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Multiple connections between Amazonia and Atlantic Forest shaped the phylogenetic and morphological diversity of *Chiasmocleis* Mehely, 1904 (Anura: Microhylidae: Gastrophryninae)

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ABSTRACT

Chiasmocleis is the most species-rich genus of Neotropical microhylids. Herein, we provide the first comprehensive multilocus phylogeny for the genus, including all but 3 of the 34 recognized species and multiple individuals per species. We discuss cryptic speciation, species discovery, patterns of morphological evolution, and provide a historical biogeographic analysis to account for the current distribution of the genus. Diversification of *Chiasmocleis* from other New World microhylids began during the Eocene, app. 40 mya, in forested areas, and current diversity seems to be a product of recurrent connections between the Atlantic Forest and Amazonia. Small-sized species evolved independently three times in *Chiasmocleis*. Furthermore, the extremely small-bodied (i.e. miniaturized) species with associated loss of digits, phalanges, and pectoral girdle cartilages evolved only once and are restricted to Amazonia. Using the phylogeny, we recognized three subgenera within *Chiasmocleis*: *Chiasmocleis* Méhely, 1904, *Relictus* subg. nov., and *Syncope* Walker, 1973. The recognition of the subgenus *Syncope* informs future research on patterns of miniaturization in the genus, and the subgenus *Relictus* highlights isolation of an endemic and species-poor lineage to the Atlantic Forest, early (about 40 mya) in the history of *Chiasmocleis*.

1. Introduction

Narrow-mouthed frogs of the family Microhylidae represent one of the largest groups of frogs, with worldwide distribution. The family shows increased rates of diversification after the Cretaceous-Paleogene mass extinction (Feng et al., 2017) leading to the 653 currently known species. Old World microhylids (570 species) are ecologically diverse (e.g., arboreal, terrestrial, or fossorial habits), whereas New World microhylids (83 species) are a group of terrestrial, leaf-litter or fossorial frogs.

The diversity of New World microhylids is taxonomically grouped into three subfamilies: (1) the monotypic Adelastinae, erected to accommodate the single species *Adelastes hylonomos* Zweifel, 1986 (Peloso et al., 2016); (2) Otophryninae Wassersug and Pyburn, 1987 with two genera and six species (*Otophryne* Boulenger, 1900 and *Synapturanus* Carvalho, 1954); and (3) Gastrophryninae Fitzinger, 1843, which includes 11 genera and 76 species and is the most diverse clade of New World microhylids (de Sá et al., 2012; Peloso et al., 2016). Only two genera (with four species each) within Gastrophryninae are distributed in North America: *Gastrophryne* Fitzinger, 1843 and *Hypopachus* Keferstein, 1867. Their combined distribution extends from the southern United States to Costa Rica. The remaining taxa occupy most of South America, with a higher diversity in the Amazonian lowlands and in the Atlantic Forest.

Molecular phylogenetic analyses of Microhylidae with dense generic sampling of Neotropical species (de Sá et al., 2012) advanced the

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understanding of Gastrophryninae relationships by placing in this subfamily several genera previously classified as *incertae sedis* (i.e., *Altigius*, *Arcovomer*, *Hyophryne*, *Melanophryne*, *Myersiella*, *Relictivomer*, *Stereocyclops*, *Syncope*, and *Synapturanus*; Frost et al., 2006; van der Meijden et al., 2007; Pyron and Wiens, 2011; Frost, 2011); more recently, relationships of *Adelastes* were tested (Peloso et al., 2016). Among the many taxonomic updates in New World microhylids, reorganizations were necessary in *Chiasmocleis* Mehely, 1904, the most species-rich genus within Gastrophryninae. *Chiasmocleis* was recovered as non-monophyletic, and taxonomic changes were necessary to have a classification consistent with the phylogeny (de Sá et al., 2012).

Chiasmocleis currently consists of 34 species, including the members of former genus *Syncope*, considered a junior synonym of *Chiasmocleis* (Peloso et al., 2014). The taxonomy of *Chiasmocleis* has had major advances in the last decade, including the description of 14 species (i.e., 41% of the diversity of the genus). Furthermore, the first complete and detailed description of the entire osteology of a *Chiasmocleis* species increased understanding of osteological diversity (Forlani et al., 2017). Previous reports on the osteology of *Chiasmocleis* were based on partial observations and descriptions (Parker, 1927; Walker and Duellman, 1974; Zweifel, 1986; Caramaschi and Cruz, 1997; Canedo et al., 2004; Peloso and Sturaro, 2008; Funk and Canatella, 2009).

Despite these advances, *Chiasmocleis* remains poorly known because of their low detectability and subtle differences in external morphology among some species, which have historically challenged observers to differentiate species (Fig. 1). Several species camouflage well among the leaf litter or underground, which makes then difficult to detect during visual surveys. Furthermore, species are fossorial or semi-fossorial, foraging on the surface for only a few days during periods of explosive breeding at the beginning of the rainy season. Thus, the breeding events are punctual and may not happen as often, or for prolonged periods, as in frog species that breed year-round (Forlani et al., 2010). Consequently, *Chiasmocleis* species are not easy to find and to collect. Success depends on being in the field at the exact time that they emerge from the ground.

In addition, given the fossorial habit and their small body and limb size, *Chiasmocleis* are expected to be poor dispersers and more likely to have marked allopatric speciation and phylogenetic structure associated with geographic distance and vicariant boundaries (e.g., rivers; Moraes et al., 2016; Tonini et al., 2014; Forlani et al., 2017). These characteristics of the genus make it an ideal system to explore hidden diversity of cryptic species and to identify unique evolutionary lineages (Fujita et al., 2012; Fouquet et al., 2012).

Chiasmocleis is ecologically diverse, occurring east of the Andes throughout South America, both in forested biomes (e.g., Atlantic Forest and Amazon) and across open environments (e.g., Cerrado and Chaco; Caramaschi and Cruz, 2001; de Sá et al., 2012; Peloso et al., 2014; Forlani et al., 2017). *Chiasmocleis* usually have marked sexual dimorphism in which males have darker chin, abundant to no dermal spines, and variable degree of foot webbing. Although some species of *Chiasmocleis* have similar external morphology and body shape, they vary in terms of body size and osteological traits (Tonini et al., 2014; Forlani et al., 2017). For instance, species formerly assigned to the genus *Syncope* exhibit digital reduction as well as bone loss and/or fusions (Walker, 1973; Silva and Meinhardt, 1999; Trueb et al., 2011; Almendáriz et al., 2017), whereas other *Chiasmocleis* species do not exhibit these patterns.

Phylogenetic relationships among species of *Chiasmocleis* are still contentious (de Sá et al., 2012; Peloso et al., 2014; Almendáriz et al., 2017). Therefore, a robust phylogeny requires a large proportion of *Chiasmocleis* species to test hypotheses on systematics, species diversity, phenotypic evolution, and historical biogeography. Because *Chiasmocleis* have multiple species distributed across the largest biomes in South America (i.e., Amazon, Cerrado, Chaco, and Atlantic Forest), the genus is a great model to understand environmental changes impacting species diversification in the Neotropics. Herein, we provide a near complete molecular phylogeny for the species of the genus *Chiasmocleis* (missing only 3 of the 34 currently recognized species). We used the phylogenetic information to understand patterns of species diversity, morphological evolution, and historical biogeography of this poorly known genus of Neotropical frogs. We found that in addition to the 34 currently valid nominal species, *Chiasmocleis* might contain as many as 22 additional cryptic lineages. The species diversity of *Chiasmocleis* has been shaped by recurrent connections between the Amazon and the Atlantic Forest. Interestingly, osteological patterns reflect the biogeographical history.

2. Material and methods

2.1. Material

Specimens and tissue samples of *Chiasmocleis* used in this study are deposited in the following collections: Departamento de Zoologia, Universidade Estadual Paulista, campus Rio Claro (CFBH); Museo Nacional de Ciencias Naturales (MNCN), Madrid, Spain, Museu de História Natural Capão da Imbuia, Curitiba (MHNCI); Museu Nacional, Rio de Janeiro (MNRJ); Museu de Zoologia, Universidade de São Paulo, São Paulo (MZUSP); Coleção Herpetológica Osvaldo Rodrigues da Cunha, Museu Paraense Emilio Goeldi, Belém, Brazil (MPEG), Coleção de Mamíferos e Tecidosnimais da Universidade Federal do Espírito Santo, Vitória, Espírito Santo (CTA and LGA); Coleção da Universidade Federal do Mato Grosso do Sul (UFMS); and Museu de Zoologia, Universidade Federal da Bahia, Salvador (UFBA); Coleção de Herpetologia da Universidade de Brasília, Brasília (CHUNB); Museo de Zoología, Pontifica Universidad Católica del Ecuador, Quito, Ecuador (QCAZ); Coleção de Tecidos e DNA da Universidade Federal do Espírito Santo (CTA); Museu de Zoologia da Universidade Estadual de Santa Cruz, Ilhéus, Bahia, Brazil (MZUESC).

2.2. Taxonomic sampling

Outgroups used are a subsampling of taxa used previously to assess microhylid relationships (de Sá et al., 2012). The current phylogeny was rooted using *Xenopus laevis* (Family Pipidae), and we included the following ranoid frog families (as distant outgroups): *Hemisus* (Family Hemisotidae), *Breviceps mossambicus, Callulina kisiwamsitu,* and *Spelaeophryne methneri* (Family Brevicipitidae), *Ptychadena anchietae* (Family Ptychadenidae), *Amietia angolonesis* and *Tomopterna tuberculosa* (Family Pyxicephalidae), *Lithobates* spp. (Family Ranidae), and *Polypedates leucomystax* (Family Rhacophoridae). We included representatives of the following subfamilies of Microhylidae: Kalophryninae (*Kalophrynus interlineatus*), Otophryninae (*Otophryne robusta*, *O. steyermarki*, and *Synapturus* sp.), Asterophryninae (*Asterophrys slateri*, *Callulops* sp., *Cophixalus* sp., *Oreophryne* sp., *Sphenophryne* sp., and *Xenorhina obesa*), Cophylinae (*Anodonthohyla* sp., *Platypelis grandis*, *Plethodontohyla* sp., and *Rhombophryne testudo*), Scaphiophryninae (*Scaphiophryne calcarata* and *S. madagascariensis*), Dyscophinae (*Dyscophus antongilii* and *D. guineti*), Microhylinae (*Glyphoglossus molossus*, *Kaloula picta* and *K. pulchra, Microhyla heymonsi* and *M. ornata, Micryletta inornata*, and *Uperodon* sp. and *U. variegatus*), and Phrynomerinae (*Phrynomantis microps*).

The analysis included all currently recognized genera of the New World Gastrophryninae. Moreover, out of the 74 species currently described in this subfamily, our taxonomic sampling includes 57 species (77%). Among the species outside our focal group (i.e., *Chiasmocleis*) are: *Arcovomer passarellii*, *Ctenophryne aequatorialis*, *C. aterrima*, *C. barbatula*, and *C. geayi*, *Dasypops schirchi*, *Dermatonotus muelleri*, *Elachistocleis bicolor*, *E. helianneae*, *E. panamensis*, *E. pearsei*, and *Elachistocleis* sp., *Gastrophryne carolinensis*, *G. elegans*, *G. olivacea*, *Hamptophryne alios*, and *H. boliviana*, *Hypopachus barberi*, *H. pictiventris*, *H. ustus*, and *H. variolosus*, *Myersiella microps*, and *Stereocyclops histrio* and *S. incrassatus*. However, our dataset is missing the monotypic

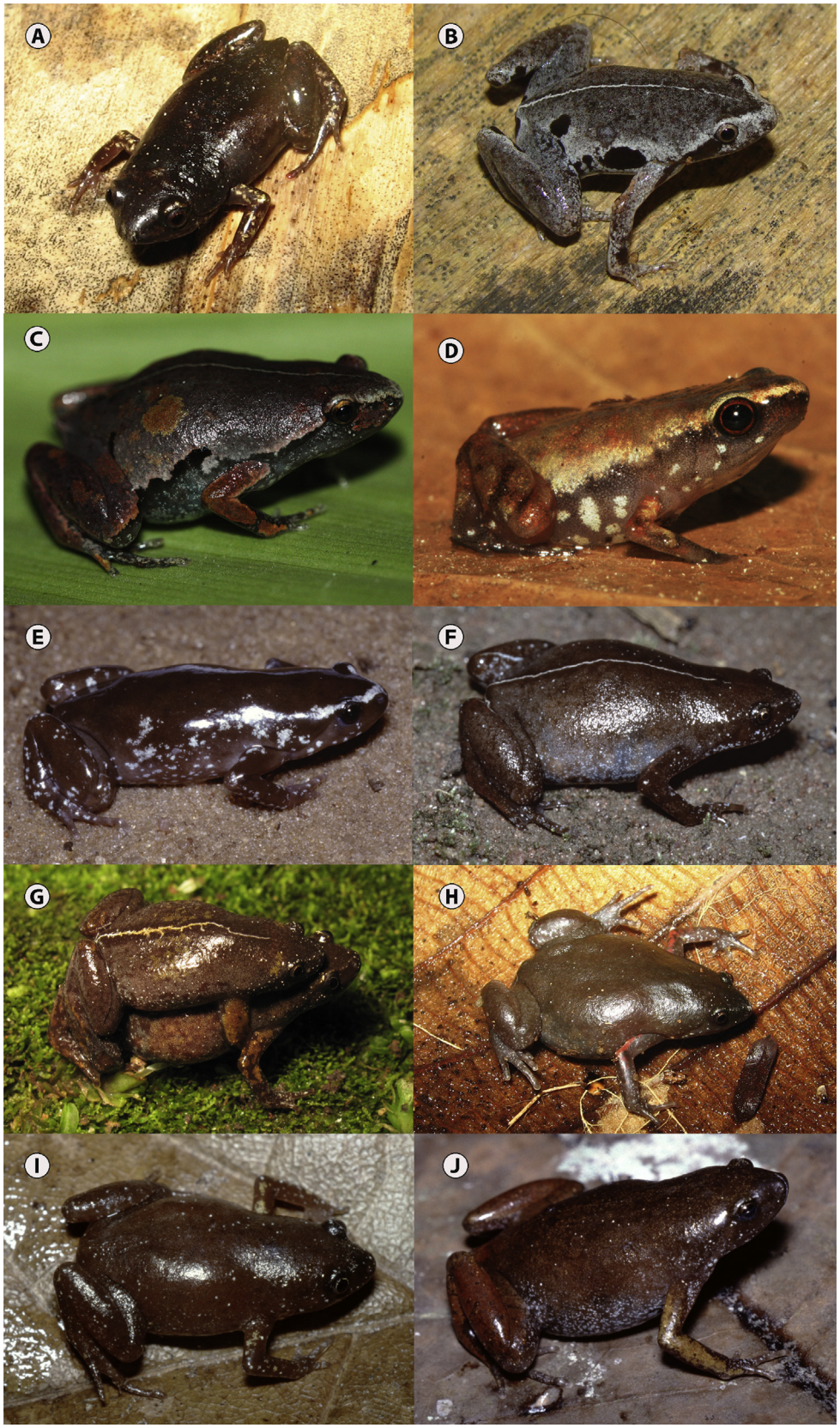

Fig. 1. Diversity of the genus *Chiasmocleis*. A. *Chiasmocleis* (*Relictus*) *gnoma*, B. *Chiasmocleis* (*Syncope*) *supercilialba*, C. *Chiasmocleis* (*Syncope*) *bassleri*, D. *Chiasmocleis* (*Syncope*) *tridactyla*, E. *Chiasmocleis* (*Chiasmocleis*) *albopunctata*, F. *Chiasmocleis* (*Chiasmocleis*) *leucosticta*, G. *Chiasmocleis* (*Chiasmocleis*) *mantiqueira*, H. *Chiasmocleis* (*Chiasmocleis*) *crucis*, I. *Chiasmocleis* (*Chiasmocleis*) *lacrimae*, J. *Chiasmocleis* (*Chiasmocleis*) *capixaba*. Photos: A, B, and H, courtesy of Marcos Freitas; C and D, courtesy of Mariela Osorno Muñoz, E, F, G, I, and J courtesy of Celio F B Haddad.

subfamily Adelastinae, one species of *Gastrophryne* (*G. mazatlanensis*), two species of *Ctenophryne* (*C. carpish* and *C. minor*), two species of *Stereocyclops* (*S. palmipes* and *S. parkeri*), and 11 species of *Elachistocleis* (*E. bumbameuboi*, *E. cesarii*, *E. erythrogaster*, *E. haroi*, *E. magnus*, *E. matogrosso*, *E. muiraquitan*, *E. piauiensis*, *E. skotogaster*, *E. surinamensis*, and *E. surumu*).

Within *Chiasmocleis* our sampling includes 31 of the 34-recognized species. The missing taxa are *C. atlantica*, *C. migueli*, and *C. sapiranga*. We sampled multiple individuals (ranging from 1 to 19) from several populations across species geographic distributions to account for phylogeographic structure and cryptic species diversity. The total number of *Chiasmocleis* samples is 213 individuals.

2.3. Molecular methodology

Total genomic DNA was extracted from ethanol-preserved liver or muscle tissues using Qiagen DNeasy kit (Valencia, California, USA). The molecular markers used to assess phylogenetic relationships are the mitochondrial ribosomal markers 12S and 16S, mitochondrial protein-coding markers NADH dehydrogenase subunit 2 (*ND2*), and Cytochrome Oxidase 1 (*COI*), nuclear ribosomal marker 28S, nuclear protein-coding markers Brain-derived neurotrophic factor (*BDNF*), Seven-in-absentia (*SIA*), and Tyrosinase (*TYR*). Markers were amplified using previously published primer sets and PCR profiles (de Sá et al., 2012; Tonini et al., 2014). PCR products were purified using USB ExoSap-IT (US78201, Amersham Biosciences, Piscataway, New Jersey, USA) and sequenced (in both primer directions) by SeqWright Corp. (Houston, Texas, USA; www.seqwright.com). Resulting chromatograms were visualized and cleaned using the program Sequencher 5.0 (Gene Codes Corp., Ann Arbor, Michigan, USA). Our dataset includes new molecular information for *Chiasmocleis* and sequences available on GenBank for Gastrophryninae (de Sá et al., 2012; Tonini et al., 2014; Forlani et al., 2017). DNA sequences generated for this study were submitted to GenBank; accession numbers are: MH884918—MH885042 (*12S*), MH9198849—91977 (*16S*), MH921195—921240 (*28S*), MH935344—935423, MH035344 (*BDNF*), MH935423—935509 (*TYR*), MH939995—940095 (*SIA*), MH940096—940116 (*ND2*), and MH940117—940164 (*COI*); GenBank records include specimen voucher numbers and locality data.

2.4. Phylogenetic analyses

We aligned each molecular marker individually with SATé v2.2.7 (Yu and Holder, 2016) using the default settings (Liu et al., 2012).

We used two approaches to estimate the phylogeny. First, we concatenated all the genes into a supermatrix and used PartitionFinder v2 (Lanfear et al., 2012) to estimate the best partition scheme and substitution models according to Bayesian Information Criterion (BIC) using the greedy algorithm (Table 1). As input to PartitionFinder analysis, we divided the protein-coding genes *ND2*, *COI*, *BDNF*, *SIA*, and *TYR* by codon position, whereas the 12S, 16S, and 28S were kept separately representing three partitions. Second, the best partition scheme was used to estimate the phylogeny using Maximum Likelihood in RAxML (Stamatakis, 2014) and Bayesian phylogenetic inference and divergence time estimation in MrBayes v3.2. In MrBayes, we unlinked across partitions the parameters for nucleotide frequency, GTR rate matrix, gamma distribution of rate variation, and the proportion of invariable sites. We used node-age calibration and the fossilized birth-death model (Heath et al., 2014) to time-calibrate the phylogeny (fixing the fossilization prior to 0 since in our data we include only extant species). This model improves precision of divergence times even when applied to extant data on extant species only (e.g., Puttick and Thomas, 2015). Finally, we rooted the phylogeny with *Xenopus laevis* and used a uniform distribution with intervals 153.1 and 192.3 my representing the divergence interval between Pipanura and Ranoidea to calibrate the root node age (Cannatella, 2015). We calibrated the node representing the initial divergence of Ranoidea using a uniform distribution with intervals 92 and 123 my and for the divergence of microhylids 66 and 94 my (after Zhang et al., 2013). We used a lognormal clock rate with the mean calculated as tree height divided by the root age (the median divergence time of Pipanura from Cannatella, 2015), and a broad offset (mean −6.16 and offset 1.004). In MrBayes, we set up eight runs, each with eight chains, of 100 million generations, sampling the posterior distribution every 2000th generation. We used the first 25% samples of the posterior distribution as burn-in. At the end of the analysis the split frequency was lower than 0.01, and we checked for convergence and Effective Sample Size (ESS) using Tracer v1.6 (Rambaut et al., 2014).

As a second approach to estimate the *Chiasmocleis* phylogeny we also used ASTRAL-III v5.5.9 (Mirarab et al., 2014; Mirarab and Warnow, 2015; Sayyari and Mirarab, 2016; Zhang et al., 2017). ASTRAL is a coalescent species-tree method that reconciles gene tree discordance by modelling the process of incomplete lineage sorting under the multispecies coalescent model (Rannala and Yang, 2003). For the analysis in ASTRAL we did not concatenate the genes; instead, we estimated the substitution models for each gene separately using jModelTest v2 (Darriba et al., 2012; Guindon and Gascuel, 2003). The best model according to BIC was used in the phylogenetic analyses. We used BEAST v2.4.2 (Bouckaert et al., 2014) to estimate gene trees, which we used as input for ASTRAL. In BEAST, we used Yule model as tree prior on gene trees, a relaxed clock model with lognormal distribution, and the mean clock rate and standard deviation were estimated using a log normal distribution. For each gene, we set up five runs of the BEAST analyses: each run had 100 million generations, sampling the posterior distribution every 5000th generation. We checked for convergence among the multiple runs for each gene using Tracer v1.6 (Rambaut et al., 2014), and Effective Sample Sizes (ESS) higher than 200 were considered suitable mixing. Converging runs were combined using LogCombiner to increase the posterior sample of trees and to improve ESS values. We re-sampled the posterior distribution at a lower frequency and applied a 25% burn-in to retain a sample of 10,000 posterior gene trees for each gene. From these, we took a sample of 100 posterior trees of each gene (i.e., 800 posterior gene trees) to estimate the species tree in ASTRAL. Then, the whole posterior distribution of trees after the burn-in (i.e., 600,000 posterior gene trees) was used to score the branches of the ASTRAL species tree. ASTRAL normalized quartet score represents the percentage of quartets observed in the gene trees that are present in the species tree. Normalized quartet score values equal to or higher than 0.8 or 80% were considered suitable. We estimated branch support as the percentage of quartets in the gene trees that agree with a branch in the species tree (Sayyari and Mirarab, 2016). We estimated local posterior probabilities for the main topology and one for each of the two alternative ones. The posterior of the three topologies adds up to one because ASTRAL assumes that the four groups around the branch are correct and, therefore, there are only three possible alternatives (Sayyari and Mirarab, 2016). The measures of branch lengths, quartet support, and alternative local posterior

Table 1
Models and partitions applied to molecular data.

Best model	Subset partitions	Subset sites
GTR + I + G	12S, 16S, ND2_1	1–873, 874–1437, 3410–4222\3
SYM + I + G	28S	1438–2192
K80 + I + G	BDNF_1, BDNF_2	2193–2811\3, 2194–2811\3
K80 + G	BDNF_3	2195–2811\3
HKY + G	COI_1, ND2_3	2812–3409\3, 3412–4222\3
SYM + I + G	COI_2	2813–3409\3
F81 + I	COI_3	2814–3409\3
GTR + G	ND2_2	3411–4222\3
SYM + I + G	SIA_1, TRY_2, TYR_1	4223–4678\3, 4679–5212\3, 4680–5212\3
K80 + G	SIA_2	4224–4678\3
GTR + I + G	SIA_3, TYR_3	4225–4678\3, 4681–5212\3

probability have been demonstrated to have high precision in simulations and empirical datasets with different levels of incomplete lineage sorting (Sayyari and Mirarab, 2016). We ran the phylogenetic analyses on Colonial One, the high-performance computer cluster at George Washington University, Washington, DC.

2.5. Species discovery

We investigated the existence of cryptic lineages using the time-calibrated phylogeny with population-level samples of *Chiasmocleis*. We used the consensus phylogeny from MrBayes analysis and a sample of 1000 posterior trees to run the Generalized Mixed Yule Coalescent model (GMYC) using the Maximum Likelihood and Bayesian versions (Reid and Carstens, 2012; Fujisawa and Barraclough, 2013). This model tests whether the diversification history could be explained under a population coalescent model or under a speciation model (e.g., Yule). Furthermore, the model assigns membership probabilities of individuals to species providing hypotheses on the species diversity within the focal group. In the maximum likelihood version (GMYC) we used the consensus topology as input to test species hypotheses, and used AIC and AIC weights to choose the better fit model on the number of potential species within *Chiasmocleis*. Moreover, we applied the multi-model comparison proposed by Powell (2012). We ran this analysis using the R package *splits* (Ezard et al., 2009; Fujisawa and Barraclough, 2013), in R version 3.4.3 (R Development Core Team, 2017).

In the Bayesian version (*bGMYC*), we used a sample of posterior trees to accommodate phylogenetic uncertainty into the species-discovery analyses in the R package *bGMYC* (Reid and Carstens, 2012). For each pair of DNA sequences, this method estimates the posterior probability that individuals are conspecific. The probability that two or more lineages were conspecific was estimated by reporting ranges of posterior probabilities among sequences from different lineages. For instance, we define 0.05 as the *ad hoc* threshold of a given individual to belong to another species cluster (e.g., representing 95% posterior probability in Bayesian analyses) and reported the number of identified species and individual relationships. In addition, as upper-bound thresholds we apply increments of 0.05 until the model identifies all the currently recognized species in the dataset as separate entities. This approach also identifies hidden and cryptic evolutionary lineages.

2.6. Historical biogeography

We used the R package BioGeoBEARS (Matzke, 2013a) to test hypotheses of the historical biogeography of *Chiasmocleis*. We used the models DEC, DIVALIKE, and BAYESLIKE to test whether biogeographic processes of dispersal, extinction, cladogenesis, and founder events have shaped the species distribution (Matzke, 2013b). To improve accuracy of estimations of ancestral areas at basal nodes of *Chiasmocleis*, we estimated the biogeographic history of the subfamily Gastrophryninae. This monophyletic subfamily is the largest microhylid radiation in the Americas, and many other gastrophrynines are sympatric with *Chiasmocleis*. Our taxonomic sample includes 77% of the currently described species and a complete generic representation of Gastrophryninae. The missing species are concentrated in the second (after *Chiasmocleis*) largest genus within the subfamily, i.e., *Elachistocleis*. Thus, estimates of ancestral areas for nodes outside the focal group (*Chiasmocleis*) should be interpreted with caution. Here, we discuss the results of ancestral estimates to *Chiasmocleis* species and the root node of the genus. Additional comprehensive studies are needed to discuss patterns of biogeography of the Gastrophryninae.

Species of Gastrophryninae are distributed across the: (1) Nearctic region in US, (2) the Mexican province, (3) tropical Middle America including areas in central and northern South America, (4) Altiplano including the high-altitude areas of the Andes slopes, (5) Amazon basin, (6) open savanna of Cerrado, (7) open savanna of Chaco, and (8) Atlantic Forest (e.g., Olson et al., 2001). We assigned species to these eight ecoregions based on distribution maps available from IUCN Red List, Amphibia Web, Map of Life, as well as our own knowledge.

We ran analyses varying the area states allowed in biogeographic reconstructions and maximum number of areas that species could occupy (unconstrained included the eight biogeographic areas and constrained included five areas). We set the maximum number of ecoregions possibly occupied by species of gastrophrynines to five since this number corresponds to the maximum number of ecoregions occupied by a Gastrophryninae species (e.g., populations formely known as *Elachistocleis "ovalis"*).

The current geographic distributions of Gastrophryninae, and *Chiasmocleis* cover non-adjacent Neotropical ecoregions (e.g., Olson et al., 2001); thus, we excluded area states (see definition below) that would estimate non-adjacent areas as next to each other. Non-adjacent areas can be reconstructed as ancestral state if intervening regions are included in the state. For example, A and B are separated by C. Thus, A, B, and C, are areas and A, B, C, AB, AC, BC, and ABC are area states. In our analyses, we removed states analogous to AB since they are non-adjacent in the above hypothetical example. We chose the best fit model based on AICc and AIC weights (Supplement Information Table 1).

3. Results

3.1. Phylogenetic systematics of Chiasmocleis

The phylogenetic trees using both concatenation and species-tree methods recover a monophyletic *Chiasmocleis,* as currently defined, with an overall structure of well-supported clades. Surprisingly, *Chiasmocleis gnoma* is the sister species of all other species in the genus. The remaining species of *Chiasmocleis* are divided into two well-supported clades: a larger one containing 22 species and a second, smaller clade consisting of 10 species, including all species formerly assigned to the genus *Syncope* Walker, 1973 (Fig. 2).

Differences between the concatenation analyses (using RAxML and MrBayes, Supplement Information Figs. 1 and 2) and the ASTRAL species tree (Supplement Information Fig. 3) are restricted to the clades: (i) *C. mantiqueira* and *C. altomontana*; (ii) *C. alagoana*, *C. cordeiroi*, *C. crucis*, and *C. schubarti*; and (iii) *C. antenori* and *C. magnova*. In the concatenation, we recover a monophyletic *C. mantiqueira* with individuals from Desengano (São Paulo state, SP), Campos do Jordão (SP), and Piquete (SP) as more closely related to each other. These samples form a clade that is the sister group to individuals of *C. mantiqueira* from Serra do Brigadeiro (Minas Gerais state, MG) and Ouro Branco (MG). However, in the ASTRAL tree we recover non-monophyletic *C. mantiqueira*, in which *C. altomontana* is the sister species to populations of *C. mantiqueira* from Minas Gerais state. The samples from São Paulo state form the sister group of *C. altomontana* and *C. mantiqueira* from MG.

Furthermore, in the concatenated analyses (Supplemental Information Figs. 1 and 2) *Chiasmocleis crucis* is the sister taxon to the clade containing *C. schubarti*, *C. alagoana*, and *C. cordeiroi*. Moreover, in the concatenation *C. alagoana* and *C. cordeiroi* are more closely related to each other than to *C. schubarti*. In the ASTRAL species tree, *C. alagoana* is the sister species to *C. cordeiroi* as well, and these species are the sister group to a clade formed by *C. crucis* and *C. schubarti*. Branch support in the concatenation was low for the phylogenetic relationships among these species, suggesting that incomplete lineage sorting might have higher impact in estimating relationships for these species. Similarly, in the concatenation and ASTRAL tree (Supplemental Information Fig. 3), the species relationships in the clade comprising *C. antenori* and *C. magnova* had low branch support and a distinct topology. Lineages in this clade have very short internodes between species, suggesting a rapid diversification resulting in poor resolution by the set of molecular markers used here for *Chiasmocleis*.

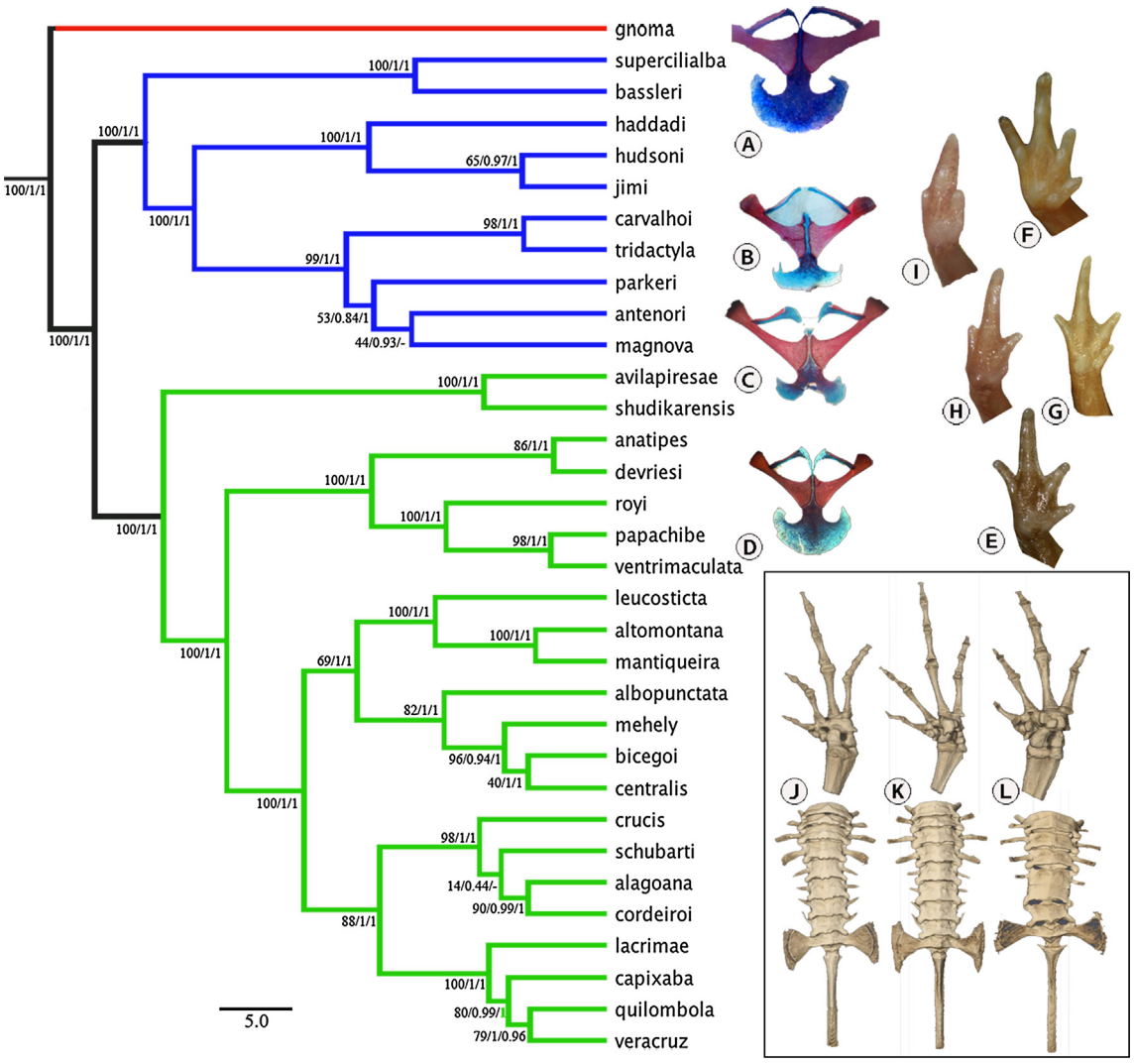

Fig. 2. Phylogeny of the genus *Chiasmocleis*: red = subgenus *Relictus*, a taxon isolated in the Atlantic Forest since the Eocene, blue = subgenus *Syncope*, an Amazonian clade that shows size reduction in body size and includes a clade of miniaturized species with reduction and/or loss of digits, and green = subgenus *Chiasmocleis*, the largest clade within the genus inhabiting Amazonia, Atlantic Forest, and the dry diagonal consisting of Chaco and Cerrado environments. Branch support indicates bootstrap estimated in RAxML, posterior probability estimated in MrBayes, and local quartet posterior probability estimated in ASTRAL, respectively. Dashes indicate that a given node was not recovered in a given method. A–D = ventral view of clear and stained pectoral girdles; E–I = ventral view of right hands; J–L = skeletal element of hands and vertebral column. Epicoracoid cartilages connecting to procoracoid cartilages and coracoid bones**:** A. *Chiasmocleis* (*Relictus*) *gnoma* = epicoracoid present and continuous between procoracoids and coracoids, B. *Chiasmocleis* (*Syncope*) *antenori* = epicoracoid connection entirely lost, *Chiasmocleis* (*Syncope*) *magnova* = epicoracoid connection partially eroded, D. *Chiasmocleis* (*Chiasmocleis*) *shudikarensis* = present and continuous. Reduction in hand digits: E = *Chiasmocleis* (*Chiasmocleis*) *corderoi*, no reduction in digits; F and G = *Chiasmocleis* (*Syncope*) *hudsoni* and *Chiasmocleis* (*Syncope*) *magnova*, reduction of digits I and IV; H and I = lost of digit I, reduction of digits II and IV. Vertebral column and right hand: J = *Chiasmocleis* (*Relictus*) *gnoma*, K = *Chiasmocleis* (*Chiasmocleis*) *quilombola*, and L = *Chiasmocleis* (*Syncope*) *antenori*. The subgenus *Syncope* shows reduction of phalanges and fusion of vertebral elements. Images of pectoral girdle of *C. gnoma* provided by Dr. Jose Pombal, other pectoral images hands by Mauricio C. Forlani, and 3D reconstructions of hands and vertebral column by Hannah VanHuss. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Cryptic species diversity

The species discovery methods using ML and Bayesian analyses are concordant in estimating similar numbers of cryptic lineages. In the ML, the number of lineages within *Chiasmocleis* ranges from 34 to 53 (Table 1). In the Bayesian analysis, the lower-bound threshold (conspecific probability = 0.05) suggests that the diversity within *Chiasmocleis* might be as low as 27 lineages, whereas the upper-bound threshold (conspecific probability = 0.85, necessary to recover all currently described species in the dataset) estimates this number as high as 55 (Fig. 3). Notably, species with marked phylogeographic structure were identified as comprising cryptic lineages even when using the lower-bound threshold, e.g., *C. bassleri*, *C. hudsoni*, *C. carvalhoi*, and *C. tridactyla*. The model also recovers closely related species in very short branches as being a single evolutionary unit (e.g., *C. papachibe* and *C. ventrimaculata*), and to recover them as separate lineages, we had to increase the conspecific threshold up to 0.85, which also increases overall diversity in the genus to 55 cryptic lineages (Fig. 3).

3.3. Historical biogeography

The DEC model accounting for range expansion through a founder event (+j) is a better fit to the data (AICc = 207.8, wAIC = 0.97; Supplemental Information Table 1). Although the biogeographic reconstruction estimated a higher probability of the Atlantic Forest as being the ancestral area of the most recent common ancestor of *Chiasmocleis* (Fig. 4), the results also show support for Amazonia as the center of origin of the genus. Nonetheless, the biogeographic history of

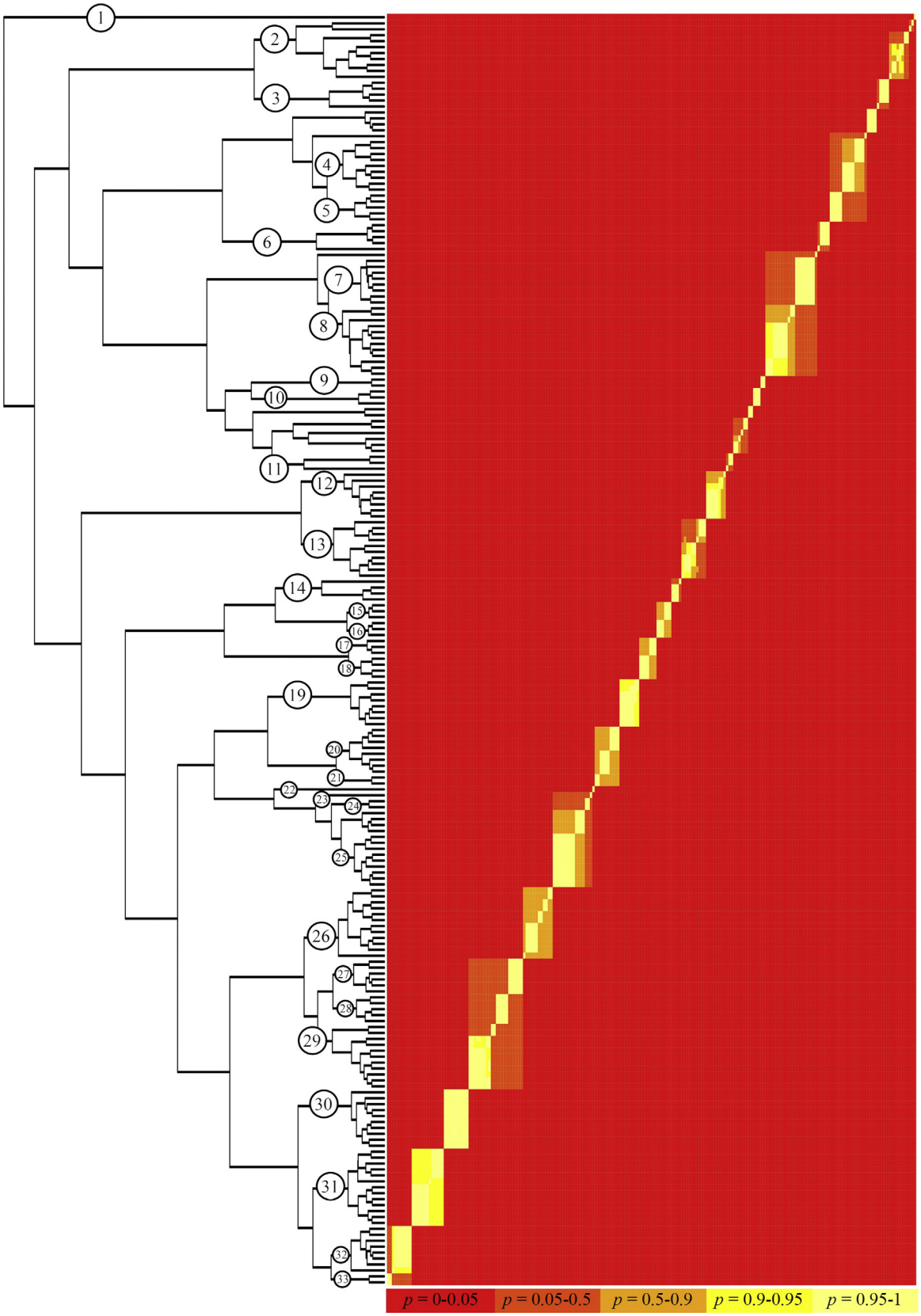

Fig. 3. Results of the species-discovery analyses using Bayesian GMYC. Numbers correspond to currently described species of *Chiasmocleis*. 1. *C. gnoma*; 2. *C.* (*Syncope*) *bassleri*; 3. *C.* (*Syncope*) *supercilialba*; 4. *C.* (*Syncope*) *jimi*; 5. *C.* (*Syncope*) *hudsoni*; 6. *C.* (*Syncope*) *haddadi*; 7. *C.* (*Syncope*) *tridactyla*; 8. *C.* (*Syncope*) *carvalhoi*; 9. *C.* (*Syncope*) *magnova*; 10. *C.* (*Syncope*) *antenori*; 11. *C.* (*Syncope*) *parkeri*; 12. *C. shudikarensis*; 13. *C. avilapiresae*; 14. *C. royi*; 15. *C. papachibe*; 16. *C. ventrimaculata*; 17. *C. devriesi*; 18. *C. anatipes*; 19. *C. leucosticta*; 20. *C. mantiqueira*; 21. *C. altomontana*; 22. *C. albopunctata*; 23. *C. mehely*; 24. *C. centralis*; 25. *C. bicegoi*; 26. *C. lacrimae*; 27. *C. veracruz*; 28. *C. quilombola*; 29. *C. capixaba*; 30. *C. crucis*; 31. *C. schubarti*; 32. *C. cordeiroi*; 33. *C. alagoana*.

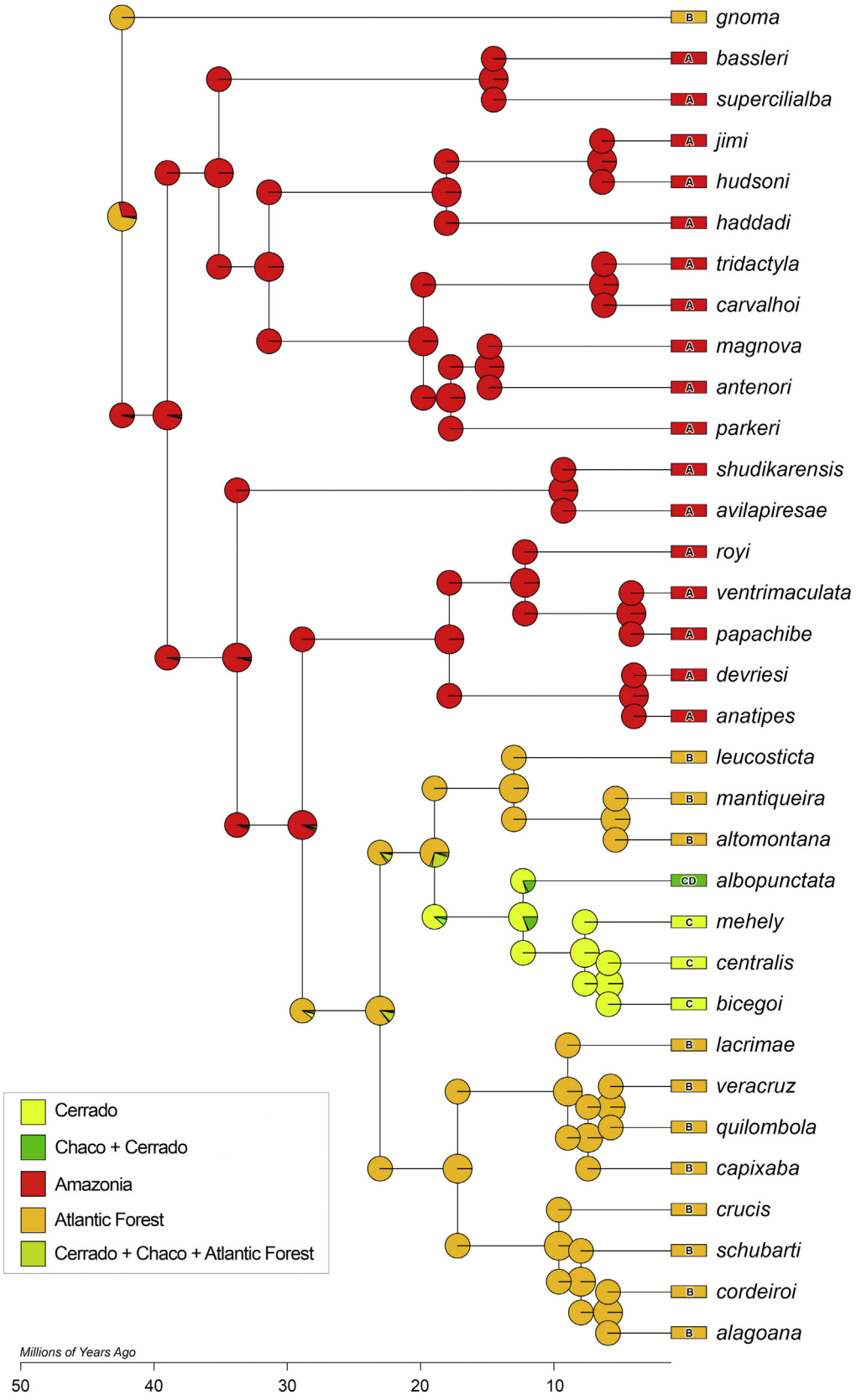

Fig. 4. Historical biogeography of *Chiasmocleis* estimated by the DEC + j model. Pie charts represent the probability of a given biogeographic area being ancestral. Letters indicate bioregions that species are known to occupy: A, Amazonia; B, Atlantic Forest; C, Cerrado; D, Chaco.

Chiasmocleis is marked by multiple connections between Amazonia and the Atlantic Forest. *Chiasmocleis gnoma* diverged from a hypothesized widespread and forest dwelling ancestor that became isolated in the Atlantic Forest at the Eocene (mean age 42.4 my, 95% HPD = 35.05–51.13). Diversification within Amazonia includes species previously assigned to *Syncope*, with miniaturized body size, bone fusions, and digit reduction or loss. In addition, typical *Chiasmocleis* species, i.e., with larger body size, without bone fusions, also diversified within Amazonia, e.g., clade including *C. avilapiresae* and *C. shudikarensis*, and another clade including *C. royi*, *C. ventrimaculata*, *C. papachibe*, *C. devriesi*, and *C. anatipes*.

During the Oligocene-Miocene (about 28.87–23.04 my) an Amazonian ancestor expanded its distribution to the Atlantic Forest, resulting in species diversification and endemicity within the Atlantic Forest biome. The Northern (i.e., *C. alagoana*, *C. crucis*, *C. cordeiroi*, and *C. schubarti*) and Central (*C. veracruz*, *C. quilombola*, *C. capixaba*, and *C. lacrimae*) Atlantic Forest clades are sister taxa and began to diversify about 17 my ago (95%HPD = 12.52–22.33). Meanwhile, the Southern Atlantic Forest clade consisting of *C. leucosticta*, *C. mantiqueira*, and *C. altomontana* has a most recent common ancestor (mean divergence of 19 my and 95%HPD = 14.03–24.24) with species endemic to the Cerrado and Chaco (i.e., *C. albopunctata*, *C. mehely*, *C. centralis*, and *C. bicegoi*).

4. Discussion

4.1. Phylogeny and species relationships of Chiasmocleis

A few studies have recently focused on understanding relationships among species of *Chiasmocleis* (de Sá et al., 2012; Peloso et al., 2014; Almendáriz et al., 2017). Phylogenetic analysis at the generic level among Gastrophryninae recovered a few species of *Chiasmocleis* (i.e., *C. bassleri*, *C. hudsoni*, *C. jimi*, and *C. magnova*) closer to species of *Syncope* than to other species of *Chiasmocleis* and transferred those species to the genus *Syncope* Walker, 1973 (de Sá et al., 2012). In contrast, *C. bassleri* was recovered as the sister taxon to the clade consisting of *Syncope* and *Chiasmocleis* (Peloso et al., 2014); consequently, the authors placed the genus *Syncope* Walker, 1973 in the synonymy of *Chiasmocleis*. More recently, Almendáriz et al. (2017) described a new and small species of Gastrophryninae from Ecuador (i.e., *Chiasmocleis parkeri*), and it also placed *C. bassleri* as the sister taxon to other smaller species of *Chiasmocleis*. Our analyses recovered *C. bassleri* and *C. supercilialba* as sister taxa; furthermore, this pair of species show a sister-taxon relationship to all other *Syncope*-like species, i.e., smaller-sized *Chiasmocleis* with bone reductions and fusions in agreement with most previous studies (i.e., de Sá et al., 2012; Almendáriz et al., 2017) (Fig. 2).

Chiasmocleis gnoma has not been included in any previous phylogenetic analyses, but the original description assigned the species to the *C. schubarti* species group (*sensu* Cruz et al., 1997). The authors hypothesized that *C. gnoma* would be closely related to other Atlantic Forest endemic species (i.e., *C. alagoana*, *C. atlantica*, *C. cordeiroi*, *C. crucis*, and *C. schubarti*) based on external morphology, e.g., vestigial or absent foot webbing (Canedo et al., 2004). Strikingly, our molecular hypothesis recovered *C. gnoma* as the sister taxon to all other species in *Chiasmocleis*. This Atlantic Forest endemic species is known only from the type locality Municipality of Una, State of Bahia, Brazil, (15°10′S, 30°03′W; Canedo et al., 2004). *Chiasmocleis gnoma* has characteristics shared with both *Syncope* and *Chiasmocleis* that would support the phylogenetic hypothesis (see below).

4.2. Taxonomy and species discovery of Chiasmocleis

Over the last half-century, with the spread of phylogenetic systematics (Henning, 1950, 1966), taxonomic changes are done to reflect phylogenetic relationships among different groups of organisms [e.g., *Bufo marinus* (Schneider, 1799), assigned to the genus *Chaunus* (Frost et al., 2006, then to the genus *Rhinella* Chaparro et al., 2007), and subsequently placed back under *Bufo* (Fouquette and Dubois, 2014) but see comment in Frost, 2017]. However, taxonomic changes should also provide information on other aspects of the biology and diversity of the organisms under study. The present analysis recovered the genus *Chiasmocleis* with a basal split separating *C. gnoma* from all other species, with the latter group forming two distinct clades. One of these clades includes most *Chiasmocleis* species (22 species) and the second clade (10 species) includes *C. bassleri* and *C. supercilialba* as the sister taxon to a clade of species formerly in the genus *Syncope* (Walker, 1973; de Sá et al., 2012). We recognized the larger clade as the subgenus *Chiasmocleis,* and for the smaller clade we resurrect the available name *Syncope* Walker, 1973 as a subgenus to reflect its unique history and phylogenetic relationships, but also to highlight the morphological patterns of miniaturization, digit reduction, and bone fusion that correlates with the tree topology. These actions require, for logical consistency, to create a subgenus for the *C. gnoma* lineage (i.e., currently represented by one living species but potentially undescribed or extinct ones as well). We create a new subgenus *Relictus* to accommodate the lineage represented by *Chiasmocleis gnoma* Canedo et al., 2004. The name derived from the Latin "*relictus*", meaning "left behind", acknowledges the long separate history of this lineage from the other two clades of *Chiasmocleis*. Thus, we recognize the following subgenera: *Chiasmocleis* [type species *Chiasmocleis* (*Chiasmocleis*) *albopunctata* (Boettger, 1885], *Syncope* [type species *Chiasmocleis* (*Syncope*) *antenori* (Walker, 1973), and *Relictus* subgen. nov. [type species *Chiasmocleis* (*Relictus*) *gnoma*]. These actions follow Articles 43, 44, and 61 of the International Code of Zoological Nomenclature (ICZN, 1999)] to accommodate the smaller-size species of *Chiasmocleis* and highlight the historical taxonomic content associated with miniaturization and evolution of osteological novelties. Moreover, *Relictus* acknowledges the long unique evolutionary history of *C. gnoma* within the Atlantic Forest.

Most of the currently valid species are recovered as monophyletic. However, we found a couple of species that deserve further taxonomic considerations. Our extensive sampling of multiple individuals allowed us to test whether previously synonymized species of *Chiasmocleis* would actually represent full species. *Chiasmocleis* (*Syncope*) *jimi* was considered a synonym of *Chiasmocleis* (*Syncope*) *hudsoni* given the morphological, behavioral, and acoustic similarities between the two species, and because they were not recovered as monophyletic (Peloso et al., 2014). However, our species discovery analysis challenges that decision. We used a lower threshold (see above 3.2), which would force the placement of several species that are morphologically distinct (e.g., *C. carvalhoi* and *C. tridactyla*; *C. papachibe* and *C. ventrimaculata*, etc.) in synonymy as single evolutionary lineages. In contrast, to recover most of the currently valid nominal species we estimate as many as 55 cryptic lineages. Among those potential evolutionarily independent lineages, we identified three clades within *Chiasmocleis* (*Syncope*) *jimi/hudsoni*. These results suggest that *Chiasmocleis* (*Syncope*) *hudsoni* and *Chiasmocleis* (*Syncope*) *jimi* could be valid species. The species-discovery analyses also show that species diversity within the *jimi/hudsoni* clade is potentially higher, as well as in other Amazon species such as *Chiasmocleis* (*Syncope*) *bassleri* and species in the *Chiasmocleis* (*Syncope*) *magnova/antenori/parkeri* clades. Previous studies had shown that *Chiasmocleis* species have marked phylogenetic structure, but slight differences in external morphology (Tonini et al., 2014; Forlani et al., 2017), and our analyses identified high levels of hidden species diversity within *Chiasmocleis*. Herein, to be consistent with our species-discovery analysis, we recognized *Chiasmocleis* (*Syncope*) *jimi* Caramaschi and Cruz, 2001, as a valid and separate species from *Chiasmocleis* (*Syncope*) *hudsoni* Parker, 1940. A more extensive sampling is needed across the range of these two species; such sampling and analyses could reveal other cryptic species hidden under or confused with these two taxa.

The type species of *Chiasmocleis* (*C.* [*Chiasmocleis*] *albopunctata*) is the sister taxon to the clade that includes *C. mehely*, *C. bicegoi*, and *C.*

centralis. *Chiasmocleis bicegoi* Miranda-Ribeiro, 1920 was placed in the synonymy of *C. albopunctata* (Boettger, 1885) given the lack of morphological differences between them (Cruz et al., 1997); however, no phylogenetic hypothesis using phenotypic characters and/or molecular data was presented. Our molecular phylogenetic hypothesis recovered samples from the vicinities of the type locality of *C. bicegoi* ("Perús" [Perus], State of São Paulo, Brazil) as the sister lineage to *C. centralis* (type locality Aruanã, State of Goiás, Brazil) instead of *C. albopunctata*. *Chiasmocleis bicegoi* and *C. centralis* form the sister group to *C. mehely* Caramaschi and Cruz, 1997, whose type locality is Estância Caiman, Municipality of Miranda, State of Mato Grosso do Sul, Brazil. The type locality of *C. albopunctata* is "Paraguay" (Bauer et al., 1996), about one thousand kilometers from the type locality of *C. bicegoi*. Thus, based on our phylogenetic hypothesis and geographic distribution of the *C. albopunctata* species complex, we resurrect *C. bicegoi* Miranda-Ribeiro, 1920 as a valid species. We acknowledge that more extensive population-level studies are necessary to describe the hidden diversity in this and other poorly known *Chiasmocleis* species. Furthermore, such studies are needed in order to identify contact zones, to estimate levels and directionality of gene flow, and to understand species evolution in a spatial-ecological context.

4.3. Patterns of morphological miniaturization in Chiasmocleis

Overall anurans normally have eight presacral vertebrae, hand with four distinct digits (phalangeal formula 2-2-3-3), feet with five digits (phalangeal formula 2-3-3-4-3), and a pectoral girdle that could be either arciferal or firmisternal (Trueb, 1973; Alberch and Gale, 1985). Ventrally, the pectoral girdle consists of two pairs of bony elements: the clavicles (anteriorly placed) and the coracoids (posteriorly located). There are also two pairs of cartilages: the procoracoids, associated with the posterior edge of the clavicles, and the epicoracoids, extending from the tip of the clavicles and broadly overlapping medially through most of their length (arciferal condition) or fusing medially (firmisternal condition), and reaching and extending between the coracoids (see Trueb, 1973 for variation). Microhylid frogs have a firmisternal pectoral girdle.

Chiasmocleis species have as ancestral states: (a) epicoracoids anteriorly wide and posteriorly thin (e.g., *C. shudikarensis*, *C. albopunctata*, and *C. leucosticta*, Fig. 2D), (b) a standard phalangeal formula (2-2-3-3, Fig. 2K) and, (c) finger I with normal size and two phalanges (e.g., *C. mantiqueira*, *C. albopunctata*, *C. crucius*, *C. alagoanus*, *C. schubarti*, *C. shudikarensis*, and *C. leucosticta*, Fig. 2e). The subgenus *Chiasmocleis* largely retains these ancestral characters.

Chiasmocleis gnoma is a small species (SVL 13.4–17.0 mm). The species has an eroded epicoracoid cartilage that is reduced to a thin connection extending from the procoracoid cartilage to and between the coracoid bones (Fig. 2a). The species has no fusions in the vertebral column (Fig. 2j) (i.e., like most species of *Chiasmocleis*, Fig. 2K); however, finger I is reduced consisting of a reduced metacarpal and a single and very small phalange (Canedo et al., 2004), i.e., one phalange lost (i.e., like most species of the subgenus *Syncope*, Fig. 2J).

Chiasmocleis subgenus *Syncope* shows a remarkable and phylogenetically traceable size reduction with miniaturized species that exhibit reduction or loss of phalanges and digits and erosion of the epicoracoid cartilages. The erosion of these cartilages ranges from partial to the complete loss of the epicoracoid connection between the anterior procoracoid cartilages and the posterior coracoid bones (epicoracoids restricted to small remains between the coracoid). Within the subgenus *Syncope*, the sister pair of *Chiasmocleis* (*Syncope*) *bassleri-supercilialba* retains more ancestral characters in contrast to the following derived traits of its sister clade: (1) an overall smaller body size and miniaturized species, including the smallest species of Neotropical microhylids (maximum SVL = 14 mm), (2) species possessing six to eight presacral vertebrae due to vertebral fusions (Fig. 2L), and (3) species with reduced and/or lost fingers I and IV (Walker, 1973) (Fig. 2H, I and L). Miniaturized species show reduction and/or loss of phalanges (e.g., *C.* [*Syncope*] *antenori* and *C.* [*Syncope*] *parkeri* [1-2-3-2] and *C.* [*Syncope*] *tridactyla* [1-1-3-1]) and have fusion of vertebrae (e.g. *C.* [*Syncope*] *antenori*, *C.* [*Syncope*] *parkeri*; *C.* [*Syncope*] *carvalhoi*, *C.* [*Syncope*] *tridactyla*, Fig. 2L). In addition, they have lost most or entirely the epicoracoid cartilage; consequently, the connection between the procoracoid cartilages and coracoid is eroded or lost (Fig. 2B and C; da Silva and Meinhardt, 1999; Walker, 1973).

The other species in this clade (e.g., *C.* [*Syncope*] *hudsoni, C.* [*Syncope*] *magnova*, and *C.* [*Syncope*] *jimi*) show digit reduction but no loss of digits (2f–g). Furthermore, the epicoracoid connection between the procoracoid cartilages and the coracoid bones among the overall smaller, but not miniaturized, *Chiasmocleis* (*Syncope*) species shows different levels of erosion, but remains visible (Fig. 2c).

4.4. Historical biogeography

Amazonian Pleistocene refugia (Haffer, 1969) have been widely used and revised as a scenario explaining biological diversification (Connor, 1986; Bush and de Oliveira, 2006; Bush, 1994; Garzón-Orduña et al., 2015). Amazonia was hypothesized to comprise multiple refugial areas during the Last Glacial Maximum (LGM) that would have maintained a stable climate and forested environments (Arruda et al., 2018). Although the geographic distribution of several *Chiasmocleis* species coincides with areas suggested as refugia, the divergence times between species pairs are older than the LGM. The same areas might have served as refugia through successive climatic cycles.

The biogeographic history of the genus is a result of multiple connections between Amazonia and the Atlantic Forest as have been shown for other South American frogs (Bush, 1994; Bush and de Oliveira, 2006; Costa, 2003; Gamble et al., 2008; Garzón-Orduña, 2015; Jaramillo et al., 2010; Ledo and Colli, 2017; Olivera-Filho and Ratter, 1995; Thomé et al., 2016). Plant diversity during the Eocene was higher than in modern Amazonia; however, pollen studies showed a distinct decline of diversity at the Eocene-Oligocene (34 mya) transition associated with a period of global cooling (Jaramillo et al., 2006). Climatic fluctuations continued through the Oligocene and Miocene (Zachos et al., 2001) and likely caused the contraction and breaking up of areas of previously continuous rainforests (Jaramillo et al., 2010).

The most likely ancestral area for the origin of *Chiasmocleis* was the Atlantic Forest, as the deepest phylogenetic split separates Atlantic Forest species *C.* (*Relictus*) *gnoma* from all congeners. However, we hypothesize that *Chiasmocleis* began to differentiate from other gastrophrynines and spread throughout forested areas during the Eocene (50–36 mya) (de Sá et al., 2012 and Fig. 4), a period when forested areas likely extended from Amazonia to the Atlantic Forest. The subsequent contraction of forested areas during the late Eocene–Oligocene (33–35 mya) may have isolated a lineage in the northern Atlantic Forest (NAF), which diverged giving rise to *Chiasmocleis* (*Relictus*) *gnoma*, a species endemic to and of apparent limited distribution within the NAF. Other descendant lineages from the Eocene *Chiasmocleis* ancestor diversified from Amazonian ancestors (Fig. 4).

The dynamics and past extension of the dry corridor today represented by the Neotropical savannas and seasonally dry forests (i.e., Cerrado, Chaco, and Caatinga) is an ongoing debate. This dry corridor is composed of open vegetation that separates the close forests of Amazonia and Atlantic Forest biodiversity hotspots (Arruda et al., 2018; Costa et al., 2018; Roig Juñent et al., 2006). Nonetheless, this dry corridor has been important in the diversification of frogs and reptiles, e.g. *Amazonphrynella* and *Dendrophryniscus*, Phyzelaphryninae, and lizards (Castroviejo-Fisher, 2014; Fouquet et al., 2012a, 2012b; Prates et al., 2016; Thomé et al., 2016a, 2016b; Sobral-Souza et al., 2015). Furthermore, it has been hypothesized that biotic interchange between these wet forests would happen mainly from the southeastern Amazon connecting to the Atlantic Forest, and less frequently via another route from the northern Amazon to the Atlantic Forest (reviewed in Ledo and

Colli, 2017). For instance, phylogenies of co-distributed taxa across the Amazon and Atlantic Forest show that several frog clades endemic to the Atlantic Forest share a most recent common ancestor with Amazonian species (Fouquet et al., 2012; Blackburn and Duellman, 2013; Caminer et al., 2017). For example, the divergence between the genera *Amazonphrynella* (Amazonia) and *Dendrophrynicus* (Atlantic Forest) was estimated to take place in mid-Eocene (≈45 mya); also, the origin of the genus *Chiasmocleis* (including *Syncope*) was estimated to occur in the mid to late Eocene (≈38 my; de Sá et al., 2012).

The biogeographic model suggests that allopatric speciation has a major role on Gastrophryninae diversification throughout the Neotropics (Supplemental Information Table 1). In *Chiasmocleis*, a range expansion with allopatric isolation of an Amazonian and an Atlantic Forest ancestor is estimated to have occurred early in the evolutionary history of the genus (late Eocene, χ = 42.5 mya, 95% HPD 35.05–51.13 mya). Given the overall small size and biology of *Chiasmocleis* species (i.e., fossorial and explosive breeders) it is unlikely that individuals could disperse across large areas. Thus, we hypothesized that in the Eocene-Oligocene the common ancestor of *Chiasmocleis* was most likely distributed throughout Amazonia and extended into the northern Atlantic Forest. The expansion and retraction of the dry corridor comprising savanna-like biomes represent an early impact on the distribution and gene exchange of *Chiasmocleis* species (Arruda et al., 2018; Costa et al., 2018).

The phylogeny suggests Amazonia as the ancestral area for all species of the subgenus *Syncope* (divergence time ~35.3 mya; 95% HPD = 28.4–42.7) and Amazonian representatives of the larger morphotype of the subgenus *Chiasmocleis*, which have no bone fusion or loss (divergence time ~34 mya; 95%HPD = 27.4–41.1) (i.e., *C. avilapiresae*, *C. shudikarensis*, *C. royi*, *C. ventrimaculata*, *C. papachibe*, *C. devriesi*, and *C. anatipes*). Furthermore, *Chiasmocleis royi*, *C. ventrimaculata*, *C. papachibe*, *C. devriesi*, and *C. anatipes* share a most recent common ancestor with species to and within Chaco, Cerrado, and Atlantic Forest (Fig. 4) that diverged ~28.9 mya (95%HPD = 22.4–35.4). In addition, an increased interchange through the southern Atlantic Forest-Amazonia route (divergence time ~19 mya; 95%HPD = 14–24.2) led to diversification of *Chiasmocleis* species within the Southern Atlantic Forest (divergence time ~13.1 mya; 95%HPD = 8.6–17.6) (i.e., *C. leucosticta*, *C. mantiqueira*, and *C. altomontana*; Fig. 4), as sister group of the species endemic to the Cerrado and Chaco (divergence time ~12.4 mya; 95%HPD = 7.7–17.1) (i.e., *C. albopunctata*, *C. mehely*, *C. centralis*, and *C. bicegoi*). Among species of *Chiasmocleis*, polyploidy is known only within the southern Atlantic Forest. *Chiasmocleis* (*Chiasmocleis*) *leucosticta* and *C. altomontana* are tetraploid species (Kasahara and Haddad, 1997; C.F.B.H. unpublished data). Furthermore, they are also the only two species of *Chiasmocleis* known that produce bubble nests for reproduction (Haddad and Hödl, 1997; C.F.B.H. unpublished data). It is very likely that these traits (i.e., tetraploidy and bubble nest) originated in the node that unites *C. leucosticta* and sister species *C. mantiqueira* and *C. altomontana*. It would be enlightening to have the chromosome number and reproductive mode of *C. mantiqueira*, as well as other *Chiasmocleis* species, to understand their role on diversification.

Moreover, the Atlantic Forest contains two more clades that became endemic to central and northern portions of the biome. Northern species (divergence time ~9.7 mya; 95%HPD = 6.3–13.1) (i.e., *C. alagoana*, *C. cordeiroi*, *C. crucis*, and *C. schubarti*) are more closely related to each other than to the central Atlantic Forest species (divergence time ~9.7 mya; 95%HPD = 6.3–12.1) (i.e., *C. veracruz*, *C. quilombola*, *C. capixaba*, and *C. lacrimae*).

Moreover, our results show that within *Chiasmocleis* evolution towards a small body size occurred independently at least three times: (a) *C. gnoma*, (b) in the clade subgenus *Syncope*, and (c) by *Chiasmocleis* endemic to the central Atlantic Forest clade (*C. capixaba*, *C. lacrimae*, *C. quilombola*, and *C. veracruz*) but not in the northern and southern ones, nor in the species endemic of Chaco, Cerrado, and Amazonia. In the central Atlantic Forest clade, the smaller body size is not correlated with osteological fusions or losses.

There are different scenarios that might explain the evolution of small body size. Within the subgenus *Syncope*, the most noticeable skeletal losses, i.e., epicoracoids of pectoral girdle, phalanges, and digits, characterize the smallest members of the genus suggesting that successful miniaturization required major rearrangements and losses in the skeletal system. Furthermore, this suggests that early in the evolutionary history of *Chiasmocleis*, the extremely small size morphotype with reduced or loss of epicoracoid cartilages and reduced fingers reached by *Chiasmocleis* in Amazonia, would be under strong negative selection in forested areas of Atlantic Forest given that no miniaturized species occurs in that Biome.

5. Conclusion

This is the first large-scale study to include a near-complete phylogeny of *Chiasmocleis* and to sample multiple populations per species. As such, it presents important, yet preliminary, information of the species diversity and evolution of the genus. Future studies may test hypotheses on hybridization and directionality in gene flow between *Chiasmocleis* species. Moreover, these studies will provide insights on phenotypic evolution across contact zones compared to regions where species occur in allopatry. For instance, the extent of species geographic distribution and contact zones in Amazonian *Chiasmocleis* are mostly unknown. Due to difficulties in accessing remote field sites, the Amazon forest is largely unexplored compared to the Atlantic Forest, Chaco, Cerrado, and Caatinga (Peloso, 2010); and the exploration of additional sites may reveal additional unnamed species of *Chiasmocleis*.

A large proportion of potential new cryptic lineages of *Chiasmocleis* identified here are endemic to Amazonia. However, about 1/3 of the named species inhabit the Atlantic Forest, an area for which a recent study demonstrated that regional conservation policies are not effective for the survival of most (i.e., 90%) amphibian species in this region (Campos et al., 2017). Furthermore, among the 26 *Chiasmocleis* species evaluated by the IUCN Red List (2017), 11 are listed as Data Deficient (five are Atlantic Forest endemics), one as Endangered, and 14 as of Least Concern. A recent study suggested that species listed as Data Deficient, on average, have higher extinction risk than species listed as Vulnerable (Tietje and Rödel, 2018).

Documentation and description of the hidden species diversity of *Chiasmocleis* represents a new frontier (Struck et al., 2018). Furthermore, natural areas must be thoroughly explored before the biodiversity vanishes along with the many other species affected by the current Sixth Mass Extinction event (Ceballos et al., 2015; Wake and Vredenburg, 2008).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2018.10.021.

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