consensus. Therefore, we included a terminal for each in the mitogenomic matrix from nearby sites to their type localities. Although with low support, three sequences clustering within the *O. yasuni* clade from Boa Vista, south bank of upper Negro River (MTR41294), and from the south bank of lower Japura River (MTR33656, 33683), Amazonas, Brazil, correspond to its first molecular records in Central Amazonia (Electronic Supplements 3 and 4). Two additional lineages phenotypically similar to *O. leprieurii* have been reported and labelled as ‘*O. leprieurii* [Ca1–2] UCS’ by Jungfer et al. (2013), but we did not include additional terminals in the mitogenomic matrix for them because of their current lack of morphological and bioacoustics information. We considered them here part of *O. leprieurii*; however, further systematic study and more variable markers are needed to delimit species and population structure within this widespread taxon.

‘*Osteocephalus mimeticus* group’

The majority rule consensus delimited two OTUs within the ‘*Osteocephalus mimeticus* group’ (Fig. 1a). One of them

Fig. 5 Dorsolateral views of *Osteocephalus*. **a** *O. lepreurii* sensu stricto, Kaw mountain, French Guiana. **b** *O. lepreurii*, AF1824, Mana, French Guiana. **c** *O. yasuni* sensu stricto, QCAZ69010, Estación Científica Yasuní, Orellana, Ecuador. **d** *O. yasuni*, QCAZ55994, Lorocachi, Pastaza, Ecuador. **e** *O. deridens*, QCAZ56031, Lorocachi, Pastaza, Ecuador. **f** *O. fuscifacies*, QCAZ59887, Parque Nacional Llanganates, Pastaza, Ecuador. **g** *O. aff. leoniae* 2, AF4513 (sequenced), San Martín, Peru. **h** *O. planiceps*, QCAZ55881, Lorocachi, Pastaza, Ecuador. Photographs: **a–b**, **g** by Antoine Fouquet; **c–f**, **h** by Santiago Ron (BIOWEB, Licence: CC BY-NC-ND 4.0)



unambiguously corresponds to *Osteocephalus mimeticus* Melin, 1941 (Fig. 3b in the Appendix) containing a sequence from its type locality. The sequences of the other OTU (*O. aff. mimeticus*) are from the Cusco department, in Peru, and form a highly divergent sister group to *O. mimeticus* sensu stricto (Fig. 1a); they substitute each other latitudinally (Electronic Supplement 3).

***Osteocephalus planiceps* group**

The majority rule consensus delimited ten OTUs within the *Osteocephalus planiceps* group (Fig. 1a). Six of them unambiguously correspond to described taxa with sequences from

their type locality or nearby sites, and/or from their known distribution: *Osteocephalus castaneicola* Moravec et al., 2009 (holotype); *O. deridens* Jungfer et al., 2000 (Fig. 5e in the Appendix); *O. fuscifacies* Jungfer et al., 2000 (Fig. 5f in the Appendix); *O. leoniae* Jungfer & Lehr, 2001; *O. planiceps* Cope, 1874 (Fig. 5h in the Appendix); and *O. vilarsii* Melin, 1941. The sample of *O. castaneicola* (MTR18640) from the east bank of lower Purus River, Amazonas, Brazil, corresponds to its first molecular record in Central Amazonia (Electronic Supplement 3 and 4). The remaining four undescribed OTUs could not be assigned to any known taxa (*O. aff. deridens*, *O. aff. leoniae* 1, *O. aff. leoniae* 2 and *O. aff. planiceps*).

The sequences of *O. aff. deridens* are from nearby Iquitos, Loreto, Peru, and form a clade which is sister to *Osteocephalus deridens* sensu stricto (Fig. 1a).

Osteocephalus aff. leoniae 1 and *O. aff. leoniae* 2 (Fig. 5g in the Appendix) are sisters to each other, and both are sisters to *O. leoniae* sensu stricto (Fig. 1a). They are distributed allopatrically (Electronic Supplement 3).

The sequences of *O. aff. planiceps* are from Serra do Divisor (border between Brazil and Peru), and form a highly divergent sister group, geographically close, to *Osteocephalus planiceps* sensu stricto (Fig. 1a; Electronic Supplement 3).

***Osteocephalus taurinus* group**

The majority rule consensus delimited two OTUs within the *Osteocephalus taurinus* group (Fig. 1a). The first OTU (labelled here as *Osteocephalus aff. taurinus*), containing two sequences from Amazonian Peru in western Amazonia, corresponds to an early diverging lineage within this group; it was previously labelled as *O. taurinus* [Ca1] CCS by Jungfer et al. (2013). The second OTU contains all the rest of sequences of *O. taurinus* Steindachner, 1862 (Fig. 3g in the Appendix) and *O. oophagus* Jungfer & Schiesari, 1995 (Fig. 3h in the Appendix) through Amazonia. Since both species differ in morphology and reproduction, this case represents another example of failure in detecting them as different given the low divergence in 16S. *Osteocephalus taurinus* is considerably larger than *O. oophagus*, *O. taurinus* males have paired, lateral vocal sacs (single and subgular in *O. oophagus* males), dorsal tubercles with queratinized tips are abundant and prominent on dorsal surfaces in *O. taurinus* males and those may be also present but less noticeable in females (very few tubercles and barely discernible in *O. oophagus* males). *Osteocephalus taurinus* is an explosive pond-breeder with large clutch sizes of ~2000 eggs and parental care absent (*O. oophagus* has a small clutch of ~30 eggs which is laid within small water cavities of trees and leaf axils of terrestrial/epiphytic plants; water cavities are used by one pair at a time which also display parental care) (Jungfer & Weygoldt, 1999; Lima et al., 2006). These differences led us to include one terminal for each of these two species from their type locality in the mitogenomic matrix (Fig. 1a). The *O. taurinus* group contains several geographically structured mtDNA lineages (Electronic Supplement 4), and four of them have been suggested to correspond to candidate species ('*O. taurinus* [Ca2–5] UCS'; Jungfer et al., 2013). However, we did not include additional terminals in the mitogenomic matrix for them because of their current lack of morphological and bioacoustics information. We considered them here part of *O. taurinus*; however, further systematic study and more variable markers are needed to delimit species and population structure within this widespread taxon.

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Author contribution DAO, CJH and AF designed the study. DAO, FPW and AF acquired samples and funding. DAO, SM and AF generated DNA sequences. DAO organized data and conducted analyses with contributions of CJH, AR and AF. DAO led the writing and preparation of figures with contributions from CJH, FPW, SRR and AF. All authors commented on the final draft.

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Data availability Information associated to all analysed DNA sequences is available in Electronic Supplement 2. Newly generated DNA sequences were deposited in GenBank under accessions: ON716130–65 (16S rRNA), and OP425862, OP572168–92 (mitogenomes). Final alignments are available in TreeBASE (<http://purl.org/phylo/treebase/phyloWS/study/TB2:S29696>).

Code availability Not applicable.

Declarations

Ethics approval Fieldwork and laboratory experiments have been conducted in accordance with relevant national legislation on the use of animals for research.

Consent to participate Not applicable.

Consent for publication Not applicable.

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