ORIGINAL ARTICLE

Reconquering the water: Evolution and systematics of South and Central American aquatic lizards (Gymnophthalmidae)

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The Cercosaurini tribe stands out from other Gymnophthalmidae lizards for including several species with morphological adaptations to aquatic lifestyle ("Crocodile-Like Morphology" – CLM). Recent molecular phylogenies of Cercosaurini demonstrated the paraphyly of CLM species, implicitly suggesting that adaptations to the aquatic life evolved more than once. However, CLM species have remained poorly sampled, and a number of uncertainties persist, such as the monophyly of *Neusticurus* and the placement of *Potamites apodemus* within the tribe. Based on a more extensive molecular and morphological data set, we propose a phylogenetic hypothesis for *Neusticurus* and investigate, for the first time, the phylogenetic position of *P. apodemus*. We recovered a monophyletic *Neusticurus* clade; however, *Neusticurus rudis* as currently understood was recovered as paraphyletic, with two lineages that also show consistent morphological diagnosis; as a result, we resurrect and provide a taxonomic redescription of *Neusticurus surinamensis* Müller, 1923. Moreover, because *P. apodemus* was recovered as sister to all other *Echinosaura*, we propose a new combination for this species (*Echinosaura apodema*). We also review the distribution of *Neusticurus* species, offering a comprehensive view of their biogeography. Lastly, our ancestral character reconstruction and dating analyses indicate that the CLM phenotype evolved four times independently during Cercosaurini's evolutionary history. We hypothesize that the CLM phenotype and subsequent adaptation to aquatic life may be linked to the development of the Pebas Lake in western Amazonia during the Miocene.

1 | INTRODUCTION

The Gymnophthalmidae family is one of the most diverse clades of Neotropical lizards, including 47 genera and 237 described species (Goicoechea et al., 2016; Uetz, 2017). By far, the most diverse of the seven tribes within this family is Cercosaurini, which includes 57% (136) of all gymnophthalmids (Uetz, 2017). Species included in that

tribe occur mostly in central and northern regions of the Andes and in Amazonia, with only a few of them reaching Central America or Brazil's coastal Atlantic Forest (Torres-Carvajal et al., 2016). Among gymnophthalmids, only within Cercosaurini, some species have evolved a striking "Crocodile-Like Morphology" (CLM) (Figures S2, S4–S7), which is found in the genera *Neusticurus*, *Gelanesaurus*, *Potamites* and *Echinosaura* (Uzzell, 1965, 1966; Doan & Castoe, 2005; Torres-Carvajal et al., 2016). This phenotype is characterized by an array of morphological adaptations to aquatic or semi-aquatic life, such as a heterogeneous dorsal scalation (e.g., presence of enlarged dorsal tubercles; although absent in some *Neusticurus* species), lateral tail compression (absent or slight in *Gelanesaurus* and *Echinosaura*), double caudal crest and a streamlined body (Uzzell, 1966). According to a recent phylogenetic analysis, aquatic genera such as *Neusticurus* and *Echinosaura* have split early from the other Cercosaurini, including non-aquatic forms (Torres-Carvajal et al., 2016). The CLM genera *Neusticurus*, *Gelanesaurus*, *Potamites* and *Echinosaura* therefore do not seem to form a clade (Pellegrino, Rodrigues, Yonenaga-Yassuda, & Sites, 2001; Castoe, Doan, & Parkinson, 2004; Doan & Castoe, 2005; Pyron, Burbrink, & Wiens, 2013; Torres-Carvajal et al., 2016). This pattern either suggests that CLM is a plesiomorphic character that appeared early in the evolutionary history of the tribe and was maintained or reversed in some lineages, or that CLM has evolved multiple times independently from non-CLM ancestors within the Cercosaurini.

An obstacle to tackle this question is that the taxonomy of lizards with CLM has been historically problematic. Previous classifications allocate CLM species in *Neusticurus* or *Echinosaura* based on morphological characteristics (Uzzell, 1965, 1966). Later, molecular evidence showed that some *Neusticurus* species were misallocated, and new, non-related genera were proposed, namely *Potamites* and *Gelanesaurus* (Doan & Castoe, 2005; Torres-Carvajal et al., 2016). Until now, only populations of two of five presently recognized species (*Neusticurus tatei*, *Neusticurus rudis*, *Neusticurus bicarinatus*, *Neusticurus racenisi* and *Neusticurus medemi*) have been included in phylogenetic analyses (Pellegrino et al., 2001; Castoe et al., 2004; Doan & Castoe, 2005; Kok et al., 2012; Pyron et al., 2013; Torres-Carvajal et al., 2016), making it difficult to assess the genus monophyly. Besides *Neusticurus*, other CLM species could be currently misallocated. This may be the case of the enigmatic Central American (Costa Rica and Panama: Lotzkat, Batista, Vargas, Hertz, & Köhler, 2012) *Potamites apodemus*, given that other congeners occur in cis-Andean South America (Chávez & Catenazzi, 2014). Originally, *P. apodemus* was described as *Neusticurus* (Uzzell, 1966) and further reallocated to *Potamites* by Castoe et al. (2004) without a detailed phylogenetic analysis.

There is also a number of issues regarding the alpha taxonomy of Cercosaurini. A good example is that of *N. rudis*. Uzzell (1966) already pointed out that there is considerable morphological variation in this species across its range, particularly in the state of the frontonasal scale (single or divided), presence of an azygous scale between the frontonasal and prefrontals, the number of chinshields in contact with the midline, size of scales on the precloacal shield, and state of femoral and precloacal pores. Despite these observations, Uzzell (1966) placed *Neusticurus dejongi* Brongersma (1927) and *Neusticurus surinamensis* Müller (1923) as junior synonyms of *N. rudis*, without examining neither the holotype of *N. rudis* nor that of *N. surinamensis*. Donnelly and Myers (1991) collected 11 specimens of *N. rudis* on the summit of Cerro Guaiquinima, a tepui (sandstone table mountain) in Venezuela, and noted that their squamation differed from the description of the holotype provided by Uzzell (1966) and Hoogmoed (1973). Avila-Pires (1995) also detected morphological variation in *N. rudis* and acknowledged that samples from Brazil, French Guiana and Suriname differ in several ways from the holotype of *N. rudis* from the base of Mount Roraima in Guyana. The phylogenetic tree provided by Kok et al. (2012, in their supplemental information) implied that the name *N. rudis* involves more than one species. It is worth to mention that two in-depth studies of *N. rudis* (Uzzell, 1966; Avila-Pires, 1995) differ greatly in the material examined. Specimens examined by Uzzell (1966) were from the the western portion of the distribution of *N. rudis* (22 specimens studied, 18 being from Venezuela, three from Guyana and one from Suriname), while samples examined by Avila-Pires (1995) were mainly collected in the eastern portion of the species' range (32 specimens mostly from Brazil, Suriname and French Guiana), including the holotype from Guyana.

In this study, based on new specimens and tissue samples of Cercosaurini species with a CLM phenotype, we examine (i) the phylogenetic relationships among all but one nominal *Neusticurus* species, testing the genus monophyly, (ii) the taxonomic status of *N. rudis* and *P. apodemus* as recognized today, and (iii) the evolution of CLM in Cercosaurini. We also offer an updated account on the morphological variation, biogeography and geographical distribution of *Neusticurus* species, providing the third known record of *N. medemi*, thus extending its range to Brazil.

2 | MATERIAL AND METHODS

2.1 | Delimitation of the geographical distribution of *Neusticurus* species

We compiled an extensive database about the distribution of *Neusticurus* species using several sources. Part of the material used was collected by us (MTR, SMS, RSR, MTJ, JC, AF, IP, FDV, JDL, MAS, CBA, PJRK) or by colleagues; in those

cases, geographical coordinates were obtained in the field using a GPS. We also used museum, literature and GBIF data (www.gbif.org), and we estimated geographical coordinates (when they were not available) from distinct sources, such as Google Earth and occurrences of other taxa from these same sites. In total, our database consists of 125 localities. Locality records were divided into four categories: verified by morphological and molecular data, only by molecular data, only by morphological data, and not verified (Figure S1).

2.2 | Molecular protocols

DNA was extracted from tissue samples (muscle or liver) and preserved in 100% ethanol. We sequenced four loci, three mitochondrial and one nuclear, using Sanger sequencing. From the mitochondrial genome, we sequenced a 761-base pair (bp) fragment of the *NADH dehydrogenase subunit 4* gene (ND4), a 332-bp fragment of the small subunit of *ribosomal RNA 12S* gene (12S) and a 456-bp fragment of the small subunit of *ribosomal RNA 16S* gene. From the nuclear genome, we sequenced a 391-bp fragment of *oocyte maturation factor mos* (c-mos) gene. Primers and PCR protocols are listed in Table S1. After amplification, PCR products were purified with Exonuclease I and Alkaline Phosphatase (ExoSAP protocol). Sequencing was performed using the BigDye Terminator 3.1 Cycle Sequencing kit (Applied Biosystems), followed by analysis in ABI Prism 310, 3700 or 3170 Genetic Analyzer Sequencers (Applied Biosystems) at the Instituto de Química, Universidade de São Paulo (IQUSP, São Paulo, Brazil). DNA sequences were combined and edited using CodonCode Aligner 5.1. After editing, sequences were verified for contamination using BLAST (http://blast. ncbi.nlm.nih.gov/Blast.cgi), and alignments were obtained using ClustalW 1.82 implemented on the CIPRES Science Gateway (https://www.phylo.org/), using a value of 10 for gap open penalty and 0.2 for gap extension penalty. For c-mos sequences, the polymorphic nucleotide positions of heterozygous terminals were coded with the IUPAC ambiguity codes. A concatenated alignment of the four markers was generated using SequenceMatrix 1.7.8 (Vaidya, Lohman, & Meier, 2010).

2.3 | Phylogenetic analyses

Our ingroup consists of 41 samples, representing *N. bicarinatus* (18 samples), *N. medemi* (five), *N. racenisi* (four), *N. rudis* (five) and *N.* cf. *rudis* (eight) (Table S2), with some of these samples also examined for morphology (Figure S1, Appendix S1). We did not include *N. tatei* in our analysis because no tissue sample was available. We additionally included one sample of *P. apodemus* from Tinamastes, San José, Costa Rica. To test the monophyly of *Neusticurus* and the position of *P. apodemus,* we included all other Cercosaurini

samples available from GenBank. These sequences mainly correspond to the database generated by Torres-Carvajal et al. (2016), consisting of sequences from 59 species in the genera *Proctoporus*, *Petracola*, *Potamites*, *Cercosaura*, *Pholidobolus*, *Macropholidus*, *Anadia*, *Oreosaurus*, *Riama*, *Gelanesaurus*, *Echinosaura* and *Placosoma*, plus three undescribed Cercosaurini species (Cercosaurini sp. 1, sp. 2 and sp. 3; Torres-Carvajal et al., 2016) (Table S2). We also used sequences from one species representative of most other tribes or subfamilies of Gymnophthalmidae, as follows: *Iphisa elegans* (Iphisini), *Loxopholis osvaldoi* (Ecpleopodini), *Bachia flavescens* (Bachiini) and *Rachisaurus brachylepis* (Rachisaurinae) (Table S2). We rooted the tree with the alopoglossid *Alopoglossus atriventris*, as *Alopoglossus* was recovered as the sister genus of a clade composed of all other gymnophthalmids (Pellegrino et al., 2001) or with *Ptychoglossus* in a clade sister to Teiidae + Gymnophthalmidae (Goicoechea et al., 2016).

We performed phylogenetic analyses under maximum likelihood (ML) and Bayesian inference (BI) on the four-gene concatenated data set. We also estimated a gene tree for each marker using BI. All ML analyses were performed using RAxML 8.2.10. (Stamatakis, 2014), using three independent searches (i.e., changing the seed number in -p for each run) for the best-scoring ML tree, each using a rapid Bootstrap and search for best-scoring ML tree (-f a and -# 1000) and using the -m GTRCAT model of nucleotide substitution. Bayesian phylogenetic analyses were performed with MrBayes v3.2. (Ronquist et al., 2012). We inferred DNA substitution models and the best partition scheme for Bayesian analyses using PartitionFinder v1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012) under the Bayesian information criterion (BIC). Two independent runs were implemented with four Bayesian Markov chain Monte Carlo (MCMC) each, starting with a random seed. Each run consisted of 10,000,000 generations sampled every 1,000 generations. Convergence between chains and adequate effective sample sizes (ESS > 200) were checked on Tracer v1.6. A 50% majority rule tree was summarized after considering a 25% value of burn-in. We considered nodes highly supported when bootstrap values were > 85 and posterior probabilities were > 0.9.

2.4 | Ancestral character estimation of CLM

We used two methods (threshold model and ML) to perform ancestral character estimation for the CLM phenotype. The threshold model (Felsenstein, 2012; Revell, 2014) was implemented with the function "ancThresh" in the R package Phytools 0.4–45, where the liability (a continuous trait) and position of thresholds (i.e., the parameter that defines when the change on character state occurs) are sampled from their joint posterior probability through a MCMC. This model intends to be more realistic about the rate of character change, since

the prevailing methods for ancestral estimation are based on continuous-time discrete-state Markov processes, where a lineage can change its state instantly, with an equal and indefinite probability of reversal—a rather undesirable characteristic that may not reflect biological reality (Revell, 2014). Species in our data set were classified as aquatic (1) or terrestrial (0) based on the literature and the morphological analyses. The best-scoring ML tree was pruned (function "drop.tip" in the ape 3.2 package in R) to obtain a tree with only one terminal per species. The resulting tree was then used as the input of ancTresh. We ran the MCMC using 5,000,000 generations sampled every 1,000 generations and assumed a burn-in value of 20%. We used the default Brownian motion (model = "BM") to the evolution of liability. We checked the ESS of log-likelihoods using the function "effectiveSize" of the coda 0.18-1 package, making sure that ESS were greater than 200.

The likelihood of ancestral character states was also estimated using the function "ace" in the package ape 3.2. This function reconstructs ancestral states for discrete characters using maximum likelihood. The same input tree as for "ancTresh" was used. We performed two analyses using different models to specify the transition probabilities between the states of a character (aquatic or terrestrial): an equal-rates model (model = "ER"), in which forward and reverse transitions between states are constrained to be equal, and an all rates different matrix (model = "ARD") where the two possible transitions between states receive distinct parameters. We tested whether there was a significant increase on the log-likelihood value of the ARD model (which uses two parameters) relative to the ER model (which uses only one parameter) through a likelihood test, using the following code line: $1-\text{pchisq}(2*\text{abs}(\text{ERmodel\$loglik} - \text{ARDmodel\$loglik})$, $df = 1$).

2.5 | Molecular dating analysis

Estimates of divergence times of CLM species were calculated after analysis of the four-gene matrix in BEAST 1.8.1. We pruned the alignment to keep only one sample per species, in order to decrease computational time. The tree model used was a Yule speciation process, adequate for interspecific analysis, with a random starting tree. Preliminary runs indicated that the model was overparameterized. Therefore, we simplified nucleotide substitution models, using HKY and empirical base frequencies for all partitions. To allow for distinct evolutionary rates among branches, we used a lognormal uncorrelated relaxed clock prior. The same node ages used by Torres-Carvajal et al. (2016) to calibrate their tree, which in turn was based on ages estimated by Zheng and Wiens (2016), were also used here, except for node referring to the *Placosoma-Neusticurus* most recent common ancestor, which we were interested in estimating and, therefore, we did not apply any constraint on it. We ran the MCMC

for 100,000,000 generations, sampling each 10,000, which yielded log and tree files with 10,000 posterior samples and trees. ESS values for parameters were checked on Tracer 1.6, and the maximum clade credibility tree was obtained using TreeAnnotator 1.8.1.

2.6 | Morphological comparisons

Morphological comparisons among *Neusticurus* species were performed after obtaining 20 meristic and 12 categorical characters (Table S3), all of them relevant to species diagnosis in *Neusticurus* as stated by previous studies. In the case of *N. bicarinatus*, we used scale counts and categorical characters available in Avila-Pires (1995). Scale counts and terminology of scales followed Uzzell (1966) and Avila-Pires (1995), except that we distinguish the counting of dorsal scales in a longitudinal row along the middorsal line (which are usually reduced scales) and number of longitudinal tubercles on dorsum, from the occipitals to the posterior margin of hind limbs. We studied specimens from three zoological collections: Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil (MZUSP), Museu Paraense Emílio Goeldi, Belém, Pará, Brazil (MPEG), and specimens housed at the Zoology Department of the Universidade de São Paulo (MTR field series). We examined a total of 107 specimens of *N. bicarinatus* (25 specimens), *N. medemi* (10, including one paratype), *N. racenisi* (17), *N. rudis* (six) and *N.* cf. *rudis* (48) (Appendix S1). Additionally, we examined the holotype of *N. rudis* (BM 1946.8.31.64), *N. tatei* (AMNH 36649) and *N. dejongi* (ZMA 10241) using high-resolution photographs.

We prepared the hemipenis of one specimen of *N.* cf. *rudis* (MZUSP 78139) to compare with the description of *N. rudis* hemipenis available in the literature (Myers & Donnelly, 2008). The hemipenis was prepared following the procedures described by Manzani and Abe (1988), modified by Pesantes (1994) and Zaher (1999). The retractor muscle was manually separated and the everted organ filled with stained petroleum jelly. The organ was immersed in an alcoholic solution of Alizarin Red for 24 hours to stain possible calcified structures (e.g., spines or spicules), in an adaptation of the procedures described by Uzzell (1973) proposed by Nunes, Fouquet, Curcio, Kok, and Rodrigues (2012). The terminology of hemipenial structures follows Dowling and Savage (1960), Savage (1997) and Myers and Donnelly (2001, 2008).

3 | RESULTS

3.1 | Phylogenetic relationships and taxonomic status of *Neusticurus rudis* and *Potamites apodemus*

Phylogenetic relationships under ML and BI yielded very similar results, with a few incongruences concerning nodes
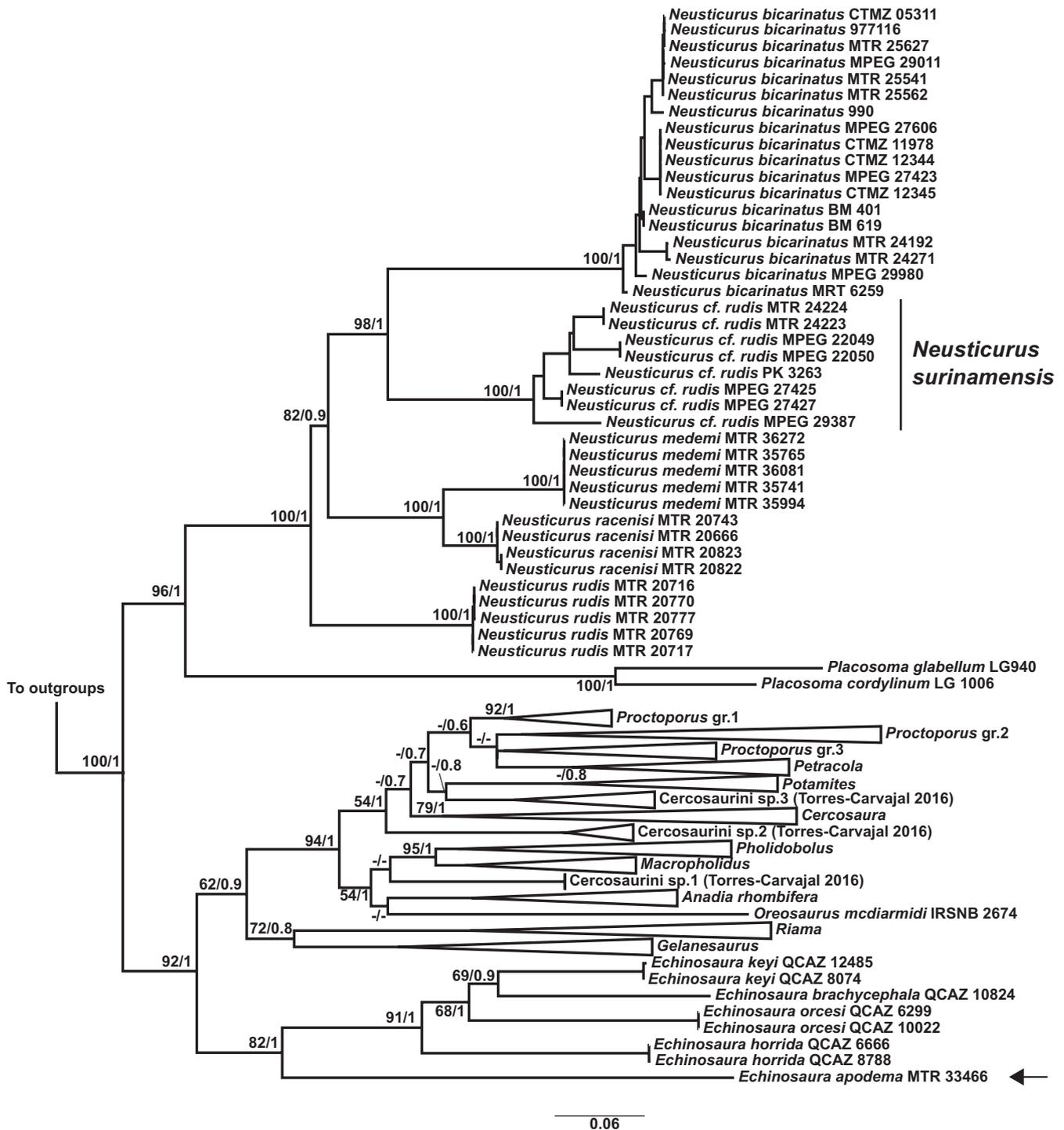
FIGURE 1 Consensus phylogram of Bayesian and maximum-likelihood results. Numbers at nodes represent bootstrap (BS)/posterior probability (PP) support values. Arrow indicates the position of *Echinosaura apodema*. *Proctoporus* gr. 1 corresponds to the species *Proctoporus chasqui*, *Proctoporus rahmi*, *Proctoporus spinalis*, *Proctoporus oreades*, *Proctoporus pachyurus* and *Proctoporus* sp. 1 (Torres-Carvajal 2016). *Proctoporus* gr. 2 corresponds to *Proctoporus iridescens*, *Proctoporus laudahnae*, *Proctoporus unsaacae*, *Proctoporus guentheri* and *Proctoporus* sp. 2 (Torres-Carvajal 2016). *Proctoporus* gr. 3 corresponds to *Proctoporus carabaya*, *Proctoporus xestus*, *Proctoporus bolivianus* and *Proctoporus sucullucu*. BS values < 50 and PP < 0.6 are indicated with "-"

that received low support values in both analyses (see below; a consensus tree is presented in Figure 1). *Placosoma* and *Neusticurus* were recovered as monophyletic and sister genera, splitting early in the Cercosaurini history, supporting the

results of previous studies (Doan & Castoe, 2005; Torres-Carvajal et al., 2016).

Internal relationships within *Neusticurus* species generally show high bootstrap (BS) and posterior probability

values (PP) and species were also recovered as monophyletic, except for *N. rudis* (sensu Uzzell, 1966; Avila-Pires, 1995), which is recovered as polyphyletic in our tree (Figure 1). More specifically, the five Pacaraima samples, a site located around 90 km SSW from the type locality of *N. rudis*, form the sister clade to all the other *Neusticurus* samples, while eight samples from the eastern Guiana Shield (*N.* cf. *rudis*) group with *N. bicarinatus* (Figure 1). *Neusticurus racenisi* and *N. medemi* were recovered as sister species, forming a clade sister to *N.* cf. *rudis* + *N. bicarinatus*. Morphologically, specimens from both *N. rudis* clades (Pacaraima and eastern Guiana Shield) diverge in several morphological characters (Table S3), including hemipenial characters, supporting the results obtained from the molecular phylogenetic analysis.

In relation to other Cercosaurini, *P. apodemus* was recovered with high support as sister to all other *Echinosaura* samples. *Echinosaura*, in turn, is sister to all remaining Cercosaurini, forming a clade with high PP value, but moderate BS value. Our analyses grouped *Gelanesaurus* and *Riama* with moderate support. The clade composed by *Proctoporus*, *Petracola*, *Potamites*, *Cercosaura*, *Pholidobolus*, *Macropholidus*, *Anadia rhombifera* and *Oreosaurus mcdiarmidi* is highly supported. However, few relationships within this clade received high support values, especially in the ML analysis (Figure 1). Nonetheless, the relationship between *Macropholidus* and *Pholidobolus* is highly supported.

The gene trees, in general, recovered similar relationships among *Neusticurus* species or yielded unresolved nodes (trees not shown). The sister relationship between *N. bicarinatus* and *N.* cf. *rudis* was recovered by 12S, ND4 and c-mos genes (unresolved in 16S). Similarly, *N. racenisi* and *N. medemi* were recovered as sister species by 12S, 16S and ND4 (the amplification of c-mos did not work for *N. medemi* specimens). The only marker that shows high PP values for the base of *Neusticurus* tree was ND4, which also yielded the tree most similar to the concatenated tree. Except for ND4, deep relationships within *Neusticurus* were unresolved (16S and c-mos) or poorly supported (12S). The position of *P. apodemus* within Cercosaurini varied according to the marker analysed. In the 16S tree, *P. apodemus* is recovered as sister to all other *Echinosaura* with high support (PP = 0.92), as in the concatenated tree. In 12S tree, however, *P. apodemus* is recovered as sister to all other *Placosoma* analysed, also with high support (PP = 0.99). In the c-mos tree, the relationship of *P. apodemus* with other Cercosaurini is unresolved, and the amplification of ND4 did not work for *P. apodemus*.

3.2 | Geographical distribution of *Neusticurus* species

We detected the presence of *N. medemi* in Brazil for the first time during one of our field surveys. This species

was previously known only from its type locality, Caño Monserero, Vaupes, and surrounding sites in Colombia (Dixon & Lamar, 1981) and reported from the base of Cerro Neblina on its Venezuelan side by McDiarmid and Paolillo (1988). The new population was found about 500 Km SE from the type locality, close to the Brazil-Colombia border, in Comunidade Cachoeirinha, on the north margin of Rio Içá, 50 Km WNW from Santo Antônio do Içá, state of Amazonas, Brazil (2°53′17″S, 68°20′35″W) (Figure S1). Local vegetation is characterized by typical lowland ombrophilous *terra-firme* forest of western Amazonia, with plateaus dissected by small streams (*igarapés*) totally covered by forest, thus with minimal light penetration. We collected 15 specimens (males, females and juveniles) in one of these *igarapés*. Most individuals were found at night, when resting on small branches above the water.

The distribution of *Neusticurus* species follows a west–east pattern in Amazonia. While *N. medemi* seems restricted to the north-west, three species (*N. racenisi*, *N. tatei* and *N. rudis*) are apparently restricted to the Pantepui region in northern South America, and *N.* cf. *rudis* occurs in the lowlands of the Eastern Guiana Shield (but not in the Pantepui region). *Neusticurus bicarinatus* seems more widely distributed throughout the Eastern Guiana Shield and the Brazilian Shield (Figure S1).

3.3 | Ancestral character estimation and dating analysis

At least three CLM genera (*Neusticurus*, *Echinosaura* and *Gelanesaurus*) diverged early within Cercosaurini. Therefore, the results of ancestral reconstruction in the most inclusive nodes of our phylogeny are essential to understand the evolution of CLM. The two models implemented differed in relation to the uncertainty degree: the threshold model gave almost equal probabilities for two phenotypes in most inclusive nodes, while ML model strongly suggested terrestrial early ancestors of Cercosaurini, and CLM phenotype evolving independently four times in each of the four aquatic genera analysed (Figure 2).

The results of the threshold model suggested that CLM is ancestral to all Cercosaurini, with *Neusticurus*, *Echinosaura* and probably *Gelanesaurus* retaining the ancestral condition, which was lost in the clade containing most of the terrestrial genera (*Macropholidus*, *Anadia*, *Proctoporus* and others), and reversed in *Potamites* (Figure 2a). The results of the threshold model can also be interpreted—given the similar probabilities—similarly to the scenario suggested by the ML model: that the evolutionary history of Cercosaurini was dominated by terrestrial ancestors, which independently evolved to aquatic habitat four times, represented by the four CLM genera (Figure 2b). Since one model (ML) strongly suggested the latter scenario, and the other shows ambiguous
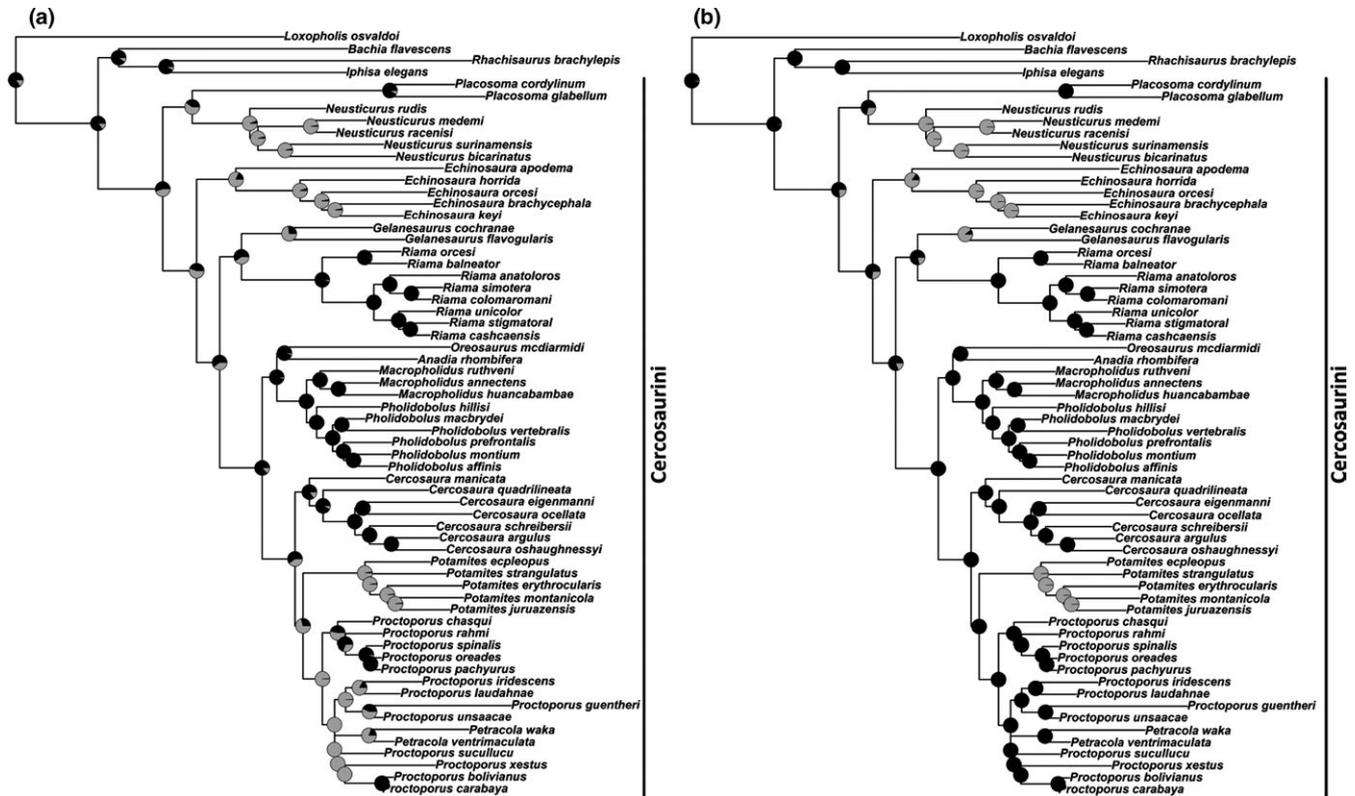
FIGURE 2 Results of the two methods used for the ancestral character reconstruction analysis of the CLM. (a) Threshold model, (b) maximum-likelihood model. Pie charts on nodes represent the probability of a CLM ancestor (grey) and non-CLM (black) for that node

results, we tentatively based our discussion assuming the "convergence" hypothesis: that CLM appeared independently four times within Cercosaurini.

BEAST analysis yielded a similar topology (not shown) to that obtained both in the BI and ML concatenated phylogenies, both in terms of the relationship among _Neusticurus_ species and the position of _P. apodemus_ (as the sister group of all other _Echinosaura_ species—PP = 0.98). Since calibration points and the data set are overall similar to the ones used in Torres-Carvajal et al. (2016), the node ages estimated here are also similar. However, our estimation of _Neusticurus_ and _Echinosaura_ crown ages are probably more accurate, given our larger taxonomic sampling. According to our results, _Echinosaura_ was the first genus to develop a CLM phenotype, starting to diversify in the middle Eocene, followed by _Neusticurus_ during the middle Oligocene, and _Potamites_ and _Gelanesaurus_, both starting to diversify during the early Miocene (Figure 3).

4 | DISCUSSION

4.1 | _Neusticurus_ and _Potamites_ taxonomy and biogeography

Our results support the idea, already introduced both by Uzzell (1966) and Avila-Pires (1995), that the large
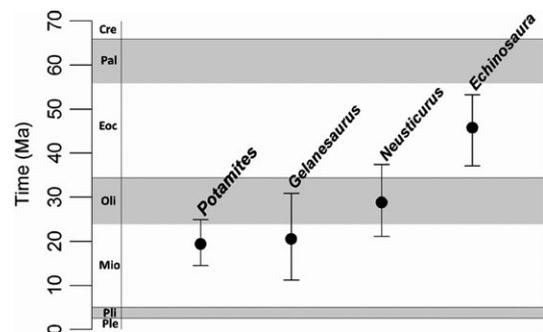

FIGURE 3 Median and 95% HPD of the divergence times of CLM species, estimated with BEAST. Ple, Pleistocene; Pli, Pliocene; Mio, Miocene; Oli, Oligocene; Eoc, Eocene; Pal, Palaeocene; Cre, Cretaceous

morphological variation observed in _N. rudis_ is in fact due to the existence of more than one species under this name. Our morphological comparisons among the holotype of _N. rudis_, our Pacaraima samples of _N. rudis_, and _N._ cf. _rudis_ unambiguously indicate that _N._ cf. _rudis_ is a different species. Therefore, instead of being a widespread species throughout the Guiana Shield, _N. rudis_ is probably restricted to the eastern Pantepui area (eastern Venezuela, Guyana, Roraima state in Brazil). Additional work is in press elucidating the taxonomic status of the Pantepui populations of _N. rudis_ (Kok et al., in press).

Although the holotype of *N. surinamensis* is lost (Uzzell, 1966; Franzen & Glaw, 2007), Müller (1923) clearly stated that the holotype possesses two frontonasal scales and two prefrontals, with an azygous scale between them (Müller, 1923: p. 295; "Frontonasalia länger als die Praefrontalia; zwischen diesen vier Schildern liegt ein kleines Schildchen"); which we freely translated as: "frontonasals longer than prefrontals; between these four shields is located a small shield"), which is one of the diagnostic characters distinguishing *N. surinamensis* from *N. rudis* (Table S3). These characteristics correspond to what we observe in all specimens of *N.* cf. *rudis* that we examined. Given our molecular results and these morphological observations, we propose to remove *N. surinamensis* Müller, 1923 from the synonymy of *N. rudis* and apply this name to specimens that have been referred here as *N.* cf. *rudis*. With this new material at hand, we are able to redefine and redescribe *N. surinamensis*. Detailed morphological comparisons between *N. rudis* and *N. surinamensis* are given within the redescription of *N. surinamensis* (the redescription of *N. surinamensis* and morphological comparisons are provided as a Supplementary Material).

As stated above, it is worth to mention that there is substantial morphological (and molecular) variation in *N. rudis* in the Pantepui region, which indicates the need of further analysis (in progress). All our specimens from Pacaraima, for instance, present the loreal scale in broad contact with the supralabials; however, in the holotype of *N. rudis*, the loreal is separated from the supralabials by the frenocular scale (Figure S4). Also, Donnelly and Myers (1991) stated that their series of 11 specimens from Cerro Guaiquinima in Venezuela differs from the holotype of *N. rudis*, but the authors did not mention in which characters. It would not be surprising if *N. rudis* ends up divided in additional taxa in the future, especially given the high levels of microendemism shown by other lizard genera distributed across Pantepui (e.g., *Arthrosaura*; Kok, 2008; or *Riolama;* Kok, 2015).

Neusticurus medemi also shows evident variation in scalation, which may be suggestive of unrecognized species under that name. In all the nine specimens examined from Rio Içá, the loreal is in broad contact with the supralabials, a condition that is not present in the holotype (Dixon & Lamar, 1981; Figure 2). Other characters analysed are slightly inconsistent with the range of values provided in the species description (Dixon & Lamar, 1981), as is the case of the number of femoral pores in females (9–12 in the type series vs. 13–18 in the Içá population) and scales on prefrontal–frontal region (19–38 vs. 15–21).

In our phylogenetic concatenated analyses, as well as in the dating analyses, *P. apodemus* is recovered as a member of *Echinosaura*, and sister to all other species of this genus. We therefore propose a new combination, *Echinosaura apodema*. This species was included in the genus *Potamites* mainly because of its external similitude (Castoe et al.,

2004). This case illustrates well the difficulties involved in a morphology-based taxonomy of CLM species due to convergent evolution. Our new arrangement is also meaningful from a biogeographical perspective. *Potamites* species have a cis-andean distribution (Chávez & Catenazzi, 2014), which contrasts with the Central American *E. apodema*. However, *Echinosaura* species show a trans-Andean distribution, occurring from Ecuador to Central America, in accordance with the geographical distribution of *E. apodema*.

The distribution of *Neusticurus* species is clearly centered in the Guiana Shield. The only species occurring outside of this region is *N. bicarinatus*, which is distributed in both the Guiana and Brazilian shields in south-eastern Amazonia (Figure S1). The lack of obvious phylogeographic structure in *N. bicarinatus*, despite its wide distribution, may indicate a relatively recent range expansion towards the Brazilian Shield. At least three species are restricted to the uplands of the Pantepui region (*N. racenisi*, *N. tatei* and *N. rudis*), with *N. rudis* being sister to the ancestor of all other species. This may indicate that the genus started to diversify in Pantepui, and posteriorly dispersed into the lowlands towards the west (*N. medemi*) and towards the east (*N. surinamensis* and *N. bicarinatus*). Remarkably, the distribution pattern and divergence times of *Neusticurus* are very similar to those observed in *Adelophryne* frogs (Fouquet et al., 2012).

4.2 | Evolution of CLM in Cercosaurini

Despite inconclusive results for the ancestral character reconstruction using the threshold model, we tentatively assume the working hypothesis that the CLM phenotype evolved four times independently in Cercosaurini, which is the scenario suggested by the ML model. It implies that morphological adaptations to aquatic or semi-aquatic lifestyle are, in fact, convergent due to similar selective forces, rather than a plesiomorphic or a synapomorphic character. Accordingly, the CLM has evolved many times independently in lizards, occurring in 11 families around the globe (Pianka & Vitt, 2003; Bauer & Jackman, 2008), for example in the genera *Dracaena* (Teiidae) and *Shinisaurus* (Shinisauridae). However, differences in the CLM phenotype among lizards and within Cercosaurini may reflect different degrees of dependence on the aquatic environment. *Gelanesaurus flavogularis*, for example, is commonly found close to creeks, but apparently does not depend on them (CBA, personal observ.), and also lacks a laterally compressed tail. In turn, three *Neusticurus* species (*N. medemi*, *N. racenisi* and *N. tatei*) do not have heterogeneous dorsal scalation, despite their close association with small creeks (see above). There is evidence of spatial and habitat segregation in *Potamites ecpleopus* and *Potamites juruazensis* when occurring in sympatry, with the later being more terrestrial than the former (Vitt & Avila-Pires, 1998). Nonetheless, the knowledge about habitat

choice and natural history of most CLM species is anecdotal at best, and robust hypothesis-testing studies still remain to be made.

Among ecological factors that may have acted in the evolution of CLM, one appealing hypothesis is that CLM adaptations, especially a laterally compressed tail and a double tail crest, may improve the locomotion in water, promoting differential survival during predatory attempts. Laterally compressed tails have shown to increase swimming speeds but reduce crawling speed in snakes (Aubret & Shine, 2008). It seems thus reasonable to hypothesize that the vertical enlargement of scales over the body and tail (the CLM phenotype) was appropriate to increase speed and direction during undulatory swimming, without affecting locomotor performance out of the water.

Historical processes that changed the landscape characteristics in north South America may have favored CLM evolution. Today, Amazonia harbours an outstanding diversity of aquatic environments such as large and small rivers, as well as floodplain forests locally known as *Igapós* or *Várzeas*. However, floodplain forests were probably much more widespread during most of the Miocene, as the western Amazonia was dominated by the Pebas Lake, an enormous system of lakes and swamps which, at the apex of its extension (middle Miocene, c. 16–11 Ma), reached 1.5 million km^2, occupying almost all the lowlands of the western Amazonia (Hoorn, Wesselingh, Hovikoski, & Guerrero, 2010). The development of the Pebas Lake is contemporaneous with the beginning of the diversification of *Potamites* and *Gelanesaurus*, which occurred during the early–middle Miocene. Also, the extant diversity of both genera is concentrated in western Amazonia (Altamirano-Benavides et al., 2013; Chávez & Catenazzi, 2014; Torres-Carvajal et al., 2016), suggesting that the great predominance of floodplain forests during the Miocene may have acted as a selection force leading to the evolution of CLM in those genera. However, the role of habitat availability is not clear in the diversification of *Neusticurus* and *Echinosaura*, whose distributions are centered in the Guiana Shield and Chocó—Central America, respectively, and which started to diversify earlier.

5 | CONCLUSION

Based on new morphological and molecular data of undersampled poorly known Cercosaurini lizards we proposed a new phylogenetic hypothesis for *Neusticurus*, with implications for this group's taxonomy and morphological evolution. The results shed light on the taxonomic status of *N. rudis*, from which *N. surinamensis* is revalidated (see Supplementary Material), as well as on the phylogenetic affinities of *E. apodema*, previously allocated to the genus *Potamites*. Moreover, our ancestral trait reconstruction

analyses suggest that the aquatic phenotype has evolved four times independently in the history of Cercosaurini. We hypothesize that both ecological and environmental selective pressures played an important role in diversification of CLM phenotype; however, the relative contribution of these factors still remains uncertain.

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REFERENCES

Altamirano-Benavides, M., Zaher, H., Lobo, L., Grazziotin, F. G., Nunes, P. M. S., & Rodrigues, M. T. (2013). A new species of lizard genus *Potamites* from Ecuador (Squamata, Gymnophthalmidae). *Zootaxa*, *3717*(3), 345–358. https://doi.org/10.11646/zootaxa.3717.3.4

Aubret, F., & Shine, R. (2008). The origin of evolutionary innovations: Locomotor consequences of tail shape in aquatic snakes. *Functional Ecology*, *22*, 317–322. https://doi.org/10.1111/j.1365-2435.2007.01359.x

Avila-Pires, T. C. S. (1995). Lizards of Brazilian *Amazonia* (Reptilia: Squamata). *Zoologische Verhandelingen*, *299*, 1–706.

Bauer, A. M., & Jackman, T. (2008). Global diversity of lizards in freshwater (Reptilia: Lacertilia). *Hydrobiologia*, *595*, 581–586.

Brongersma, L. D. (1927). *Neusticurus dejongi*, sp.n., a new lizard from Surinam. *The Annals and Magazine of Natural History*, *20*, 543–545. https://doi.org/10.1080/00222932708655486

Castoe, T. A., Doan, T. M., & Parkinson, C. L. (2004). Data partitions and complex models in Bayesian analysis: The phylogeny of Gymnophthalmid lizards. *Systematic Biology*, *53*(3), 448–469. https://doi.org/10.1080/10635150490445797

Chávez, G., & Catenazzi, A. (2014). A new Andean lizard of the genus *Potamites* (Sauria, Gymnophthalmidae) from Manu National Park, southeastern Peru. *Zootaxa*, *3774*(1), 45–56. https://doi.org/10.11646/zootaxa.3774.1.3

Dixon, J. R., & Lamar, W. W. (1981). A new species of microteiid lizard (genus *Neusticurus*) from Colombia. *Journal of Herpetology*, *15*(3), 309–314. https://doi.org/10.2307/1563434

Doan, T. M., & Castoe, T. A. (2005). Phylogenetic taxonomy of the Cercosaurini (Squamata: Gymnophthalmidae), with new genera for species of *Neusticurus* and *Proctoporus*. *Zoological Journal of Linnean Society*, *143*, 405–416. https://doi.org/10.1111/j.1096-3642.2005.00145.x

Donnelly, M. A., & Myers, C. W. (1991). Herpetological results of the 1990 Venezuelan Expedition to the summit of Cerro Guaiquinima, with new tepui reptiles. *American Museum Novitates*, *3017*, 1–54.

Dowling, H. G., & Savage, J. M. (1960). A guide to the snake hemipenis: A survey of basic structure and systematic characteristics. *Zoologica*, *45*, 17–28.

Felsenstein, J. (2012). A comparative method for both discrete and continuous characters using the threshold model. *American Naturalist*, *179*, 145–156. https://doi.org/10.1086/663681

Fouquet, A., Loebmann, D., Castroviejo-Fisher, S., Padial, J. M., Orrico, V. G. D., Lyra, M. L., … Rodrigues, M. T. (2012). From Amazonia to the Atlantic forest: Molecular phylogeny of Phyzelaphryninae frogs reveals unexpected diversity and a striking biogeographic pattern emphasizing conservation challenges. *Molecular Phylogenetics and Evolution*, *65*, 547–561. https://doi.org/10.1016/j.ympev.2012.07.012

Franzen, M., & Glaw, F. (2007). Type catalogue of reptiles in the Zoologische Staatssammlung München. *Spixiana*, *30*(2), 201–274.

Goicoechea, N., Frost, D. R., de la Riva, I., Pellegrino, K. C. M., Sites-Jr, J., Rodrigues, M. T., & Padial, J. M. (2016). Molecular systematics of teioid lizards (Teiioidea/Gymnophthalmoidea: Squamata) based on the analysis of 48 loci under tree-alignment and similarity-alignment. *Cladistics*, https://doi.org/10.1111/cla.12150

Hoogmoed, M. S. (1973). Notes on the herpetofauna of Surinam IV. The lizards and amphisbaenians of Surinam. *Biogeographica*, *4*, 1–419.

Hoorn, C., Wesselingh, F. P., Hovikoski, J., & Guerrero, J. (2010). The development of the Amazonian mega-wetland (Miocene, Brazil, Colombia, Peru, Bolivia). In C. Hoorn, & F. P. Wesselingh (Eds.), *Amazonia: Landscape and species evolution. A look into the past* (pp. 123–142). Chichester: Wiley-Blackwell.

Kok, P. J. R. (2008). A new highland species of *Arthrosaura* Boulenger, 1885 (Squamata: Gymnophthalmidae) from Maringma tepui on the border of Guyana and Brazil. *Zootaxa*, *1909*, 1–15.

Kok, P. J. R. (2015). A new species of the Pantepui endemic genus *Riolama* (Squamata: Gymnophthalmidae) from the summit of Murisipán-tepui, with the erection of a new gymnophthalmid subfamily. *Zoological Journal of the Linnean Society*, *174*, 500–518. https://doi.org/10.1111/zoj.12241

Kok, P. J. R., MacCulloch, R. D., Means, D. B., Roelants, K., Van Bocxlaer, I., & Bossuyt, F. (2012). Low genetic diversity in tepui summit vertebrates. *Current Biology*, *22*, R589–R590. https://doi.org/10.1016/j.cub.2012.06.034

Kok, P. J. R., Bittenbinder, M. A., van den Berg, J. K., Marques-Souza, S., Nunes, P. M. S., & Laking, A., … Rodrigues, M. T. (2018). Integrative taxonomy of the gymnophthalmid lizard Neusticurus rudis Boulenger, 1900 identifies a new species in the eastern Pantepui region, north-eastern South America. *Journal of Natural History*. *52*, 1029–1066

Lanfear, R., Calcott, B., Ho, S. Y. W., & Guindon, S. (2012). PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, *29*, 1695–1701. https://doi.org/10.1093/molbev/mss020

Lotzkat, S., Batista, A., Vargas, J., Hertz, A., & Köhler, G. (2012). *Reptilia*, *Squamata*, *Gymnophthalmidae*, *Potamites apodemus* (Uzzel, 1966): Distribution extension and first records from Panama. *Check List*, *8*(2), 302–306. https://doi.org/10.15560/8.2.302

Manzani, P. R., & Abe, A. S. (1988). Sobre dois novos métodos de preparo do hemipenis de serpentes. *Memorias do Instituto Butantan (Sao Paulo)*, *50*, 15–20.

McDiarmid, R. W., & Paolillo, A. (1988). Herpetological collections: Cerro de la Neblina. In C. Brewer-Carías (Ed.), *Cerro de la Neblina. Resultados de la expedición 1983–1987* (pp. 667–670). Caracas, Venezuela: Fudeci.

Müller, V. L. (1923). Neue order seltene Reptilien und Batrachier der Zoologischen Sammlung des bayerischen Staates. *Zoologischer Anzeiger*, *57*(7/8), 145–156.

Myers, C. W., & Donnelly, M. A. (2001). Herpetofauna of the Yutajé-Corocoro massif, Venezuela: Second report from the Robert G. Goelet American Museum-Terramar expedition to the Northwestern tepuis. *Bulletin of the American Museum of Natural History*, *261*, 1–85. https://doi.org/10.1206/0003-0090(2001)261<0001:HOTYCM>2.0.CO;2

Myers, C. W., & Donnelly, M. A. (2008). The summit herpetofauna of Auyantepui, Venezuela: Report from the Robert G. Goelet American Museum-Terramar Expedition. *Bulletin of the American Museum of Natural History*, *308*, 1–147. https://doi.org/10.1206/308.1

Nunes, P. M. S., Fouquet, A., Curcio, F. F., Kok, P. J. R., & Rodrigues, M. T. (2012). Cryptic species in *Iphisa elegans* Gray, 1851 (Squamata: Gymnophthalmidae) revealed by hemipenial morphology and molecular data. *Zoological Journal of the Linnean Society*, *166*, 361–376. https://doi.org/10.1111/j.1096-3642.2012.00846.x

Pellegrino, K. C. M., Rodrigues, M. T., Yonenaga-Yassuda, Y., & Sites, J. W. Jr (2001). A molecular perspective on the evolution of microteiid lizards (Squamata, Gymnophthalmidae), and a new classification for the family. *Biological Journal of Linnean Society*, *74*, 315–338. https://doi.org/10.1111/j.1095-8312.2001.tb01395.x

Pesantes, O. S. (1994). A method for preparing the hemipenis of preserved snakes. *Journal of Herpetology*, *28*, 93–95. https://doi.org/10.2307/1564686

Pianka, E. R., & Vitt, L. (2003). *Lizards: Windows to the evolution of diversity*. Oakland, CA: University of California Press.

Pyron, R. A., Burbrink, F. T., & Wiens, J. J. (2013). A phylogeny and revised classification of *Squamata*, including 4161 species of lizards and snakes. *BMC Evolutionary Biology*, *13*(93), 1–53.

Revell, L. J. (2014). Ancestral character estimation under the threshold model from quantitative genetics. *Evolution*, *68*(3), 743–759. https://doi.org/10.1111/evo.12300

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., … Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *6*(3), 539–542. https://doi.org/10.1093/sysbio/sys029

Savage, J. M. (1997). On terminology for the description of the hemipenes of squamate reptiles. *Herpetological Journal*, *7*, 23–25.

Stamatakis, A. (2014). RaxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Torres-Carvajal, O., Lobos, S. E., Venegas, P. J., Chávez, G., Aguirre-Peñafiel, V., Zurita, D., & Echevarría, A. (2016). Phylogeny and biogeography of the most diverse clade of South American gymnophthalmid lizards (Squamata, Gymnophthalmidae, Cercosaurinae). *Molecular Phylogenetics and Evolution*, *99*, 63–75. https://doi.org/10.1016/j.ympev.2016.03.006

Uetz, P. (2017). *The reptile database*. Retrieve from http://www.reptile-database.org. accessed May 17, 2017.

Uzzell, T. (1965). Teiid lizards of the genus *Echinosaura*. *Copeia*, *1965*(1), 82–89. https://doi.org/10.2307/1441244

Uzzell, T. (1966). Teiid lizards of the genus *Neusticurus* (Reptilia, Sauria). *Bulletin of the American Museum of Natural History*, *132*(5), 277–328.

Uzzell, T. (1973). A revision of lizards of the genus *Prionodactylus*, with a new genus for *P. leucostictus* and notes on the genus *Euspondylus* (Sauria, Teiidae). *Postilla*, *159*, 1–67. https://doi.org/10.5962/bhl.part.11535

Vaidya, G., Lohman, D. J., & Meier, R. (2010). SequenceMatrix: Concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics*, *27*(2), 171–180.

Vitt, L. J., & Avila-Pires, T. C. S. (1998). Ecology of two sympatric species of *Neusticurus* (Sauria: Gymnophthalmidae) in the western Amazon of Brazil. *Copeia*, *1998*(3), 570–582. https://doi.org/10.2307/1447787

Zaher, H. (1999). Hemipenial morphology of the South American xenodontine snakes, with a proposal for a monophyletic Xenodontinae and a reappraisal of colubroid hemipenes. *Bulletin of the American Museum of Natural History*, *240*, 1–168.

Zheng, Y., & Wiens, J. J. (2016). Combining phylogenomic and supermatrix approaches, and a time-calibrated phylogeny for squamate reptiles (lizards and snakes) based on 52 genes and 4162 species. *Molecular Phylogenetics and Evolution*, *94*, 537–547. https://doi.org/10.1016/j.ympev.2015.10.009

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