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High-throughput DNA sequencing of museum specimens sheds light on the long-missing species of the *Bokermannohyla claresignata* group (Anura: Hylidae: Cophomantini)

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The two species of the *Bokermannohyla claresignata* species group (Anura: Hylidae) have not been collected for the last four decades. It is the only species group of the hyline tribe Cophomantini that has not yet been analysed genetically. Its phylogenetic position is thus uncertain, and it has a combination of adult and larval character states that make this group a crucial missing piece that hinders our understanding of Cophomantini phylogenetics and character evolution. We obtained DNA sequences from a museum larval specimen of *Bok. claresignata*, using specialized extraction methods and high-throughput DNA sequencing, and combined the molecular phylogenetic results with available phenotypic information to provide new insights into the taxonomy and phylogenetic relationships of its species group. Our phylogenetic results place *Bok. claresignata* as sister to the *Boana pulchella* group, supporting its inclusion in *Boana*, together with *Bokermannohyla clepsydra*. In light of this new finding, we recognize a newly defined *Boana claresignata* group to accommodate these species, thus resolving both the polyphyly of *Bokermannohyla* and

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the paraphyly of *Boana*. Considering the phylogenetic relationships of the *Boana claresignata* group, we also discuss the evolution of suctorial tadpoles and mature oocyte/egg pigmentation in Cophomantini.

ADDITIONAL KEYWORDS: archival DNA – *Boana pulchella* group – Hylinae – museum – phylogenetics – taxonomy.

INTRODUCTION

During the last three centuries, museums and other natural history collections have housed an important record of the biodiversity of our planet. These collections have traditionally been used to address crucial issues in taxonomy, phylogenetic systematics, biogeography, conservation, ecology and evolutionary biology (Wandeler *et al.*, 2007; Habel *et al.*, 2014; Schmitt *et al.*, 2018). Biological collections have also become essential repositories of genetic samples, which have also allowed studies of molecular variation across time and space (e.g. Yeates *et al.*, 2016). However, much of the material stored in these collections was not available for genetic analysis until recently, when the advent of high-throughput sequencing (HTS) and the improvement of DNA extraction protocols changed this scenario (Gilbert *et al.*, 2007; Miller *et al.*, 2008; Briggs *et al.*, 2009; Gansauge & Meyer, 2013; Hofreiter *et al.*, 2014; Kehlmaier *et al.*, 2017). These technological advances have allowed access to degraded DNA from preserved samples, including species that are extinct or have disappeared from the wild.

Knowledge on phylogenetic relationships of the hylid subfamily Hylinae has increased substantially in the last 15 years (e.g. Faivovich *et al.*, 2005, 2018; Wiens *et al.*, 2010; Pyron, 2014; Duellman *et al.*, 2016). Of the seven currently recognized hyline tribes (Faivovich *et al.*, 2018), Cophomantini is among those that have received comparatively more attention (e.g. Coloma *et al.*, 2012; Faivovich *et al.*, 2013; Guayasamin *et al.*, 2015; Caminer & Ron, 2014; Berneck *et al.*, 2016; Fouquet *et al.*, 2016; Orrico *et al.*, 2017; Peloso *et al.*, 2018; Rojas-Runjaic *et al.*, 2018; Pinheiro *et al.*, 2019). Exemplar species of the genera *Myersiohyla*, *Nesorohyla* and specimens of most species groups recognized in *Aplastodiscus*, *Boana*, *Bokermannohyla* and *Hyloscirtus* were included in phylogenetic analyses, sometimes with reasonably good or nearly complete taxonomic sampling (Faivovich *et al.*, 2013; Berneck *et al.*, 2016; Rojas-Runjaic *et al.*, 2018; Pinheiro *et al.*, 2019). The only exception persisting in Cophomantini in terms of a species group that has not been available for molecular phylogenetic analysis is the *Bokermannohyla claresignata* species group.

The *Bok. claresignata* group was recognized by Faivovich *et al.* (2005) for the former *Hyla claresignata* A. Lutz & B. Lutz, 1939 group. Bokermann (1972) suggested that the nominal species and *Hyla clepsydra* A. Lutz, 1925, both known from a few montane localities in the Atlantic Forest of south-eastern Brazil, were closely related and that they might further be related to the then *Hyla circumdata* group. Although not explicitly stated by Bokermann (1972), the evidence for the first hypothesis possibly was the difficulty in differentiating both species in addition to the unique characters of their tadpoles. Lutz (1973) grouped both species as 'Montane Southeastern Forms of *Hyla*' without any additional comment, nor recognizing any affinity among them. Based on the remarks by Bokermann (1972), Jim & Caramaschi (1979) included *Hyla claresignata* and *Hyla clepsydra* in the former *Hyla circumdata* group. However, subsequent workers on this group did not consider those species (Cardoso & Haddad, 1982; Caramaschi & Feio, 1990; Pombal & Haddad, 1993). The *Hyla claresignata* group was first recognized with that name by Cei (1980, 1987) in the context of the supposed presence of the nominal species in the Province of Misiones, north-eastern Argentina. Duellman *et al.* (1997) referred to the *Hyla claresignata* group as containing the nominal species and *Hyla clepsydra* when discussing stream-adapted tadpoles in South American hylids.

Based on their phylogenetic results, Faivovich *et al.* (2005) erected the genus *Bokermannohyla* for the former *Hyla circumdata*, *Hyla martinsi* and *Hyla pseudopseudis* species groups. Given that tissue samples of the former *Hyla claresignata* group were not available, these authors only tentatively included its species in *Bokermannohyla* as the *Bokermannohyla claresignata* group, based on the comments by Bokermann (1972) and Jim & Caramaschi (1979). Faivovich *et al.* (2005) noticed that the putative synapomorphies of this group (larvae with oral disc surrounded by marginal papillae, and 7/12–8/14 labial tooth rows) could be considered as such only if assumed to be reversals to the plesiomorphic states of these characters that occur in the stream-adapted larvae of basal Cophomantini (at that time, *Hyloscirtus* and *Myersiohyla*). From this perspective, the phylogenetic position of the *Bok. claresignata* group is a crucial missing piece in our understanding of Cophomantini phylogenetics, not only for assessing the monophyly of *Bokermannohyla* but also for the evolution of tadpole morphology and the biogeography of the group.

Bokermannohyla clepsydra (A. Lutz, 1925) was briefly described based on one adult male from 'Serra da Bocaina', State of São Paulo, Brazil (subsequently restricted to Fazenda do Bonito, Serra da Bocaina, São José do Barreiro, São Paulo, by Bokermann, 1966).

Bokermannohyla claresignata was described based on one adult female from the Fazenda of Messrs. Guinle, Teresópolis, State of Rio de Janeiro, Brazil (type locality; subsequently modified to 'garden of Mr. Guinle, at the entrance of Granja Guarany, Teresópolis' by Lutz, 1973) and two subadult males from Serra da Bocaina, State of São Paulo. Lutz & Orton (1946) reported on tadpoles of *Bok. claresignata*, collected in fast-flowing rivers in the Parque Nacional da Serra dos Órgãos, around Teresópolis (Beija Flor, Garrafão, Paquequer) and Theodoro de Oliveira (then in the outskirts of Nova Friburgo, 50 km north-east of Teresópolis, and today a neighbourhood of that city). Subsequently, B. Lutz (1949a) provided an extended description of adults on the basis of the type series and, apparently, other adult specimens (Lutz, 1949a: 794 refers to 'one of the allotypes [sic] and several other specimens of the collection were found sleeping on gravatás' [a common name given to several species of bromeliads]). She also recounted the information on larval morphology of Lutz & Orton (1946) and added new notes on its natural history. Cochran (1955) described a male paratype of *Bok. claresignata* and the holotype of *Bok. clepsydra* (a male). Bokermann (1972) reported on a collection of 30 adult specimens and tadpoles of *Bok. clepsydra* from the Campo de Fruticultura, Serra da Bocaina, a locality 5 km north-west (air line) from the type locality, and three newly collected adult specimens and two tadpole series of *Bok. claresignata* from Teresópolis.

In her influential revision of Brazilian hylines, Lutz (1973) provided descriptions of the type material of both species and summarized information on these species from her previous publications and from the study by Cochran (1955). In the account of *Bok. claresignata*, she mentioned additional adult specimens collected in Serra dos Órgãos, near Teresópolis. Furthermore, she referred to having found tadpoles similar to those of this species in Marumbi, State of Paraná.

The reports of Bokermann (1972) and Lutz (1973) were the last references to freshly collected specimens of *Bok. claresignata* and *Bok. clepsydra*. The material that they studied is now housed mostly in the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ) and the Museu de Zoologia da Universidade de São Paulo (MZUSP). Owing to the proximity of the type locality of *Bok. claresignata* (Teresópolis, State of Rio de Janeiro) to the city of Rio de Janeiro and its many academic institutions, it has been among the most intensely collected areas in the Atlantic Forest during the last 50 years. However, to our knowledge, no adults or tadpoles of this species have been found since the larvae collected in 1964 (lot MZUSP 80128).

Besides the specimens collected by Bokermann during the 1960s and reported by Bokermann (1972), *Bok. clepsydra* was found for the last time in 1980 next to the Paraty-Cunha road, between the states of Rio de Janeiro and São Paulo. Fieldwork along the Paraty-Cunha road and in Parque Nacional Serra da Bocaina, including the area formerly occupied by the Campo de Fruticultura da Serra da Bocaina, during the last 12 years has been unsuccessful in finding adults or tadpoles.

Given that *Bok. clepsydra* and *Bok. claresignata* were not collected in the last four and five decades, respectively, there are no well-preserved tissue samples for molecular analyses available in collections. In this study, we report the production of sequences from a museum specimen of *Bok. claresignata* with the use of high-throughput DNA sequencing and, together with the study of the morphology of preserved museum specimens, provide new insights into the taxonomy and phylogenetic relationships of this species and *Bok. clepsydra*.

MATERIAL AND METHODS

Museum specimens and vocalizations

For this study, we had access to adults and larvae of *Bok. claresignata* and *Bok. clepsydra* available in the Adolpho Lutz collection (AL-MN, housed in the Museu Nacional, Universidade Federal do Rio de Janeiro), the collections of the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ), the Museu de Zoologia, Universidade de São Paulo (MZUSP) and the Departamento de Zoologia, Instituto de Biologia, Universidade Federal do Rio de Janeiro (ZUFRJ).

For osteological observations, specimens were cleared and double stained with Alcian Blue and Alizarin Red S following the protocol of Taylor & Van Dike (1985). Muscle anatomy was studied through gross dissections and with the help of topical application of an iodine/potassium iodide solution (Bock & Shear, 1972). The terminology used for osteology, nuptial pads and tadpole external morphology is that of Trueb (1973, 1993), Luna *et al.* (2018) and Altig & McDiarmid (1999), respectively. Tadpoles were staged using the table of Gosner (1960). The taxonomy of Hylidae, including the recognition of tribes in the subfamily Hylinae, follows the recent discussion by Faivovich *et al.* (2018).

Vocalizations were recorded by Werner C. A. Bokermann with a UHER 4000 analog recorder, at a tape velocity of 9.5 cm/s (data from Fonoteca Neotropical Jacques Vielliard, accession number 31798). Original recordings were digitized with a MOTU Ultra Lite Mk3 sound board and analysed with RAVEN v.1.5 (Cornell Lab of Ornithology, Ithaca, NY, USA). Spectrograms were generated with: window type Hann, 512 samples, 3 dB filter bandwidth 270 Hz; time grid with 75% overlap and 128 hop size; frequency grid with discrete Fourier transform (DFT)

size 512 samples and grid spacing 188 Hz. For call analysis, we adopted the call-centred approach and measured the following parameters, following Köhler *et al.* (2017): note duration, note interval, minimum frequency, maximum frequency, peak frequency (using the Peak Frequency tool of RAVEN), bandwidth 90% (−10 dB; using the Bandwidth 90% tool of RAVEN), minimum frequency at 5% of energy (using the Frequency 5% tool of RAVEN) and maximum frequency at 95% of energy (using the Frequency 95% tool of RAVEN).

See the Supporting Information (Appendix S1) for a list of specimens examined.

Processing of archival DNA of the historical sample

We obtained a tissue sample from a tadpole of *Bok. claresignata* from the type locality housed at the amphibian collection of the Museu Nacional, Rio de Janeiro, Brazil (MNRJ 54331: Brazil: Rio de Janeiro: Teresópolis: Parque Nacional Serra dos Órgaos: Rio Soberbo, acima de Barreira). This specimen was collected on 21 January 1953 by Bertha Lutz and most probably fixed and stored in 70% ethanol, the preservative liquid in which it is still stored (Supporting Information, Appendix S2: Fig. S1). The voucher larval specimen is identical to those described for *Bok. claresignata* based on a metamorphic series by Lutz & Orton (1946) and Bokermann (1972) from nearby localities. Given that the larvae of the two species of the *Bok. claresignata* group are unique among larvae of *Boana* (Duellman *et al.*, 1997; present study) and among larval forms of Brazilian anurans, the identification of this tadpole as *Bok. claresignata* is straightforward.

We extracted total DNA, converted it to a single-stranded library, captured the mitochondrial genome and sequenced the enriched library on an Illumina Nextseq 500 platform following the pipeline described below. All stages of DNA extraction and library preparation, before polymerase chain reaction (PCR) amplification for library indexing, were carried out in the dedicated historical DNA facilities at the University of Potsdam, following established guidelines (Fulton & Shapiro, 2019).

In summary, we extracted DNA from 48 mg of tail muscle tissue, following the protocol of Dabney *et al.* (2013) and Rohland *et al.* (2004). Tissue was first washed with a phosphate-buffered saline solution, digested using the non-destructive buffer of Rohland (2004), and DNA was purified using the silica column-based method described by Dabney *et al.* (2013). The tissue pellet remaining after non-destructive digestion was then re-digested using a proteinase K-based buffer and DNA purified using the silica column with the aim of maximizing total DNA recovery.

The DNA extractions were converted into Illumina sequencing libraries using protocols based on single-stranded DNA (ss-library; Gansauge & Meyer, 2013; Korlevic *et al.*, 2014). The protocol included treatment with uracil-DNA glycosylase and endonuclease VIII for uracil excision and DNA cleavage of abasic sites and the use of the Klenow fragment of DNA polymerase I for the fill-in reaction. We also performed a quantitative PCR experiment to determine the optimal number of cycles for subsequent libraries, indexing as described by Basler *et al.* (2017).

An initial assessment of DNA preservation, relative endogenous DNA content and contamination was made by low-level (~1 million reads) shotgun sequencing of the libraries on an Illumina Nextseq 500 sequencing platform, using 500/550 High Output v.2.5 (75 cycles SE) kits at the University of Potsdam, following the procedures described by Paijmans *et al.* (2017). Owing to low abundance of endogenous DNA fragments in the sequencing libraries (see Results), we performed two rounds of in-solution hybridization capture to enrich for mitochondrial DNA fragments, using DNA baits made in-house (see next subsection).

DNA baits and mitochondrial capture

To prepare baits for mitochondrial in-solution hybridization capture enrichment, we first extracted total DNA of fresh tissue from one specimen of a species from another genus of the tribe Cophomantini, *Aplastodiscus arildae* (Cruz & Peixoto, 1987) CFBH30829 (Célio F. B. Haddad Collection, Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista 'Júlio de Mesquita Filho', Rio Claro, São Paulo, Brazil), using the DNeasy Blood & Tissue kit (QIAGEN Inc.). We amplified the partial mitogenome in two overlapping fragments (missing control region) using primers 12SAL + NFSer11650 and FCOIII9400L + FCB15200H (Zhang *et al.*, 2013) and the TaKaRa LA Taq (©2019 Takara Bio Inc.). Amplification products were sheared by sonication using a Covaris S220 System to ~150 bp and converted into dual indexing double-stranded DNA Illumina sequencing libraries (Henneberger *et al.*, 2019). Target capture was performed following Gonzalez-Fortes & Paijmans (2019) using a 10:1 proportion of ssDNA library to baits. We sequenced the enriched libraries as described previously. The *A. arildae* library used as bait was also sequenced and the mitogenome assembled to serve as a reference sequence for mapping sequencing reads from the historical sample (see next subsection).

Sequencing data processing and assembly of mitogenome sequences

Sequenced reads from both shotgun and enriched libraries were quality trimmed using CUTADAPT v.1.16 (Martin, 2011), with a minimum read length of 30 bp. PCR duplicates were removed from the trimmed reads using TALLY (Davis *et al.*, 2013), and average library fragment size was estimated.

Mitogenomes of human, mouse and other species analysed previously in the same clean laboratory, and library adapters (see Supporting Information, Appendix S3), were screened as potential sources of contamination using FastQScreen v.0.13.0 (Wingett & Andrews, 2018). We included the raw transcriptome of *Aplastodiscus leucopygius* (Cruz & Peixoto, 1985) (NCBI Sequence Read Archive accession number: BioProject PRJNA625657, BioSample SAMN14604402) as a phylogenetically close reference to estimate the relative endogenous content, although this method will represent an underestimate because large parts of the genome are not transcribed.

For the mitochondrial genome assembly, we combined all trimmed and non-duplicated sequences from the different libraries, because they were prepared from the same individual. We mapped all reads against the human mitogenome using BWA (Li & Durban, 2009) to exclude potential contaminants and then proceeded with the unmapped reads. We assembled the mitochondrial genome through iterative mapping using MITObim v.1.9 (Hahn *et al.*, 2013). We implemented MITObim using default parameters apart from the mismatch value, where we used zero and a kmer baiting of 15. The newly generated mitogenome of *A. arildae* (GenBank accession number MT358316) was used as the seed. The final '.caf' file output was visualized in GENEIOUS v.11.0.5 (Kearse *et al.*, 2012), and we estimated the coverage of mitochondrial contigs. Only sequences with a coverage higher than ten and from markers available for other Cophomantini were used for phylogenetic inferences. Biosample metadata are available in the NCBI Sequence Read Archive under BioProject accession number PRJNA624823.

Taxonomic sampling

Our dataset includes the taxonomic sampling of Cophomantini of Faivovich *et al.* (2013), to which we added all species of that tribe for which sequences were produced subsequently by Almendáriz *et al.* (2014), Caminer & Ron (2014), Guayasamin *et al.* (2015), Berneck *et al.* (2016), Fouquet *et al.* (2016), Orrico *et al.* (2017), Peloso *et al.* (2018), Rojas-Runjaic *et al.* (2018), Ron *et al.* (2018) and Pinheiro *et al.* (2019) to a total of 136 terminals. As outgroups, we used the same exemplars of hylid diversity included by Pinheiro *et al.* (2019), and the trees were rooted with *Phrynomedusa dryade* Baêta, Giasson, Pombal & Haddad, 2016.

Character sampling

We included ≤ 7486 bp per terminal from the same gene fragments used by Faivovich *et al.* (2013): the mitochondrial genes cytochrome *b* (*CYTB*), 12S rRNA–tRNA^{VAL}–16S rRNA (H1), tRNA^{LEU}, NADH dehydrogenase subunit 1 and tRNA^{ILE} (*ND1*), and also fragments of the nuclear genes seven in absentia homolog 1 (*SIAH*), exon 1 of rhodopsin (*RHOD*), tyrosinase (*TYR*), recombination activating gene 1 (*RAG1*), exon 2 of chemokine receptor 4 (*CXCR4*), and 28S ribosomal. Sequences were aligned using MAFFT v.7 (Katoh & Standley, 2013). For the protein-coding genes (i.e. *CYTB*, *ND1*, *SIA*, *RHOD*, *TYR*, *RAG1* and *CXCR4*) we used the G-INS-I strategy and for the non-coding genes H1 and 28S we used AUTO-FFT-NS-2. All other alignment parameters were set as default. Alignments were edited using BioEdit (Hall, 1999), and sequence files were merged with SequenceMatrix (Vaidya *et al.*, 2011). See the Supporting Information (Appendix S4) for a complete list of GenBank accession numbers and voucher information of sequences produced for this study.

Phylogenetic analyses

The phylogenetic analysis was done following the parsimony optimality criterion (Farris, 1983; Goloboff, 2003; Goloboff & Pol, 2005; Kluge & Grant, 2006; Grant & Kluge, 2009) with TNT (Goloboff *et al.*, 2008) using equally weighted parsimony. Searches used the new technology search under level 50, which included sectorial searches, tree drifting and tree fusing (Goloboff, 1999), hitting the best length 500 times and submitting the resulting trees to a final round of tree bissection and reconection (TBR) branch swapping. Parsimony jackknife (Farris *et al.*, 1996) absolute frequencies were estimated from 1000 replicates, hitting the minimum length five times (search level 15) with new technology searches (Goloboff, 1999) in each replicate, because preliminary analyses of the original data matrix showed that minimum length is hit with this search strategy. Analyses were done considering gaps either as a fifth state or as missing data, for comparison with results of maximum likelihood (ML) analyses.

Maximum likelihood analyses were conducted using RAxML v.8.2.10 (Stamatakis, 2014) on the concatenated dataset, using the GTRGAMMA model. All RAxML analyses were performed using the CIPRES Science Gateway online server (Miller *et al.*, 2010). Ribosomal genes and first, second and third codon positions for each protein-coding gene were treated as separate partitions. Best-fitting combinations for these partitions were selected using the corrected Akaike Information

Criterion with PartitionFinder v.2.1.1 (Lanfear *et al.*, 2016), using the greedy algorithm (Lanfear *et al.*, 2012). Searches included 1000 runs using the rapid hill-climbing algorithm (Stamatakis *et al.*, 2007). Non-parametric bootstrapping values (Felsenstein, 1985) were estimated using 1000 pseudoreplicates. Trees were visualized and edited in FigTree v.1.4.3 (Rambaut, 2016). Ancestral character state reconstructions were done with non-additive or unordered (Fitch, 1971) optimizations in TNT.

RESULTS

SEQUENCING AND MITOGENOME ASSEMBLY OF HISTORICAL *BOK. CLARESIGNATA*

Shotgun sequencing of the non-destructive and proteinase K extraction libraries resulted in a total of 1 488 782 reads. After trimming and excluding duplicate reads, 444 509 reads remained (for results for each library, see Supporting Information, Appendix S3). The average fragment size was 37 bp, after excluding adapters and small reads. FastqScreen analyses revealed no obvious contamination except for a few reads that mapped to the human genome (41 in total). Only 0.41–0.46% of reads in each library mapped to the *Aplastodiscus* transcriptome, suggesting low relative endogenous content. Most reads did not map to any reference genomes. A Basic Local Alignment Search Tool (BLAST) search at the US National Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) on unmapped reads revealed many unidentified or bacterial fragments.

Sequencing of libraries after capture resulted in a total of 2 040 483 reads (454 339 reads after quality controls). We pooled all quality trimmed and non-duplicated reads from shotgun and captured libraries to assemble the mitochondrial DNA. We were unable to assemble the complete mitogenome of *Bok. claresignata*, but recovered ~1580 bp arranged in 11 contigs with coverage higher than ten from the 12S and 16S rRNAs. The final read pool of mitochondrial DNA sequences recovered by MITObim was 23 225 reads, with 15 146 reads mapped to the 12S–tRNA^{VAL}–16S mitochondrial region. These sequences were used for downstream analyses. We also recovered small fragments from some tRNAs or coding genes (*ND2*, *COI* and *COIII*), but they were not included in our phylogenetic dataset.

PHYLOGENETIC ANALYSES

The phylogenetic analysis with TNT considering gaps as a fifth state resulted in 96 trees of 29 455 steps (Fig. 1; Supporting Information, Appendix S5). The strict

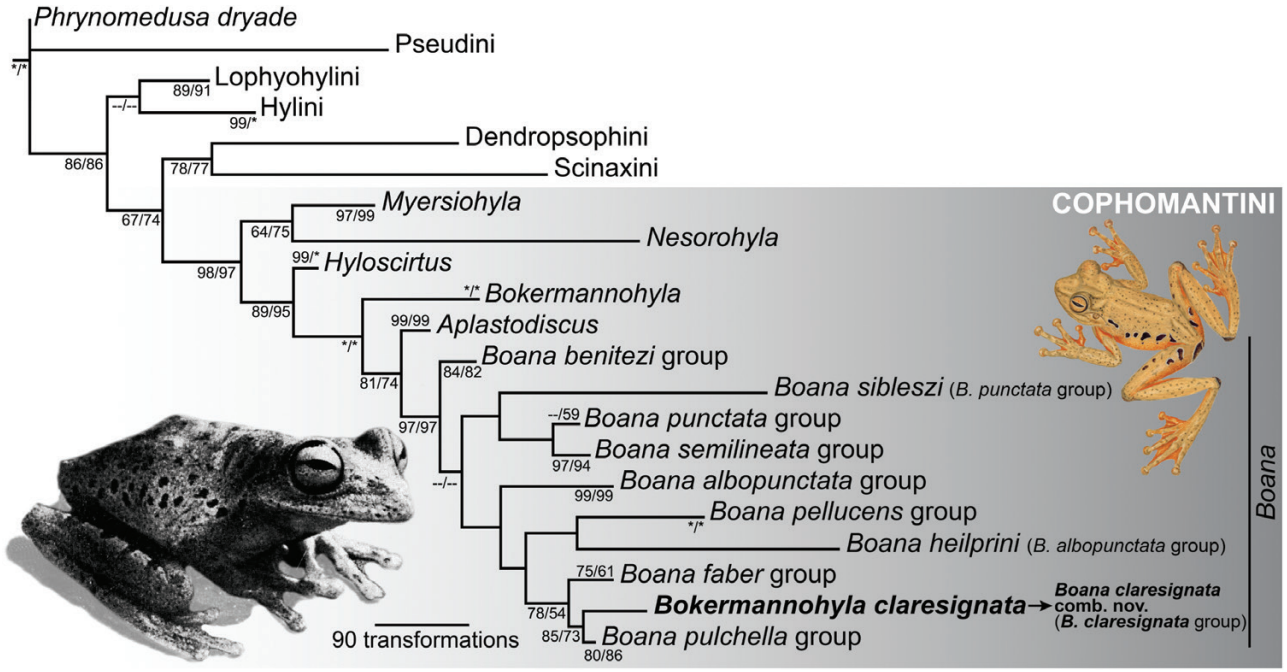

Figure 1. One of the 96 most parsimonious trees (29 455 steps; considering gaps as a fifth state). None of the nodes that are shown collapses in the strict consensus. Most genera of Cophomantini and species groups of *Boana* are condensed. See the Supporting Information (Appendix S5) for a complete topology. Numbers below nodes are jackknife frequencies with gaps considered as a fifth state / as missing data. An asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. The photograph of *Boana claresignata* is taken from Lutz (1948). The watercolour depicts the living holotype of this species and is part of the collection of the Departamento de Vertebrados, Museu Nacional, Rio de Janeiro, Brazil [artist Paulo Sandig (according to Lutz, 1949a); undated].

consensus was highly resolved, with all conflict among the most parsimonious topologies (MPTs) restricted to: (1) relationships among the *Hyloscirtus bogotensis* and *Hyloscirtus jahni* groups with the clade including the *Hyloscirtus armatus* and *Hyloscirtus larinopygion* groups; (2) internal relationships of the *Hyloscirtus bogotensis* group; and (3) internal relationships of the *Boana semilineata* group. The parsimony analysis considering gaps as missing data resulted in 24 equally parsimonious trees of 28 103 steps. The strict consensus was mostly congruent with that obtained with gaps considered as a fifth state, and the conflict among MPTs was also similar, with additional internal conflict in the *Boana albopunctata* group. The ML results (Supporting Information, Appendix S6) were congruent in terms of the most supported groups with the parsimony analyses. *Bokermannohyla claresignata* was nested in *Boana*, as the sister taxon of the *Boana pulchella* group, with 85% jackknife support (73% when gaps were considered as missing data; 98% bootstrap in the ML analysis); the monophyly of the *Boana pulchella* group was supported with 80% jackknife (86% when gaps were considered as missing data; 93% bootstrap in the ML analysis).

DISCUSSION

HIGH-THROUGHPUT DNA SEQUENCING AS A TOOL TO SOLVE LONG-STANDING TAXONOMIC AND PHYLOGENETIC QUESTIONS

The successful sequencing of museum fluid-preserved specimens, made possible by advances in extraction and sequencing techniques, is an important step towards solving countless phylogenetic and taxonomic problems (e.g. Evans *et al.*, 2019). This is especially crucial for amphibians, because the global decline in amphibian populations has resulted in the disappearance of hundreds of species since the 1980s (Stuart *et al.*, 2004), making it impossible to obtain fresh specimens for DNA extraction and analysis of these lost species.

By sequencing a museum sample of *Bok. claresignata*, we have filled an important gap in the evolutionary history of the Cophomantini tribe. We succeeded in retrieving phylogenetically informative sequences from the sample, but found that the DNA was fragmented despite apparently being only fixed in ethanol, without any known history of formalin exposure of the specimen. This is consistent with results obtained by Ruane & Austin (2017) and McGuire *et al.* (2018), suggesting that old specimens stored in 70% ethanol might be as challenging for DNA extraction as formalin-fixed, ethanol-stored specimens. Nevertheless, obtaining only partial fragments of mitochondrial DNA might be sufficient to solve important issues that hinder the progress of knowledge on the diversity and evolution of certain taxonomic groups.

COPHOMANTINI: CONGRUENCE WITH PREVIOUS RESULTS

Relationships among most genera of Cophomantini have remained stable since the study by Faivovich *et al.* (2005), with the occasional non-monophyly of *Myersiohyla* in some analyses (e.g. Wiens *et al.*, 2010). Pinheiro *et al.* (2019) recently solved this problem with the erection of the genus *Nesorohyla* for the former *Myersiohyla kanaima* (Goin & Woodley, 1969) and the redefinition of *Myersiohyla*. Our results are congruent with previous studies in that *Myersiohyla* and *Nesorohyla* are early diverging genera, with the position of the latter being poorly supported, in this case as sister to the former in the parsimony analysis (Fig. 1; Supporting Information, Appendix S4). Our results for *Hyloscirtus* are congruent with the recent hypotheses of Rojas-Runjaic *et al.* (2018) and Ron *et al.* (2018) in the recognition of four species groups (the *Hyloscirtus armatus*, *Hyloscirtus bogotensis*, *Hyloscirtus jahni* and *Hyloscirtus larinopygion* groups).

The relationships of *Aplastodiscus* are congruent with those reported by Berneck *et al.* (2016). The internal relationships of *Boana* are congruent with the recent phylogenetic hypothesis of Pinheiro *et al.* (2019), in terms of most well-supported groups. An important difference is the reduction in support (73–85% jackknife support) for the monophyly of the *Boana pulchella* group as redefined by Faivovich *et al.* (2004), which was recovered with higher values (99–100% support, in both bootstrap and jackknife) in previous analyses (Faivovich *et al.*, 2004, 2005, 2013; Wiens *et al.*, 2010; Duellman *et al.*, 2016; Pinheiro *et al.*, 2019).

BOK. CLARESIGNATA AND *BOK. CLEPSYDRA* AS *BOANA*

Our phylogenetic analyses recover *Bok. claresignata* deeply nested in *Boana*, as the sister taxon of the *Boana pulchella* group, with 85% jackknife support (Fig. 1; Supporting Information, Appendix S5). Although we included only *Bok. claresignata* in our analysis, the monophyly of this species and *Bok. clepsydra*, in what has been called the *Bok. claresignata* group, is supported by phenotypic evidence. For this reason, we consider that the recovered position of *Bok. claresignata* can be extended to *Bok. clepsydra*.

Our result conflicts with the tentative taxonomic placement of the former *Hyla claresignata* group in *Bokermannohyla* by Faivovich *et al.* (2005), but it does not imply more phenotypic character conflict than did its placement in *Bokermannohyla*. Although *Boana* is diagnosable from the other genera of Cophomantini (combination of prepollical spine, projected or not, with expanded sacral diapophyses), no phenotypic synapomorphies are known for this genus.

An expanded sacral diapophysis occurs in *Aplastodiscus*, *Boana* and *Hyloscirtus*, as opposed to a round or unexpanded diapophysis in *Bokermannohyla*, *Myersiohyla* and, homoplastically, in a few species deeply nested in *Hyloscirtus* (unknown in *Nesorohyla* and in the *Hyloscirtus jahni* group; Kizirian *et al.*, 2003; Coloma *et al.*, 2012; A.C.C. Lourenço, M. Rivera-Correa, J. Faivovich and P.D.P. Pinheiro, pers. obs.). The origin of the expanded sacral diapophyses optimizes ambiguously in Cophomantini, being equally parsimonious to interpret it as a synapomorphy of the common ancestor of *Aplastodiscus*, *Boana*, *Bokermannohyla* and *Hyloscirtus*, with a reversal in *Bokermannohyla*, or two independent origins in *Hyloscirtus* and the common ancestor of *Aplastodiscus* and *Boana*. The expanded diapophysis occurs also in *Bok. claresignata* (MNRJ 24028) and *Bok. clepsydra* (MZUSP 112612).

Besides the molecular evidence, there is one character state that supports the position of the former *Hyla claresignata* group as the sister taxon of the *Boana pulchella* group. This is the absence of the slip of the m. depressor mandibulae that originates at the level of the dorsal fascia of the m. levator scapulae (Supporting Information, Appendix S2: Fig. S2; see also Pinheiro *et al.*, 2018: fig. 2). J. Faivovich and P.C.A. Garcia (in Faivovich *et al.*, 2005) identified this character state as a synapomorphy of the *Boana pulchella* group. Subsequent observations indicate that in Cophomantini it is homoplastic only with the clade including *Boana atlantica* (Caramaschi & Velosa, 1996), *Boana cinerascens* (Spix, 1824) and *Boana punctata* (Schneider, 1799) (P.D.P. Pinheiro, pers. obs.)

Pinheiro *et al.* (2018) stated that the absence of the anterolateral process of the hyoid plate is known so far only in the *Boana pulchella* group. An anterolateral process is also absent in *Bok. clepsydra* (MZUSP 112612) but present in *Bok. claresignata* (MNRJ 24028). The taxonomic distribution of this character state requires more study; it is still unknown in a number of species of the *Boana pulchella* group [*Boana aguilari* (Lehr, Faivovich & Jungfer, 2010), *Boana balzani* (Boulenger, 1898), *Boana cambui* (Pinheiro, Pezzuti, Leite, Garcia, Haddad & Faivovich, 2016), *Boana melanopleura* (Boulenger, 1912) and *Boana palaestes* (Duellman, De la Riva & Wild, 1997)], and the anterolateral process is present in *Boana freicanecae* (Carnaval & Peixoto, 2004) (P.D.P. Pinheiro, pers. obs.); therefore, its polarity is still not clear.

The hyoid plates of *Bok. claresignata* and *Bok. clepsydra* lack the posterolateral processes. The available evidence indicates that this process is absent in some species of the *Boana pulchella* group (Pinheiro *et al.*, 2018: fig 3D) and present at least in some species of the *Boana albopunctata* and *Boana faber* groups (Pinheiro *et al.*, 2018: fig. 3A–C). Although it could be another putative synapomorphy supporting the monophyly of the former *Hyla claresignata* group and the *Boana pulchella* group, its taxonomic distribution still requires more study.

A NEW SPECIES GROUP OF *BOANA*

Our results require the transfer of the former *Hyla claresignata* and *Hyla clepsydra* from *Bokermannohyla* to *Boana*, to resolve the polyphyly of *Bokermannohyla* and the paraphyly of *Boana*. For this reason, we recognize them as *Boana claresignata* (A. Lutz & B. Lutz, 1939) **comb. nov.** and *Boana clepsydra* (A. Lutz, 1925) **comb. nov.** Although these species are being included in a separate species group, future studies should focus on the monophyly of the *Boana pulchella* group. Whether the decrease in support for this group compared with all previous phylogenetic studies is attributable to a lack of informative sequences or to a genuine lack of evidence for its monophyly is still unclear. Although there is abundant phenotypic evidence for the monophyly of the *Boana claresignata* group, it also shares with the *Boana pulchella* group the only putative phenotypic synapomorphy so far known for this group (the absence of the origin of the m. depressor mandibulae in the dorsal fascia at the level of the m. levator scapulae). Thus, the monophyly of the *Boana pulchella* group is currently supported only by molecular data.

THE *BOANA CLARESIGNATA* GROUP

Diagnosis: The *Boana claresignata* group can be diagnosed by: (1) projected prepollical spine; (2) nuptial pads present, single, light-coloured, without macroscopically evident epidermal projections (Supporting Information, Appendix S2: Fig. S3); (3) palpebral membrane without reticulation; (4) tympanum diameter/eye diameter of the two species combined 0.23–0.54 (N = 26), see comments below; (5) mental gland not evident macroscopically; (6) posterolateral process of the hyoid plate absent; (7) m. depressor mandibulae without origin in the dorsal fascia at the level of the m. levator scapulae (Supporting Information, Appendix S2: Fig. S2); (8) transparent parietal and visceral peritonea; (9) tadpole with expanded snout; (10) larval nostril oval, reduced (diameter 0.02–0.03 body length), with a small medial projection; (11) spiracle located below the mid-body line; (12) fins low and parallel to the tail muscle proximally, increasing their height at the medial third of the tail; (13) larval oral disc enlarged (0.80–0.87 of maximum body width); (14) larval oral disc surrounded by a continuous row of marginal papillae; (15) labial

tooth-row formula (LTRF) with seven to nine anterior rows and 11–14 posterior rows (7–9/11–14); (16) upper jaw sheath M-shaped, with lateral processes laterally directed; (17) presence of a medial shelf on the anterior jaw sheath; and (18) unpigmented oocytes (2.1–3.3 mm in diameter).

Comparison with other species of Boana: In the context of *Boana*, the presence of light-coloured nuptial pads without macroscopically evident epidermal projections, the unpigmented mature oocytes and the characters related to the larval oral disc are synapomorphies of the *Boana claresignata* group. The occurrence of these character states differentiates this group from all other species of *Boana*. All these characters, in combination with the projected prepollical spine, distinguish the species in the *Boana claresignata* group from all other genera of Cophomantini. The unpigmented mature oocytes are homoplastic with *Boana heilprini* (Noble, 1923), with the *Boana benitezi* group (when mature oocytes/eggs are known) and with some species of *Myersiohyla* and *Hyloscirtus*. In *Boana*, nuptial pads with dark-coloured epidermal projections are known to occur only in the *Boana semilineata* group (Faivovich *et al.*, 2006). The palpebral membrane without reticulation differentiates the *Boana claresignata* group from the *Boana semilineata* group (present in this species group; Faivovich *et al.*, 2006; Peloso *et al.*, 2018). The absence of a macroscopically evident mental gland differentiates of the *Boana claresignata* group from species of the *Boana benitezi*, *Boana punctata* and *Boana semilineata* groups and from *Boana heilprini* (present in these species; Faivovich *et al.*, 2006; Brunetti *et al.*, 2015). The absence of the posterolateral process of the hyoid plate differentiates the *Boana claresignata* group from at least some species of the *Boana albopunctata* and *Boana faber* groups (Pinheiro *et al.*, 2018: fig. 3A–C). The absence of the origin of the m. depressor mandibulae in the dorsal fascia at the level of the m. levator scapulae is shared only with species of the *Boana pulchella* and *Boana punctata* groups.

The tympanum diameter/eye diameter ratio in the *Boana claresignata* group is 0.23–0.54, with the larger value represented by the few available specimens of *Boana claresignata* and the smaller by all those of *Boana clepsydra* (see comments below). Although the size of the tympanum might be taxonomically relevant for some comparisons, it becomes more challenging to interpret when considering all the diversity in *Boana*. A large tympanum is present among the species of the *Boana albopunctata*, *Boana faber*, *Boana pellucens*, *Boana punctata* and *Boana semilineata* groups, with a combined tympanum diameter/eye diameter ratio varying from 0.48 [*Boana wavrini* (Parker, 1936); Hoogmoed, 1990] to 0.98 [*Boana rosenbergi* (Boulenger, 1898); Duellman, 1970]. In the *Boana benitezi* group, this ratio varies from 0.25 [*Boana ornatissima* (Noble, 1923); Hoogmoed, 1979] to 0.51 [*Boana nympha* (Faivovich, Moravec, Cisneros-Heredia & Köhler, 2006); Faivovich *et al.*, 2006]. In the *Boana pulchella* group, the sister taxon of the *Boana claresignata* group, it varies from 0.35 [*Boana caipora* (Antunes, Faivovich & Haddad, 2008); Antunes *et al.*, 2008] to 0.68 [*Boana joaquini* (Lutz, 1968); Garcia *et al.*, 2003]. Several species of this group also have a smaller tympanum, measuring less than half of the eye diameter [e.g. *Boana cambui*, *Boana ericae* (Caramaschi & Cruz, 2000) and *Boana semiguttata* (A. Lutz, 1925); Caramaschi & Cruz, 2000; Garcia *et al.*, 2007; Pinheiro *et al.*, 2016].

The large oral disc with complete marginal papillae and the LTRF of the larvae of the *Boana claresignata* group (7–9/11–14) differentiate them from all other species of *Boana* with known tadpoles. The LTRF is higher than any other known larvae of *Boana*. Until now, the highest known LTRF was from *Boana heilprini* at 6/9 (Noble, 1927; Galvis *et al.*, 2014; Díaz *et al.*, 2015), followed by *Boana benitezi* at 5/8 (this larva was only tentatively assigned to *Boana benitezi*; Myers & Donnelly, 1997), *Boana hutchinsi* (Pyburn & Hall, 1984) at 4/7, *Boana jimenezi* (Señaris & Ayarzagüena, 2006), *Boana curupi* (Garcia, Faivovich & Haddad, 2007) and *Boana stellae* (Kwet, 2008) at 3/5 (Faivovich, 1996; Myers & Donnelly, 1997; Myers & Donnelly, 2008; Widholzer & Castroviejo-Fisher, 2018). Most species of *Boana* have an LTRF of 2/3 or 2/4 (Kolenc *et al.*, 2008). The same applies to the large oral disc, which is almost equivalent to the body width in the tadpoles of the *Boana claresignata* clade [oral disc width (ODW) 0.80–0.87 of body width (BW)], whereas it is smaller in the remaining species of *Boana* (e.g. ODW/BW = 0.48–0.60 in *Boana heilprini*; ODW/BW = 0.60 in *Boana curupi*, ODW/BW = 0.50–0.53 in *Boana jimenezi*; and ODW/BW = 0.50 in *Boana stellae*; smaller than 50% of the body width in most species of *Boana*; Faivovich, 1996; Myers & Donnelly, 2008; Kolenc *et al.*, 2008; Díaz *et al.*, 2015; Widholzer & Castroviejo-Fisher, 2018; T. L. Pezzuti, pers. obs).

The M-shaped upper jaw sheath, with long lateral processes laterally directed and a shelf on its medial portion, differentiates the tadpoles of the *Boana claresignata* group from the other species of *Boana* (upper jaw sheath arc shaped, lateral processes medially directed and medial shelf absent in most species of *Boana*; Kolenc *et al.*, 2008). The shapes of body and tail of the tadpoles of the *Boana claresignata* group are also unique in the genus, being adapted to rheophilic microhabitats in swift or torrential mountain streams. This suctorial morphology (see comments below) comprises a depressed body with an expanded snout and low fins that are parallel to the

tail muscle proximally, increasing their height at the medial third of the tail (the other tadpoles of the genus have a benthic-like morphology).

Characterization: Bokermann (1972) and Lutz (1973) provided appropriate characterizations of the two species of the *Boana claresignata* group. To complement the observations of these authors, we add the occurrence of a light-coloured nuptial pad (Supporting Information, Appendix S2: Fig. S3), the absence of a macroscopically evident mental gland and the occurrence of unpigmented mature oocytes.

Contents: Boana claresignata (A. Lutz & B. Lutz, 1939) and *Boana clepsydra* (A. Lutz, 1925).

Natural history: Lutz & Lutz (1939), Lutz & Orton (1946) and Lutz (1949a) provided observations on the natural history of adults and larvae of *Boana claresignata* and *Boana clepsydra*, which were summarized by Lutz (1973). The available information on adults of *Boana claresignata* is restricted to the three specimens of the type series and several specimens raised from tadpoles in captivity. Bokermann (1972) provided information from the series of adults and the tadpoles that he collected. All observations of adults of the two species are in agreement that they inhabit epiphytic bromeliads growing on the sides of swift or torrential mountain streams. All collecting localities are ~400–1200 m a.s.l. The males of *Boana clepsydra* call from the bromeliads or perched from branches suspended 1–2 m above streams or brooks. Courtship, amplexus and oviposition remain unknown. Lutz (1949a) and our observations on the female holotype of *Boana claresignata* (AL-MN 1971), which has a lateral incision on the left flank, and on another female of this species (MZUSP 117074), indicate that mature oocytes are unpigmented (AL-MN 1971, 2.7–3.3 mm, mean = 3.07 mm, SD = 0.22 mm, N = 10; MZUSP 117074, 2.1–2.7 mm, mean = 2.26 mm, SD = 0.26 mm, N = 5). A female of *Boana clepsydra* (MZUSP 112626) also has unpigmented mature oocytes evident through the skin. Given that the oocytes are distorted owing to preservation, the reported diameter should be taken cautiously.

Tadpoles were reported in fast-flowing streams, using their oral discs to cling to the rocky streambed (Lutz & Lutz, 1939; Bokermann, 1972). Tadpoles of *Boana claresignata* were, in all instances, attached to the rocks, more frequently vertically, but also horizontally, near the bottom of the stream, not rising to the surface (Lutz & Orton, 1946). When disturbed, tadpoles of *Boana clepsydra* were observed swimming against the current (Bokermann, 1972).

Vocalization: The advertisement call of *Boana clepsydra* was briefly described by Bokermann (1972). We re-analysed his recording of two males (WCAB 42553 and 42554; currently MZUSP 112613 and 112614, respectively) available at Fonoteca Neotropical Jacques Vielliard (accession number 31798). The recording of MZUSP 112614 is of lower quality (probably, the individual was more distant); we describe them separately.

As mentioned by Bokermann (1972), *Boana clepsydra* emits groups of three to five calls, with each call corresponding to one tonal note (Fig. 2A, B). Notes are irregularly spaced and with a considerable interval between them. The call has bands, similar to a harmonic structure (Fig. 2B, C), but the peak frequency of each band is not necessarily an exact multiple of the fundamental peak frequency and may vary within each band (see values below). The call has a characteristic metallic or high-pitched tone.

The first individual (MZUSP 112613) emitted five calls in 15.48 s (N = 1). Each call lasted 130–169 ms (144.4 ± 16.6 ms) and was separated by intervals of 2.60–4.17 s (3.68 ± 0.72). The fundamental frequency, which was also the dominant, was between 2053.8 and 3010.9 Hz, with the peak frequency at 2437.5 Hz. The minimum frequency at 5% of energy was 2250 Hz (N = 5); maximum frequency at 95% of energy was 2625 Hz (N = 5); and bandwidth 90% was 375 Hz (N = 5). Up to four additional bands were present: the first one had peaks at 4500 (N = 1), 4875 (N = 1) or 5062.5 Hz (N = 3); the second had peaks at 7125 (N = 1) or 7500 Hz (N = 4); the third one, which was absent in the first note and was the band with lower energy, had peaks at 9000 (N = 1), 9937.5 (N = 2) or 10 125 Hz (N = 1); and the fourth and highest band had peaks at 12 000 (N = 1), 12 375 (N = 1) or 12 562.5 Hz (N = 3).

The second individual (MZUSP 112614) emitted three calls in 7.58 s (N = 1). Each call lasted 59–89 ms (73 ± 15 ms), and they were separated by intervals of 3.51–3.85 s. The fundamental frequency, which was also the dominant, was between 2050.6 and 2885 Hz, with the peak frequency at 2437.5 Hz (N = 3). The minimum frequency at 5% of energy was 2250 Hz (N = 3); maximum frequency at 95% of energy was 2625 Hz (N = 3); and bandwidth 90% was 375 Hz (N = 3). Up to four additional bands were present: the first one had peaks at 4500 (N = 2) or 4875 Hz (N = 1); the second one had peaks at 7312.5 (N = 2) or 7687.5 Hz (N = 1); the third one, which was absent in the third note, was found to have a peak at 9562 (N = 1) or 9750 Hz (N = 1) Hz; and the fourth and higher band was found with peaks at 12 000 (N = 1), 12 187.5 (N = 1) or 12 750 Hz (N = 1).

The call of *Boana clepsydra* can be distinguished from those of *Boana aguilari*, *Boana balzani*, *Boana bandeirantes* (Caramaschi & Cruz, 2013), *Boana*

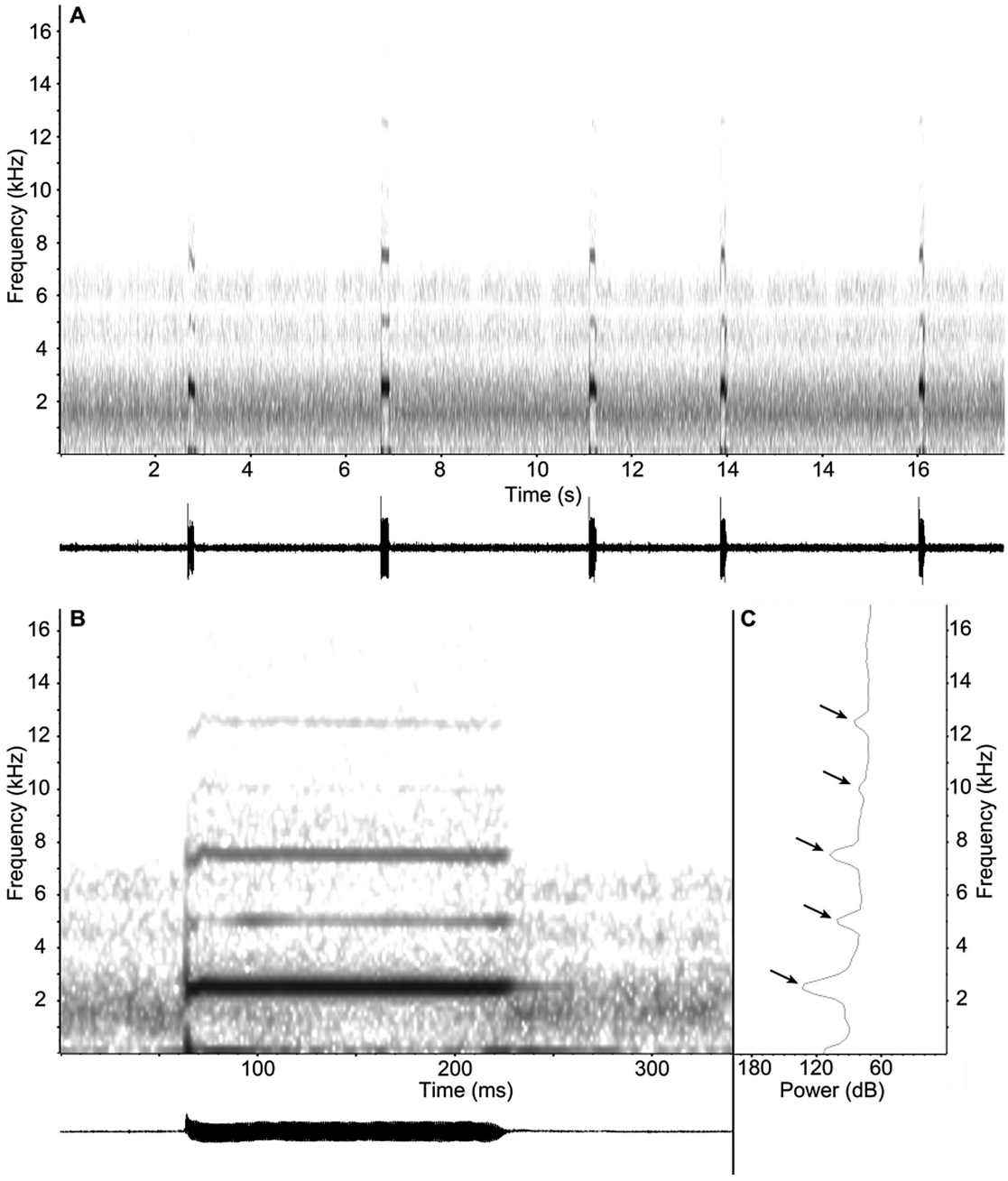

Figure 2. Advertisement call of *Boana clespsydra* (MZUSP 112613). This recording was described by Bokermann (1972) and is now housed in Fonoteca Neotropical Jacques Vielliard (accession number 31798). A, audiospectrogram of a sequence of five calls; Discrete Fourier transform (DFT) size 512 samples; waveform below. B, detail of the second note from A; waveform below. C, power spectrum of the note shown in B. Black arrows point to the dominant frequency (bottom) and additional, harmonic-like bands (see main text). Recording made by Werner C. Bokermann on the margin of the Ponte Alta River, at Campo de Fruticultura, Serra da Bocaina, São Paulo, Brazil, 6 November 1968, 21.00 h, 17 °C; a male calling from 1.5 m above the ground.

beckeri (Caramaschi & Cruz, 2004), *Boana botumirim* (Caramaschi, Cruz & Nascimento, 2009), *Boana caipora*, *Boana cipoensis* (Lutz, 1968), *Boana curupi*, *Boana cymbalum* (Bokermann, 1963), *Boana ericae*, *Boana gladiator* (Köhler, Koscinski, Padial, Chaparro, Handford, Lougheed & De la Riva, 2010), *Boana guentheri* (Boulenger, 1886), *Boana jaguariaivensis* (Caramaschi, Cruz & Segalla, 2010), *Boana latistriata*

(Caramaschi & Cruz, 2004), *Boana leptolineata* (Braun & Braun, 1977), *Boana marianitae* (Carrizo, 1992), *Boana melanopleura*, *Boana polytaenia* (Cope, 1870), *Boana stellae* and *Boana stenocephala* (Caramaschi & Cruz, 1999) by having notes with a tonal structure (pulsed notes in these species; Haddad *et al.*, 1988; Heyer *et al.*, 1990; Duellman *et al.*, 1997; Garcia *et al.*, 2007; Acioli & Toledo, 2008; Antunes *et al.*, 2008; Garcia & Haddad, 2008; Kwet, 2008; Caramaschi *et al.*, 2009; Köhler *et al.*, 2010; Lehr *et al.*, 2010; Guerra *et al.*, 2017; Forti *et al.*, 2019). From, *Boana caingua* (Carrizo, 1991), *Boana cambui*, *Boana cordobae* (Barrio, 1965), *Boana goiana* (Lutz, 1968), *Boana phaeopleura* (Caramaschi & Cruz, 2000), *Boana pulchella* and *Boana riojana* (Koslowsky, 1895), the call of *Boana clepsydra* can be distinguished by being composed by one note (call composed by > 1 note in the other species; Barrio, 1965a; Guimarães *et al.*, 2001; Pinheiro *et al.*, 2012, 2016; Baraquet *et al.*, 2013; Batista *et al.*, 2015). The non-pulsed structure of the call of *Boana clepsydra* differentiates it from those of the closely related *Boana pellucens* and *Boana faber* groups, which have calls with a pulsed structure (Fouquette, 1961; Bokermann, 1967; Duellman, 1970, 2001; Bokermann & Sazima, 1973; Kluge, 1981; Heyer *et al.*, 1990; Loebmann *et al.*, 2008; Martins *et al.*, 2009).

Comments: Lutz & Orton (1946) described the occurrence of green bones in postmetamorphic specimens of *Boana claresignata*, indicating impregnation with biliverdin (Barrio, 1965b), which also happens in several other species of *Boana* from various species groups (e.g. Lutz, 1949b, 1973; Duellman, 1970; Hoogmoed, 1979; Caminer & Ron, 2014; Taboada *et al.*, 2017: tab. 1). There are no observations regarding the persistence of biliverdin in adults of *Boana claresignata*, nor any comment regarding its occurrence in *Boana clepsydra*. Lutz & Orton (1946) reported that specimens of *Boana claresignata* release a characteristic odour of crushed plants when handled, which also happens in several other species of Cophomantini (see Faivovich *et al.*, 2013; Brunetti *et al.*, 2015, 2016, 2019). There are no reports on odour from *Boana clepsydra*.

The occurrence of nuptial pads in *Boana claresignata* and *Boana clepsydra* is reported here for the first time. The nuptial pad is easily observed in *Boana clepsydra* (see Supporting Information, Appendix S2: Fig. S3). There are few available males of *Boana claresignata* in collections, and all of these are poorly fixed. In these, the nuptial pad is evident only through the occurrence of some yellowish acini in the same position as in *Boana clepsydra* and, for that reason, we interpret that they are present.

Gallardo (1961) reported the occurrence of *Hyla claresignata* in the Province of Misiones, Argentina. For this reason, Cei (1980) included an account of that species based on the published information and included it in the *Hyla claresignata* group, without further comments. Carrizo (1992) re-identified the specimens studied by Gallardo (1961) as *Hyla semiguttata* A. Lutz, 1925, a species that Cei & Roig (1961) recorded in Misiones. The populations of this species from Argentina were subsequently shown to be a different, new species (*Boana curupi*) by Garcia *et al.* (2007).

Cochran (1955) included a description of a male paratype of *Boana claresignata* from 'Bonito, Serra da Bocaina, Rio de Janeiro' and the holotype of *Boana clepsydra* (a male). The latter is an important reference because the holotype specimen, which Cochran (1955: 88) described at that time as a 'badly faded and mutilated specimen' with 'an immaculate drab over its entire surface', is now reduced to what seems to be an even worse condition to the point that almost no relevant characters are discernible (AL-MN 976; Supporting Information, Appendix S2: Fig. S4). One notable point of her description of *Boana clepsydra* is her comment on the occurrence of 'a pair of lateral external vocal sacs'. She did not compare *Boana claresignata* and *Boana clepsydra*.

Bokermann (1972) noticed that adults of *Boana claresignata* and *Boana clepsydra* were similar, to the point that initially he suspected that the latter corresponded to males of the former species until he collected females of *Boana clepsydra*. He described the variation in coloration pattern, vocalization and tadpoles of *Boana clepsydra* and compared it with three newly collected adult specimens and two tadpole series of *Boana claresignata* from Teresópolis. He differentiated *Boana claresignata* from *Boana clepsydra* based on snout shape in profile (rounded in *Boana claresignata*, truncate in *Boana clepsydra*), tympanum size and position (larger in *Boana claresignata*, in a more posterior position), hindlimb size (smaller in *Boana claresignata*), larval body shape (rounder in *Boana claresignata*, flatter in *Boana clepsydra*), ridges on jaw sheaths (present in *Boana claresignata*, absent in *Boana clepsydra*), larval tail size (proportionally larger in *Boana claresignata*), larval eye size (slightly larger in *Boana clepsydra*) and larval coloration pattern (larger blotches in larvae of *Boana claresignata*). He did not comment on the vocal sac morphology of *Boana clepsydra* nor did he include it as a diagnostic character. Lutz (1973) stressed that *Boana claresignata* and *Boana clepsydra* share a small tympanum, inhabit bromeliads and are montane species, but stated that *Hyla claresignata* showed no marked affinities with other species, being unaware of the paper by Bokermann (1972) and emphasizing differences in dorsal pattern and Cochran's (1955) reference to a double vocal sac in the holotype of *Boana clepsydra*.

The information on variation in the dorsal pattern in *Boana clepsydra* provided by Bokermann (1972) clearly shows that it does not allow differentiation of this species from *Boana claresignata*. Our observations on all specimens of *Boana clepsydra* in the MNRJ and MZUSP collections corroborate that statement (Supporting Information, Appendix S2: Fig. S5).

The analyses of available specimens from both species corroborate the differences reported by Bokermann (1972) regarding the snout shape in profile. *Boana claresignata* has a rounder snout than *Boana clepsydra*, which has a shorter and truncated snout. Although in both species there is intraspecific variation in snout shape, we do not see an overlap.

Boana claresignata has a tympanum diameter/eye diameter (TD/ED) ratio of 0.39–0.54 in females (0.44 ± 0.06; $N = 4$) and 0.41–0.57 in males (0.48 ± 0.08, $N = 6$) and a tympanum diameter/head length (TD/HL) ratio of 0.11–0.15 in females (0.13 ± 0.02; $N = 4$) and 0.12–0.22 in males (0.16 ± 0.03; $N = 6$). In contrast, *Boana clepsydra* has a TD/ED ratio of 0.30–0.38 in females (0.34 ± 0.06; $N = 2$) and 0.23–0.38 in males (0.31 ± 0.04; $N = 23$) and a TD/HL ratio of 0.09–0.11 in females (0.10 ± 0.01; $N = 2$) and 0.08–0.12 in males (0.10 ± 0.01; $N = 23$). Although these measurements tend to corroborate the larger tympanum size in *Boana claresignata* noticed by Bokermann (1972), the values of the ratios that express tympanum size are continuous. Considering the low number of specimens of *Boana claresignata* available, this difference in tympanum size should be viewed cautiously, because the lack of overlap between intervals for the ratios could well be a consequence of the small sample size for that species.

With the caveat of the low sample size for *Boana claresignata*, we observed that in this species the lower margin of the tympanic ring is slightly below the level of the lower margin of the eye. In *Boana clepsydra*, the lower margin of the tympanic ring is at or above the level of the lower margin of the eye.

Measurements of hindlimbs revealed that in females of both species and in males of *Boana clepsydra*, the sum of thigh length and tibia length is slightly larger than snout–vent length. The hindlimb length/snout–vent length ratio of *Boana claresignata* is 1.01–1.08 in females (1.02 ± 0.07; $N = 4$) and 0.88–1.01 in males (0.94 ± 0.05; $N = 6$), and in *Boana clepsydra* it is 1.03–1.04 in females (1.03 ± 0.01; $N = 2$) and 1.01–1.11 in males (1.05 ± 0.03; $N = 23$).

The study of male specimens of *Boana clepsydra* ($N = 22$) reveals that the vocal sac is single and subgular. This observation differs from the description by Cochran (1955) of the vocal sac in the now highly deteriorated male holotype as paired and lateral. The vocal sac is now unrecognizable in the holotype.

Our study of the paratypes of *Boana claresignata*, the only specimens of this species from Serra da Bocaina, indicates that the reasonably preserved specimen (AL-MN 2088) falls within the variation found in the series of topotypes of *Boana clepsydra* (Supporting Information, Appendix S2: Figs S6, S7). Therefore, we consider that these paratypes of *Boana claresignata* are misidentified specimens of *Boana clepsydra*, indicating that there is no evidence of the occurrence of *Boana claresignata* in the Serra da Bocaina.

Lutz (1973) reported the collection of tadpoles similar to those of *Boana claresignata* in Marumbi, State of Paraná (~550 km south-west from Serra da Bocaina). These tadpoles are the lot MNRJ 68427 (nine tadpoles in stages 25–28 and 38, and one postmetamorphic specimen; Supporting Information, Appendix S2: Fig. S8), with the exact locality given as 'Rio Taquaral em Marumbi, Serra do Mar, Estrada de ferro Curitiba-Paranaguá'. We interpret this locality as areas nearby the Marumbi railway station (25°26′19″S; 48°55′12″W; 500 m a.s.l.). We corroborate the striking similarity of these larvae to those of *Boana claresignata* and *Boana clepsydra*, but note the presence of conspicuous lateral projections in the upper jaw sheath that we did not find in larvae of the other species. Pending more studies in morphological variation of larvae in the two described species, for the time being, we consider the larvae to belong to an unidentified species of the *Boana claresignata* group.

Distribution: Both species are endemic to the Atlantic Forest in south-eastern Brazil. *Boana clepsydra* is known only from three nearby localities in the section of the Serra do Mar mountain range known as Serra da Bocaina. These are the type locality Fazenda do Bonito and the former Campo de Fruticultura da Serra da Bocaina, currently within the limits of the Parque Nacional da Serra da Bocaina, in the State of São Paulo. The third locality is in the south-western boundary of the national park, in the State of Rio de Janeiro, along the road between Paraty (State of Rio de Janeiro) and Cunha (State of São Paulo), ~57 km south-west (air line) from the other localities. *Boana claresignata* is known from the Parque Nacional da Serra dos Órgãos in Teresópolis, where it has been collected in some swift-flowing streams (Beija Flor, Garrafão, Paquequer and Soberbo), and from Nova Friburgo, both in the State of Rio de Janeiro.

Suctorial tadpoles in Cophomantini

Tadpoles of the *Boana claresignata* group have several modified characters typical of a suctorial morphology (suctorial guild, type II; Altig & Johnston, 1989). They have the posterior portion of the body markedly depressed and an expanded snout that supports the

ventral and large oral disc. The expanded snout, which forms a rim surrounding the anterior part of the body, is composed of loose connective tissue between the anterior portion of the trabecular horns and the tip of the snout (Lutz & Orton, 1946). In Cophomantini, a similar condition has been described and illustrated for the *Hyloscirtus armatus* group (Haas & Richards, 1998). It may also be present in some species of *Myersiohyla* [i.e. *Myersiohyla neblinaria* Faivovich, McDiarmid & Myers, 2013 and *Myersiohyla inparquesi* (Ayarzagüena & Señaris, 1994); interpreted from the illustrations, Ayarzagüena & Señaris, 1994; Faivovich *et al.*, 2013] and, possibly, in the *Hyloscirtus jahni* group (whether the larva of this species has the same snout structure is not clear from the information provided by La Marca, 1985). This specialization, already described in other suctorial species of Pelodryadinae (Gradwell, 1973, 1975), might act in the engagement or disengagement of the oral disc in the substrate (Gradwell, 1975) or at least in absorbing shocks against the rocky streambed (Lutz & Orton, 1946). In the current phylogenetic hypothesis of Cophomantini, an expanded snout would have evolved at least three times in the tribe (in some species of *Myersiohyla*, the *Hyloscirtus armatus* group and the *Boana claresignata* group) and would optimize ambiguously in *Hyloscirtus*, if also present in *Hyloscirtus jahni*.

Many of the character states present in the larvae of the *Boana claresignata* group (enlarged oral discs, lack of gaps in the marginal papillae, high values of LTRF and jaw sheaths laterally expanded) have also been described for stream-dwelling tadpoles of *Myersiohyla* (Ayarzagüena & Señaris, 1994; Faivovich *et al.*, 2013) and *Hyloscirtus* (e.g. Duellman & Altig, 1978; La Marca, 1985; Cadle & Altig, 1991; Lötters *et al.*, 2005; Sánchez, 2010; Coloma *et al.*, 2012). Several of these character states have been considered as putative synapomorphies of the former *Bok. claresignata* group (Faivovich *et al.*, 2005), and of *Hyloscirtus* (Duellman *et al.*, 1997), and were subsequently inferred as plesiomorphic conditions within Cophomantini (Faivovich *et al.*, 2005). However, the phylogenetic position of *N. kanaima* and the morphological differences of its larvae (i.e. smaller larval oral disc, lower LTRF and an anterior gap on marginal papillae; MacCulloch & Lathrop, 2005; Pinheiro *et al.*, 2019) have resulted in changes (i.e. 2/4 as the ancestral LTRF) and some ambiguities (i.e. in the presence/absence of gaps on marginal papillae) in the optimizations of larval ancestral states in the tribe (Pinheiro *et al.*, 2019).

Faivovich *et al.* (2005) inferred that transformations of oral disc characters (e.g. reduction of oral disc size and number of tooth rows and the presence of an anterior gap in the marginal papillae) could have evolved in a common ancestor of and of *Bokermannohyla*, *Aplastodiscus* and *Boana*. Besides the ambiguity generated by the tadpole of *N. kanaima*, stream-related features have been described for some tadpoles of *Aplastodiscus*, *Boana* and *Bokermannohyla* (e.g. oral disc surrounded by marginal papillae without gaps, and an increase in LTRF above 2/4 in the *Aplastodiscus sibilatus* (Cruz, Pimenta and Silvano, 2003), *Bok. pseudopseudis* and *Bok. martinsi* groups, and some species of the *Boana albopunctata*, *Boana benitezi*, *Boana punctata*, *Boana pulchella* and *Boana semilineata* groups; Pyburn & Hall, 1984; Faivovich, 1996; Myers & Donnelly, 1997, 2008; Leite & Eterovick, 2010; Mercês & Juncá, 2010; Lins *et al.*, 2018). The plesiomorphic states of these characters in *Boana* are still uncertain, in part because of the ambiguities commented on above, but also because the relationships among most species groups of *Boana* are poorly supported (Faivovich *et al.*, 2013; Pinheiro *et al.*, 2019; our results). Regardless, only the *Boana claresignata* group and *Boana heilprini* have tadpoles with enlarged oral discs and LTRF that show an extreme of development when compared with closely related taxa (*Boana heilprini*, LTRF 4–6/6–9; Noble, 1927; Galvis *et al.*, 2014; Díaz *et al.*, 2015), indicating that in these particular cases the characters related to the suctorial morphology had evolved independently.

Unpigmented mature oocytes in *Boana*

Nali *et al.* (2014) reviewed the occurrence of pigmentation in mature oocytes/eggs of *Boana*. At that time, unpigmented mature oocytes were known to occur in *Boana heilprini* (Nali *et al.*, 2014), *Boana lemai* (Rivero, 1972) (Duellman, 1997), *Boana nympha* and *Boana roraima* (Duellman & Hoogmoed, 1992) (Faivovich *et al.*, 2006). The recognition of the *Boana claresignata* group adds another case of unpigmented mature oocytes. The optimization of egg pigmentation in our phylogenetic hypothesis indicates that unpigmented mature oocytes are plesiomorphic in *Boana* and that the pigmented animal pole evolved in the sister taxon of the *Boana benitezi* group (Fig. 3; Supporting Information, Appendix S7). However, this inference should be taken cautiously, because the relationships among most species groups of *Boana* are poorly supported (Fig. 1; Supporting Information, Appendix S5). Regardless, unpigmented mature oocytes evolved independently in the *Boana claresignata* group and in *Boana heilprini*.

From the three independent occurrences of unpigmented mature oocytes, the reproductive mode is known only superficially in *Boana heilprini*. Based on observations and published records of this

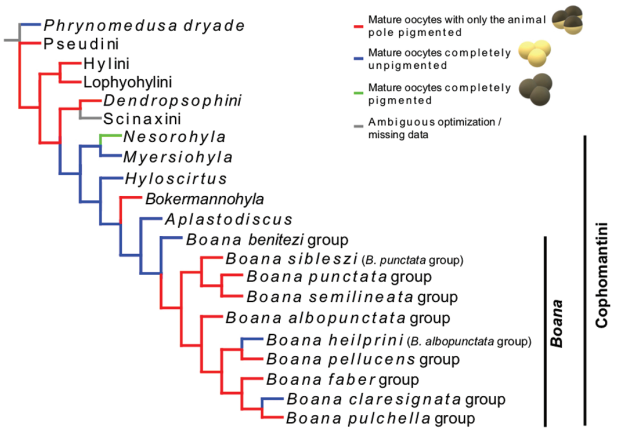

Figure 3. Ancestral character state reconstruction of animal pole pigmentation in mature oocytes/eggs of Cophomantini. Most genera of Cophomantini and species groups of *Boana* are condensed. See the Supporting Information (Appendix S7) for the optimization in the complete topology.

species, Landestoy (2013) suggested that amplexus and oviposition take place in flooded streamside burrows, apparently not constructed by the male. The reproductive mode remains mostly unknown in the *Boana benitezi* group, where there is a single record of an amplectant pair of *Boana lemai* in a plastic bag that deposited eggs in a leaf (Duellman, 1997), without any observation in the field. From the other genera of Cophomantini where unpigmented mature oocytes/eggs are known to occur (*Aplastodiscus*, *Hyloscirtus* and *Myersiohyla*), reproduction is better known in *Aplastodiscus* (e.g. Haddad & Sawaya, 2000; Haddad *et al.*, 2005; Zina & Haddad, 2006). In this genus, unpigmented eggs are placed in hidden, flooded streamside burrows built by the male; the eggs develop, and the tadpoles in early stages are eventually released into the stream, where they complete their development. In the ten species of *Hyloscirtus* where mature oocytes/eggs are known, these are unpigmented (for a review, see Faivovich *et al.*, 2013). From these, observations are only available for *Hyloscirtus platydactylus* (Boulenger, 1905), for which La Marca (1985) describes egg clutches on the apex of leaves of Melastomataceae and Laureaceae, but does not provide information on whether they were overhanging streams.

Although Bokermann (1972) and Lutz (1949a, 1973) refer to the close association of adults with epiphytic bromeliads, the place of oviposition of *Boana claresignata* and *Boana clepsydra* remains unknown. The fact that other Cophomantini that also have unpigmented eggs have different reproductive modes also limits any inference about it.

CONCLUSION

The two described species of the former *Hyla claresignata* group, *Hyla claresignata* and *Hyla clepsydra*, have not been collected in the last 55 and 40 years, respectively. As such, this remained the last known putative clade in Cophomantini that had never been included in a phylogenetic analysis, and its relationships with other taxa were far from clear. The access to DNA sequences of museum specimens of *Hyla claresignata* through high-throughput DNA sequencing provides valuable information. The phylogenetic analysis of the resulting DNA sequence allowed us to recover the position of this species, revealing that it should be associated with *Boana*. The combination of these results with an extensive discussion of available phenotypic evidence supports the transfer of this species to *Boana* together with *Boana clepsydra*, where both are included in a newly diagnosed *Boana claresignata* group. The inclusion of this group in a phylogenetic context sheds further light on the evolution of some morphological characters in Cophomantini.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Studied specimens.
Appendix S2. Some characters and specimens of the *Boana claresignata* group referred to in the main text.
Appendix S3. Laboratory data and results of sequencing analyses.
Appendix S4. GenBank accession numbers for the sequences used in this study.
Appendix S5. Complete parsimony tree.
Appendix S6. Maximum likelihood tree.
Appendix S7. Ancestral character state reconstruction of animal pole pigmentation in mature oocytes/eggs of Cophomantini. As in Figure 3, but showing the complete topology.