High throughput DNA sequencing of museum specimens sheds light on a species that has been missing for the last 55 years: *Bokermannohyla claresignata* (Anura: Hylidae: Cophomantini)

Abstract

The two species of the *Bokermannohyla claresignata* species group (Anura: Hylidae) have not been collected for at least the last three decades, and it is the only species group of the hyline tribe Cophomantini that has not yet been included in phylogenetic analyses. Its phylogenetic position is uncertain, and it has a combination of adult and larval character states that make this group a critical missing piece hindering our understanding of Cophomantini phylogenetics and character evolution. Here 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 the sister taxon of the *Boana pulchella* group, supporting its inclusion in *Boana,* together with *Bok. clepsydra*. In the light of this new finding, we recognized a newly defined *Boana claresignata* group to accommodate these species, thus resolving both the polyphyly of *Bokermannohyla* and the paraphyly of *Boana*. Considering the phylogenetic relationships of the *Boana claresignata* group, we further discuss the evolution of suctorial tadpoles and mature oocyte/egg pigmentation in Cophomantini.

Keywords: Archival DNA - museum - phylogenetics - taxonomy -Hylinae - *Boana pulchella* group

INTRODUCTION

During the last three centuries museums and other natural history collections have housed an immense record of the planet's biodiversity. These collections have traditionally been used to address critical issues in taxonomy, phylogenetic systematics, biogeography, and conservation, as well as 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 (Hofreiter *et al.*, 2001; Gilbert *et al.*, 2007; Miller *et al.*, 2008; Briggs *et al.*, 2009; Gansauge & Meyer, 2013; Kehlmaier *et al.*, 2017). These technological advances have allowed access to degraded DNA from preserved samples, including species that are extinct or have disappeared in the wild.

Knowledge on phylogenetic relationships of the hylid subfamily Hylinae has increased substantially in the last 14 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 comparatively have received 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; Rojas-Runjaic *et al.*, 2018; Peloso *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; Pinheiro *et al.*, 2019; Rojas-Runjaic *et al.*, 2018). The only exception that persists in Cophomantini in terms of a species group that had 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* group. Bokermann (1972) suggested that the nominal species and *H. clepsydra* A. Lutz, 1925, both known from a few montane localities in the Atlantic Forest of Southeastern Brazil, were closely related and that they may further be related to the then *Hyla circumdata* group. Although not explicitly stated by this author, the evidence for the first hypothesis possibly was the difficulty in differentiating both species and as well as the unique characters of their tadpoles. He did not advance evidence to support affinities with the *Hyla circumdata* group. 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 *H. clepsydra* in the former *H. circumdata* group. However, subsequent workers on this group did not consider those species (Cardoso & Haddad, 1982; Caramaschi & Feio, 1990; Pombal & Haddad, 1993). The *H. 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, in Northeastern Argentina. Duellman *et al.* (1997) referred to the *H. claresignata* group as containing the nominal species and *H. clepsydra* when discussing stream-adapted tadpoles in South American hylids.

Faivovich *et al.* (2005) based on their phylogenetic results, erected the genus *Bokermannohyla* for the former *Hyla circumdata*, *H. martinsi*, and *H. pseudopseudis* species groups. As tissue samples of the former *H. claresignata* group were not available, these authors only tentatively included its species in *Bokermannohyla* as the *Bok. 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 only be considered as such 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* (A. Lutz & B. Lutz, 1939) was described based on one adult female from the Fazenda of Messrs. Guinle, Teresópolis, State of Rio de Janeiro, Brazil (Type locality), 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 Serra da dos Órgãos, around Teresópolis (Beija Flor, Garrafão, Paquequer) and Theodoro de Oliveira (then in the outskirts of Nova Friburgo—50 km NE from Teresópolis—today a neighborhood 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 alotypes [sic] and several other specimens of the collection were found sleeping on gravatás" [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 NW (airline) 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, B. Lutz (1973) provided descriptions of the type material of both species and summarized information on these species from her previous publications and 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 B. Lutz (1973) were the last published references to 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). Due 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 1982 in the road Paraty-Cunha, between the states of Rio de Janeiro and São Paulo (C.A.G. Cruz pers. comm.). These specimens were housed in the Eugenio Izecksohn Collection, Universidade Federal Rural de Rio de Janeiro, Seropédica, Rio de Janeiro, Brazil, but they could not be located (H.R. da Silva, pers. comm.). Fieldwork in the road Paraty-Cunha, and in the Parque Nacional Serra da Bocaina, including the area formerly occupied by the Campo de Fruticultura da Serra da Bocaina, during the last 12 years had been unsuccessful in finding adults or tadpoles.

Since *Bok. clepsydra* and *Bok. claresignata* were not collected in the last three 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 *B. claresignata* with the use of high throughput DNA sequencing and provide new insights into the taxonomy and phylogenetic relationships of this species and *B. clepsydra*.

MATERIAL AND METHODS

MUSEUM SPECIMENS AND VOCALIZATIONS

For this study, we had access to available adult and larvae of *Bok. claresignata* and *Bok. clepsydra* available in the collections of the Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ), the Museu de Zoologia, Universidade de São Paulo (MZUSP), and the Adolpho Lutz collection (AL-MN, housed in the Museu Nacional, Universidade Federal do Rio de Janeiro). 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 employed 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 of Faivovich *et al.* (2018). Vocalizations were recorded 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 analyzed with Raven 1.5 (Cornell Lab of Ornithology, Ithaca, NY). Spectrograms were generated with window type Hann, 512 samples, 75% overlap, and 128 hop size. See 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* housed at the amphibian collection of the Museu Nacional, Rio de Janeiro, Brazil (MNRJ 54331). This specimen was collected in January 21, 1953 by Bertha Lutz and most probably fixed and stored in 70% ethanol.

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 amplification (PCR) 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 purified using the silica-column based method described in 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 archival DNA recovery.

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 Klenow fragment of DNA polymerase I for the fill-in reaction. We also performed a quantitative PCR experiment to determine optimal number of cycles for subsequent libraries indexing as described in Basler *et al*. (2017).

An initial assessment of archival 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 v2.5 (75 cycles SE) kits at the University of Potsdam, following the procedures described in 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 in-house made DNA baits (see below).

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 another genus from tribe Cophomantini, *Aplastodiscus arilde* CFBH30829 (Célio F. B. Haddad Collection, Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, State of São Paulo), using the DNeasy Blood & Tissue kit (QIAGEN Inc.). We amplified the partial mitogenome in two overlapping fragments 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 approximately 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 *Aplastodiscus arilde* library used as bait was also sequenced and the mitogenome was assembled to serve as reference sequence for mapping sequencing reads from the historical sample (see below).

SEQUENCING DATA PROCESSING AND ASSEMBLY OF MITOGENOME SEQUENCES

Sequenced reads from both shotgun and enriched libraries were quality-trimmed using cutadapt vers. 1.16 (Martin 2011) with 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 previously analyzed in the same clean laboratory, and library adapters (see Appendix S2), were screened as potential sources of contamination using FastQScreen v0.13.0 (Wingett & Andrews,

2018) as suggested in Straube *et al* (submitted). We included the raw transcriptome of *Aplastodiscus leucopygius* (GenBank accession number **[to be provided upon acceptance of this Ms]**) as a phylogenetically close reference to estimate the relative endogenous content, although this method will represent an underestimate since large parts of the genome are not transcribed.

For mitochondrial genome assembly, we combined all trimmed and non-duplicated sequences from the different libraries, since they were prepared from the same individual. We mapped reads 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 v1.9 (Hahn *et al.*, 2013). We implemented MITObim using default parameters apart from the mismatch value where we used zero and kbait of 15. The newly generated mitogenome of *Aplastodiscus arilde* (GenBank accession number **[to be provided upon acceptance of this Ms]**) was used as seed. The final ".caf" file output was visualized in Geneious v11.0.5 (Kearse *et al.*, 2012) and we estimate coverage of mitochondrial contigs. Only sequences with coverage higher than 10 and from markers available for other Cophomantini were used for phylogenetic inferences.

TAXONOMIC SAMPLING

Our dataset included the taxonomic sampling of Cophomantini of Faivovich *et al.* (2013), to which we added all the species of that tribe for which sequences were produced subsequently by Caminer & Ron (2014), Guayasamin *et al*. (2015), Fouquet *et al.* (2016), Berneck *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) for a total of 132

terminals. As outgroups, we employed the same exemplars of hylid diversity included by Pinheiro *et al.* (2019), and the trees were rooted with *Phrynomedusa dryade*. See Supplementary Material 1 for GenBank accession numbers.

CHARACTER SAMPLING

We included up to 7486 base pairs (bp) per terminal from the same gene fragments employed by Faivovich *et al.* (2013): the mitochondrial genes cytochrome b (CYTB), 12S rRNA, $tRNA^{VAL}$, and 16S rRNA (H1), $tRNA^{LEU}$, NADH dehydrogenase subunit 1, and $tRNA^{ILE}$ (ND1) mitochondrial genes, 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 nuclear genes. Sequences were aligned using MAFFT V7 (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 Appendix S3 for a complete list of GenBank accession numbers and voucher information of sequences produced for this study.

PHYLOGENETIC ANALYSES

Numerous authors have discussed the rationale for using the parsimony optimality criterion (Farris, 1983; Goloboff, 2003; Goloboff & Pol, 2005; Kluge & Grant, 2006, Grant & Kluge, 2009). The phylogenetic analysis was done 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 TBR branch swapping. Parsimony Jackknife (Farris et al., 1996) absolute frequencies were estimated from 1,000 replicates, hitting minimum length five times (search level 15) with new technology searches (Goloboff, 1999) in each replicate, since 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.

Maximum likelihood (ML) analyses were conducted using RAxML v8.2.10 (Stamatakis, 2014) on the concatenated dataset, employing 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 v2.1.1 (Lanfear *et al.*, 2016), using the greedy algorithm (Lanfear *et al.*, 2012). Searches included 1,000 runs using the rapid hill-climbing algorithm (Stamatakis *et al.*, 2007). Non-parametric bootstrapping values (Felsenstein, 1985) were estimated using 1,000 pseudoreplicates. Trees were visualized and edited in FIGTREE 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 Prot-K libraries resulted in a total of 1488782 reads. After trimming and excluding duplicated reads, 444509 reads remained (see Appendix S2 for results for each library). The average fragment size was 37 bp, after excluding adapters and very small reads. FastqScreen analyses revealed no obvious contamination except few reads that mapped to the human genome (41 in total). Only 0.41% to 0.46% of reads in each library mapped to the *Aplastodiscus* transcriptome, suggesting low relative endogenous content. Most reads have not mapped to any reference genomes. A blast search in NBCI (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 2040483 reads (454339 reads after cleaning). We pooled all quality trimmed and non-duplicated reads shotgun and captured libraries to assembly the mitochondrial DNA. We were unable to assemble the complete mitogenome of *B. claresignata*, but recovered around 1580 bp arranged in 11 contigs with coverage higher than 10 from the 12S and 16S rRNAs. The final readpool of mitochondrial DNA sequences recovered by MITObim was 23225 reads, being that 15146 reads mapped to 12S-tRNVval-16S mitochondrial fragment. These sequences were used for downstream analyses. We also recovered small fragments from some tRNAs or coding genes (ND2, COI, 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 29455 steps (Fig. 1; Appendix S4). The strict consensus is highly resolved, with all conflict among the most parsimonious topologies (MPT) restricted

to (1) relationships among the *Hyloscirtus bogotensis* and *H. jahni* groups with the clade including the *H. armatus* and *H. larinopygion* groups, (2) internal relationships of the *H. 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 28103 steps. The strict consensus is mostly congruent with that obtained with gaps considered as a fifth state, and the conflict among MPTs is also similar, with additional internal conflict in the *B. albopunctata* group. The ML results (Appendix S5) are congruent in terms of the most supported groups with the parsimony analyses. *Bokermannohyla claresignata* is nested in *Boana*, as the sister taxon of the *B. pulchella* group, with 85% jackknife support (73% when gaps are considered missing data; 98% bootstrap in the ML analysis); the monophyly of the *B. pulchella* group is supported with 80% jackknife (86% when gaps are considered 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 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 so-called lost species.

By sequencing a museum sample of *Bok. claresignata*, we are filling 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 very fragmented despite being apparently fixed in ethanol only, 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 may be as challenging for DNA extraction as formalin-fixed, ethanol stored specimens. On the other hand, obtaining only partial fragments of mitochondrial DNA may 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 results of 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 *M. kanaima* and the redefinition of *Myersiohyla*. Our results are congruent with previous studies in that *Myersiohyla* and *Nesorohyla* are the two earlier diverging, with the position of the latter being poorly supported, in this case as sister taxon of the former in the parsimony analysis (Fig. 1; Appendix S4). Our results for *Hyloscirtus* are congruent with the recent hypothesis of Rojas-Runjaic *et al.* (2018) and Ron *et al.* (2018) in the recognition of four species groups (the *H. armatus*, *H. bogotensis*, *H. jahni*, and *H. larinopygion* groups). However, it differs from the first study and that of Almendáriz *et al.* (2014) in that the *H. armatus* group is supported as the sister taxon

of the *H. larinopygion* group with 86% jackknife (78% when gaps are treated as missing data; 86% in the ML analysis). The monophyly of the *H. armatus* and *H. larinopygion* groups was obtained as well by Guayasamin *et al.* (2015) and by Pinheiro *et al.* (2019), although with 61% bootstrap and 91% jackknife respectively. We note that our analysis differs from those of Almendáriz *et al.* (2014), Guayasamin *et al.* (2015), Rojas-Runjaic *et al.* (2018), and Ron *et al.* (2018) in that we included sequences of nuclear genes.

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 *B. 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). See discussion below.

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

Our phylogenetic analyses recover *Bok. claresignata* deeply nested in *Boana*, as the sister taxon of the *B. pulchella* group with 85% jackknife support (Fig. 1; Appendix S4). Although we only included *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 (see below); for this reason, we consider that the recovered position of *Bok. claresignata* is extensive 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 still 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 *H. jahni* group; Kizirian *et al.*, 2003; Coloma *et al.*, 2012; Lourenço, Rivera-Correa, Faivovich, and 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 *Hyloscirtus*, *Aplastodiscus*, *Boana*, and *Bokermannohyla*, with a reversal in the latter genus, or two independent origins, in *Hyloscirtus* and the common ancestor of *Aplastodiscus* and *Boana*. The expanded diapophysis occurs as well 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 *B. pulchella* group. One of these 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 (Appendix S6: Fig. S1). Faivovich and Garcia (in Faivovich et al., 2005) identified this character state as a synapomorphy of the *B. pulchella* group. Subsequent observations indicate that in Cophomantini it is homoplastic only with the clade including *B. atlantica*, *B. cinerascens*, and *B. punctata* (Pinheiro pers. obs.)

Pinheiro et al. (2018) stated that the absence of the anterolateral process of the hyoid plate is so far only known in the *B. pulchella* group. An anterolateral process is absent as well in *Bok. claresignata* (MNRJ 24028) but present in *Bok. clepsydra* (MZUSP 112612). The taxonomic distribution of this character state requires more study: it is still unknown in a number of species of the *B. pulchella* group (*B. aguilari*, *B. balzani*, *B. cambui*, *B. melanopleura*, *B. palaestes*), and the anterolateral process is present in *B. freicanecae* (Pinheiro pers. obs.), so 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 *B. pulchella* group (Pinheiro et al., 2018: fig 3D), and present at least in some species of the *B. albopunctata* and *B. faber* groups (Pinheiro et al. 2018: fig 3A–3C). Although it could be another putative synapomorphy supporting the monophyly of the former *Hyla claresignata* group and the *B. 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 *H. 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) **new combination** and *Boana clepsydra* (A. Lutz, 1925) **new combination**. While these species are being included in a separate species groups, future studies should focus on the monophyly of the *B. pulchella* group. Whether the decrease in support for this group compared to all previous phylogenetic studies is due to a lack of informative sequences, or actual lack of evidence for its monophyly is still unclear. Although there is abundant phenotypic

evidence for the monophyly of the *B. claresignata* group (see below), it also shares with the *B. 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 *B. pulchella* group is now only supported by molecular data.

The *Boana claresignata* group

Diagnosis: The *B. claresignata* group can be diagnosed by (1) projected prepollical spine; (2) nuptial pads present, single, light-colored, without macroscopically evident epidermal projections (Appendix S6: Fig. S2); (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* (Appendix S6: Fig. S1); (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 7–9 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; (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-colored nuptial pads without macroscopically evident epidermal projections, the unpigmented mature oocytes, and the characters related to the larval oral disc are synapomorphies of the *B. claresignata* group. The occurrence of these character states differentiates this group from all other species of *Boana*. Further, all these 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 *B. heilprini*, with the *B. benitezi* group (when mature oocytes/eggs are known), and with some species of *Myersiohyla* and *Hyloscirtus*. In *Boana*, nuptial pads with dark-colored epidermal projections are known to occur only in the *B. semilineata* group (Faivovich et al., 2006). The palpebral membrane without reticulation differentiates the *B. claresignata* group from the *B. semilineata* group (present in this species group; Faivovich et al. 2006; Peloso et al., 2018). The absence of a mental gland macroscopically evident differentiates the *B. claresignata* group from species of the *B. benitezi*, *B. punctata,* and *B. semilineata* groups, and also from *B. 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 *B. claresignata* group from al least some species of the *B. albopunctata* and *B. faber* groups (Pinheiro et al. 2018: fig 3A–3C). 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 *B. pulchella* and *B. punctata* groups.

The tympanum diameter/eye diameter ratio in the *B. claresignata* group is 0.23–0.54, with the larger value represented by the few available specimens of *B. claresignata*, and the smaller by all those of *B. clepsydra* (see comments below).

While tympanum size 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 *B. albopunctata*, *B. faber*, *B. pellucens*, *B. punctata*, and *B. semilineata* groups, with a combined tympanum diameter/eye diameter ratio varying from 0.48 (*B. wavrini*; Hoogmoed, 1990) to 0.98 (*B. rosenbergi*; Duellman, 1970). In the *B. benitezi* group, this ratio varies from 0.25 (*B. ornatissima*; Hoogmoed, 1979) to 0.51 (*B. nympha*; Faivovich et al., 2006). In the *B. pulchella* group, the sister taxon of the *B. claresignata* group, it varies from 0.35 (*B. caipora*; Antunes et al., 2008) to 0.68 (*B. joaquini*; Garcia et al. 2003). Several species of this group also have smaller tympanum, measuring less than half of eye diameter (e.g., *B. cambui*, *B. ericae*, and *B. semiguttata*; 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 *B. heilprini* with 6/9 (Noble, 1927; Galvis et al., 2014; Díaz et al., 2015), followed by *B. benitezi* with 5/8 (this larva was only tentatively assigned to *B. benitezi*; Myers & Donnelly, 1997), *B. hutchinsi* with 4/7, *B. jimenezi*, *B. curupi* and *B. stellae* with 3/5 (Pyburn & Hall, 1984; Faivovich, 1996; Myers & Donnelly, 1997; Myers & Donnelly, 2008; Widholzer & Castroviejo-Fisher, 2018). Most species of *Boana* have LTRF 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 *B. claresignata* clade (oral disc width 0.80–0.87 body width), while it is smaller in the remaining species of *Boana* (e.g. ODW/BW = 0.48–0.60 in *B. heilprini*; ODW/BW = 0.60 in *B. curupi*,

ODW/BW = 0.50–0.53 in *B. jimenezi*; ODW/BW = 0.50 in *B. stellae*; smaller than 50% of the body width in most species of *Boana*; Faivovich, 1996; Myers & Donnelly, 1998; Kolenc et al., 2008; Díaz et al., 2015; Widholzer & Castroviejo-Fisher, 2018; Pezzuti pers. obs).

The M-shaped upper jaw sheath with long lateral processes laterally directed and a shelf on its medial portion, differentiate the tadpoles of the *B. 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 shape of body and tail of the tadpoles of the *B. claresignata* group are also unique in the genus, being adapted to reophilic microhabitats in swift or torrential mountain streams. This suctorial morphology (see comments below) comprises a very depressed body with an expanded snout, low fins which 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 *B. claresignata* group. To complement the observations of these authors, we add the occurrence of a light-colored nuptial pad (Appendix S6: Fig. S2), 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 natural history of adults and larvae of *B. claresignata* and *B. clepsydra,* which were summarized by Lutz (1973). The available information on adults of *B. 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 agree in that they inhabit epiphytic bromeliads growing on the sides of swift or torrential mountain streams. All collecting localities are approximately 400–1200 m above sea level (a.s.l.) The males of *B. 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 *B. 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, $\bar{X}$= 3.07, SD = 0.22, n = 10; MZUSP 117074 2.1–2.7 mm, $\bar{X}$ = 2.26, SD = 0.26, n = 5). A female of *B. clepsydra* (MZUSP 112626) also has unpigmented mature oocytes evident through the skin. As the oocytes are quite distorted due 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 *B. 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 *B. clepsydra* were observed swimming against the current (Bokermann, 1972).

Vocalization: The advertisement call of *B. clepsydra* was briefly described by Bokermann (1972). We reanalyzed his recording of two unvouchered males (available at Fonoteca Neotropical Jacques Vielliard, accession number 31798). The recording of one of these males has a lower quality (probably the individual was more distant); we describe them separately.

As mentioned by Bokermann (1972) *B. clepsydra* emits groups of three to five calls, each call corresponding to one tonal note (Fig 2A, 2B). Notes are irregularly spaced and with a considerable interval between them. The call has bands, similar to a harmonic structure (Fig 2B, 2C), 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 emitted five calls in 15.48 s (n = 1). Each call (note) lasts 130–169 ms (144.4 ± 16.6) and is separated by intervals of 2.60–4.17 s (3.68 ± 0.72). The fundamental frequency, which is also the dominant, is 2053.8–3010.9 Hz, with the peak frequency at 2437.5 Hz. Up to four additional bands are present, the first one with peak at 4500 (n = 1), 4875 (n = 1), or 5062.5 Hz (n = 3). The second with peak at 7125 (n = 1) or 7500 Hz (n = 4). The third one, which is absent in the first note, and is the band with lower energy, with peak at 9000 (n =1), 9937.5 (n = 2), or 10125 Hz (n = 1). The fourth and higher band have it peaks at 12000 (n = 1), 12375 (n = 1), or 12562.5 Hz (n = 3).

The second individual emitted three calls lasting 7.58 s (n = 1). Each call (note) lasts 59–89 (73 ± 15) ms, and they are separated by intervals of 3.51–3.85 s. The fundamental frequency, which is also the dominant, is between 2050.6–2885 Hz, with the peak frequency at 2437.5 Hz (n =2) or 2625 Hz (n = 1). Up to four additional bands are present, the first one with peak at 4500 (n = 2) or 4875 (n = 1). The second one with peak at 7312.5 (n = 2) or at 7687.5 Hz (n = 1). The third one, which was absent in the third note, was found with peak at 9562 (n =1) or 9750 (n = 1) Hz. The fourth and higher band was found with peak at 12000 (n = 1), 12187.5 (n = 1), or 12750 Hz (n = 1).

The call of *B. clepsydra* can be distinguished from those of *B. aguilari*, *B. balzani*, *B. bandeirantes*, *B. beckeri*, *B. botumirim*, *B. caipora*, *B. cipoensis*, *B. curupi*, *B. cymbalum*, *B. ericae*, *B. gladiator*, *B. guentheri*, *B. jaguariaivensis, B. latistriata*, *B. leptolineata*, *B. marianitae, B. melanopleura, B. polytaenia*, *B. stellae,* and *B. stenocephala* by having notes with a tonal structure (pulsed notes in these species; Haddad et al., 1988; Heyer et al., 1990; Duellman et al., 1997; Acioli & Toledo, 2008; Garcia et al., 2007, Antunes et al., 2008; Garcia & Haddad, 2008; Kwet. 2008; Caramaschi et al., 2009; Köhler et al., 2010; Lehr et al., 2010; Forti et al., 2019; Guerra et al., 2017; PDP Pinheiro, pers. obs.). From *B. albonigra*, *B. caingua*, *B. cambui*, *B. cordobae*, *B. goiana*, *B. phaeopleura*, *B. pulchella*, and *B. riojana*, the call of *B. clepsydra* can be distinguished by an interval between notes longer than 2 s (interval between notes lower than 1 s in the other species; Barrio, 1965a; Guimarães et al., 2001; Pinheiro et al., 2012, 2016; Baraquet et al., 2013; Batista et al., 2015; PDP Pinheiro, pers. obs.). The non-pulsed structure of the call of *B. clepsydra* differentiates it from those of the closely related *B. pellucens* and *B. faber* groups, that have calls with pulsed structure (Fouquette, 1961; Bokermann, 1967; Bokermann & Sazima, 1973; Duellman, 1970, 2001; Heyer et al., 1990; Kluge, 1981; Loebmann et al., 2008; Martins et al., 2009).

Comments: Lutz & Orton (1946) described the occurrence of green bones in postmetamorphic specimens of *B. claresignata*, indicating impregnation with biliverdin (Barrio, 1965b), as it happens in several other species of *Boana* from various species groups (e.g., Hoogmoed, 1979; Duellman, 1970; Lutz, 1949b; 1973; Caminer & Ron, 2014). There are no observations regarding the persistence of biliverdin in adults of *B. claresignata*, nor any comment regarding its occurrence in *B. clepsydra*. Lutz & Orton (1946) reported that specimens of *B. claresignata* release a

characteristic smell of crushed plants when handled, as it happens in several other species of Cophomantini (see Faivovich et al. 2013; Brunetti et al., 2015, 2016). There are no reports on smell from *B. clepsydra*.

The occurrence of nuptial pads in *B. claresignata* and *B. clepsydra* is reported here for the first time. The nuptial pad is easily observed *in B. clepsydra* (see Appendix S6: Fig. S2). There are few available males of *B. claresignata* in collections, and all of these are very poorly fixed. In these, the nuptial pad is only evident through the occurrence of some yellowish acini in the same position as in *B. 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 including 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 and Roig (1961) recorded for Misiones. The populations of this species from Argentina were subsequently shown to be a different, new species by Garcia et al. (2007).

Cochran (1955) included a description of a male paratype of *B. claresignata* from "Bonito, Serra da Bocaina, Rio de Janeiro," and the holotype of *B. clepsydra* (a male). The latter is an important reference because the holotype specimen, that Cochran (1955: 88) described at that time as "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; Appendix S6: Fig. S4). One notable point of her description of *B. clepsydra* is her comment on the occurrence of "a pair of lateral external vocal sacs." She did not compare *B. claresignata* and *B. clepsydra*.

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

The information on variation in the dorsal pattern in *B. clepsydra* provided by Bokermann (1972) clearly shows that it does not allow differentiating this species from *B. claresignata*. Our observations on all specimens of *B. clepsydra* in the MNRJ and MZUSP collections corroborate that statement.

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 *B. clepsydra*, which has a shorter and truncated snout.

Boana claresignata has tympanum diameter/eye diameter ratio (TD/ED) of 0.39–0.54 (females; 0.44 ± 0.06; n = 4) and 0.41–0.57 (males; 0.48 ± 0.08, n = 6), and a tympanum diameter/head length ratio (TD/HL) of 0.11–0.15 (females; 0.13 ± 0.02; n = 4) and 0.12–0.22 (males; 0.16 ± 0.03; n = 6). *Boana clepsydra*, instead, have TD/ED 0.30–0.38 (females; 0.34 ± 0.06; n = 2) and 0.23–0.38 (males; 0.31 ± 0.04; n = 23), and TD/HL 0.09–0.11 (females 0.10 ± 0.01; n = 2) and 0.08–0.12 (males; 0.10 ± 0.01; n = 23). While these measurements tend to corroborate the larger tympanum size in *B. claresignata* noticed by Bokermann (1972), the values of the ratios that express tympanum size are continuous. Considering the low number of available specimens of *B. claresignata*, this difference in tympanum size should be taken cautiously, as the lack of overlapping between intervals for the ratios could well be a consequence of the reduced 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 tympanum is slightly below the level of the lower margin of the eye. In *B. clepsydra* the lower margin of the tympanum is well above the lower margin of the eye.

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

The study of male specimens of *B. clepsydra* (n = 22) revealed that the vocal sac is single and subgular. This observation differs from Cochran's (1955) description 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 *B. claresignata*, the only specimens of this species from Serra da Bocaina, indicates that the reasonably preserved specimen (AL-MN 2088) has the rounded snout that we associate with *B. clepsydra*. Therefore, we consider that these paratypes of *B. claresignata* are misidentified specimens of *B. clepsydra*, indicating that there is no evidence of the occurrence of *B. claresignata* in the Serra da Bocaina.

Lutz (1973) reported the collection of tadpoles similar to those of *B. claresignata* in Marumbi, State of Paraná (~ 550 km SW from Serra da Bocaina). These tadpoles are the lot MNRJ 68427 (nine tadpoles in stages 25–28, and 38, and one postmetamorphic specimen; Appendix S6: Fig. S3), 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.). While we corroborate their striking similarity with larvae of *B. claresignata* and *B. clepsydra* (Appendix S5), we do not have evidence to associate it specifically with one of these species: for the time being, we consider them as larvae from an unidentified species of the *B. claresignata* group.

Distribution: Both species are endemic of the Atlantic Forest in Southeastern 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 (22°44'44" S, 44°33'41" W, 1220 m a.s.l.), and the former Campo de Fruticultura da Serra da Bocaina (22°43'49.48"S, 44°35'56.01"W, 1500 m

a.s.l.), currently within the limits of the Parque Nacional da Serra da Bocaina, in the State of São Paulo. The third locality is in the southwestern 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), approximately 57 km SW (airline) from the other localities. *Boana claresignata* is known from the Parque Nacional Serra dos Órgãos in Teresópolis, where it has been collected in some swift streams (Beija Flor, Garrafão, Paquequer, 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 a very 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 a 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., *M. neblinaria* and *M. inparquesi*; interpreted from the illustrations, Ayarzagüena & Señaris, 1993; Faivovich *et al.*, 2013), and possibly in the *Hyloscirtus jahni* group (it is not clear from the information provided by La Marca, 1985, if the larva of this species has the same snout structure). This specialization, already described in other suctorial species of Pelodryadinae (Gradwell, 1973; 1975), may act in the engagement or disengagement of the oral disc in the substrate (Gradwell, 1975), or at least 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 *B. claresignata* clade), and even optimize ambiguously in *Hyloscirtus*, if present as well in *H. jahni*.

Many of the character states present in the larvae of the *B. 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, 1993, 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), *Hyloscirtus* (Duellman *et al.,* 1997), and subsequently were inferred as plesiomorphic conditions of 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) from other tadpoles of early diverging lineages of the tribe, 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 presence of an anterior gap on marginal papillae) could have evolved in a common ancestor of *Bokermannohyla*, *Aplastodiscus*, and *Boana*. Besides the ambiguity generated by the tadpole of *Nesorohyla 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 *A. sibilatus* group, *Bok. pseudopseudis* and *Bok. martinsi* groups, and some species of the *Boana albopunctata*, *B. benitezi*, *B. punctata*, *B. pulchella*, and *B. semilineata* groups; Pyburn & Hall, 1984; Faivovich, 1996; Myers & Donnelly, 1997, 1998; Leite & Eterovick, 2010; Mercês & Juncá, 2010; Lins et al., 2016). The plesiomorphic states of these characters in *Boana* are still uncertain, in part because of the ambiguities commented 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 *B. claresignata* group and *B. heilprini*, have tadpoles with enlarged oral discs and LTRF that show an extreme of development when compared to closely related taxa (*B. heilprini*, LTRF 4-6/6-9; Noble, 1927; Galvis *et al.,* 2014; Díaz *et al.,* 2015), indicating that in these particular cases the suctorial related characters 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 *B. heilprini* (Nali et al., 2014), *B. lemai* (Duellman, 1997), *B. nympha*, and *B. roraima* (Faivovich et al., 2006). The recognition of the *B. claresignata* group, adds another case of unpigmented mature oocytes. The optimization of egg pigmentation in our phylogenetic hypothesis indicates that the unpigmented mature oocytes are plesiomorphic in *Boana* and that the pigmented animal pole evolved in the sister taxon of the *B. benitezi* group (Fig. 3). This inference, however, should be taken cautiously, as the relationships among most species groups of *Boana* are poorly

supported (Fig. 1, Appendix S4). Regardless, unpigmented mature oocytes evolved independently in the *B. claresignata* group and in *B. heilprini*.

From the three independent occurrences of unpigmented mature oocytes, the reproductive mode is only superficially known in *B. heilprini*. Based on observations and published records of this species, Landestoy (2013) suggested that amplexus and oviposition take place in streamside-flooded burrows, apparently not constructed by the male. The reproductive mode remains mostly unknown in the *B. benitezi* group, where there is a single record of an amplectant pair of *B. 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*, the 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 a hidden, flooded streamside burrows built by the male; the eggs develop, and the tadpoles in early stages are eventually released in the stream where they complete their development. In the ten species of *Hyloscirtus* where mature oocytes/eggs are known, these are unpigmented (see Faivovich *et al.*, 2013 for a review). From these, observations are only available for *H. platydactylus*, were La Marca (1985) describes egg clutches on the apex of leaves of Melastomataceae and Laureaceae, but but did not provide information on whether they were overhanging streams.

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

CONCLUSION

The species of the former *Hyla claresignata* group, *Hyla claresignata* and *H. clepsydra*, have not been collected in the last 55 and 37 years, respectively. As such, it 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 provided 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 *B. clepsydra*, where both are included in a newly diagnosed *Boana claresignata* group. The inclusion of this group in a phylogenetic context further sheds light on the evolution of some morphological characters in Cophomantini.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

- Appendix S1: Studied specimens.
- Appendix S2: Laboratory data and results of sequencing analyses.
- Appendix S3: GenBank accession numbers for the sequences employed in this study.
- Appendix S4: Complete maximum parsimony tree.
- Appendix S5: Maximum likelihood tree.
- Appendix S6: Some characters and specimens of the *Boana claresignata* group referred in the main text.

FIGURE LEGENDS

Fig. 1. The strict consensus of the 96 most parsimonious trees (29455 steps), treating gaps as fifth state. Most genera of Cophomantini and species groups of *Boana* are condensed. See Appendix S4 for a complete topology. Numbers below nodes are

jackknife frequencies with gaps treated as fifth state or a missing data. The asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. The photograph of *Boana claresignata* is taken from Lutz (1948). The watercolor depicts the living holotype of this species and is part of the collection of the Departamento de Vertebrados, Museu Nacional, Rio de Janeiro, Brazil. Author unidentified. Undated.

Fig. 2. Advertisement call of an unvouchered male of *Boana clespsydra.* This recording was made by W.C.A. Bokermann, described by Bokermann (1972), and now housed in Fonoteca Neotropical Jacques Vielliard (accession number 31798). A. Audiospectrogram of a sequence of five calls. Waveform below. B. Detail of the second note from A; waveform below. C. Power spectrum of note shown in B. Black arrows point the dominant frequency (bottom) and additional, harmonic-like bands (see 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; Nov. 6, 1968, 21 hs, 17º C, a male calling from 1.5 m above the ground.

Fig. 3. Ancestral character state reconstruction of animal pole pigmentation in mature oocytes/eggs of Cophomantini in the strict consensus of the 96 most parsimonious trees (29455 steps), treating gaps as fifth state. Most genera of Cophomantini and species groups of *Boana* are condensed. See Appendix S7 for the optimization in the complete topology.

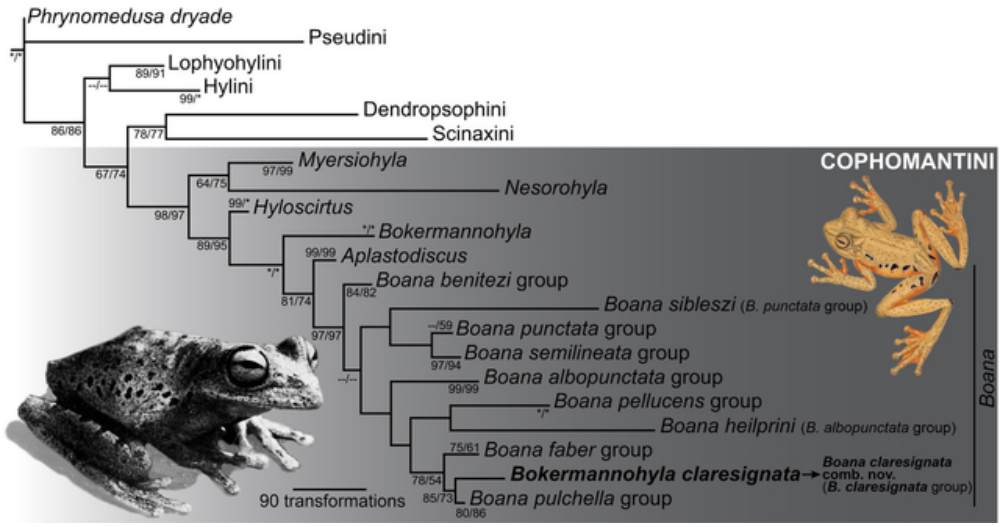

Fig. 1. The strict consensus of the 96 most parsimonious trees (29455 steps), treating gaps as fifth state.

27x14mm (600 x 600 DPI)

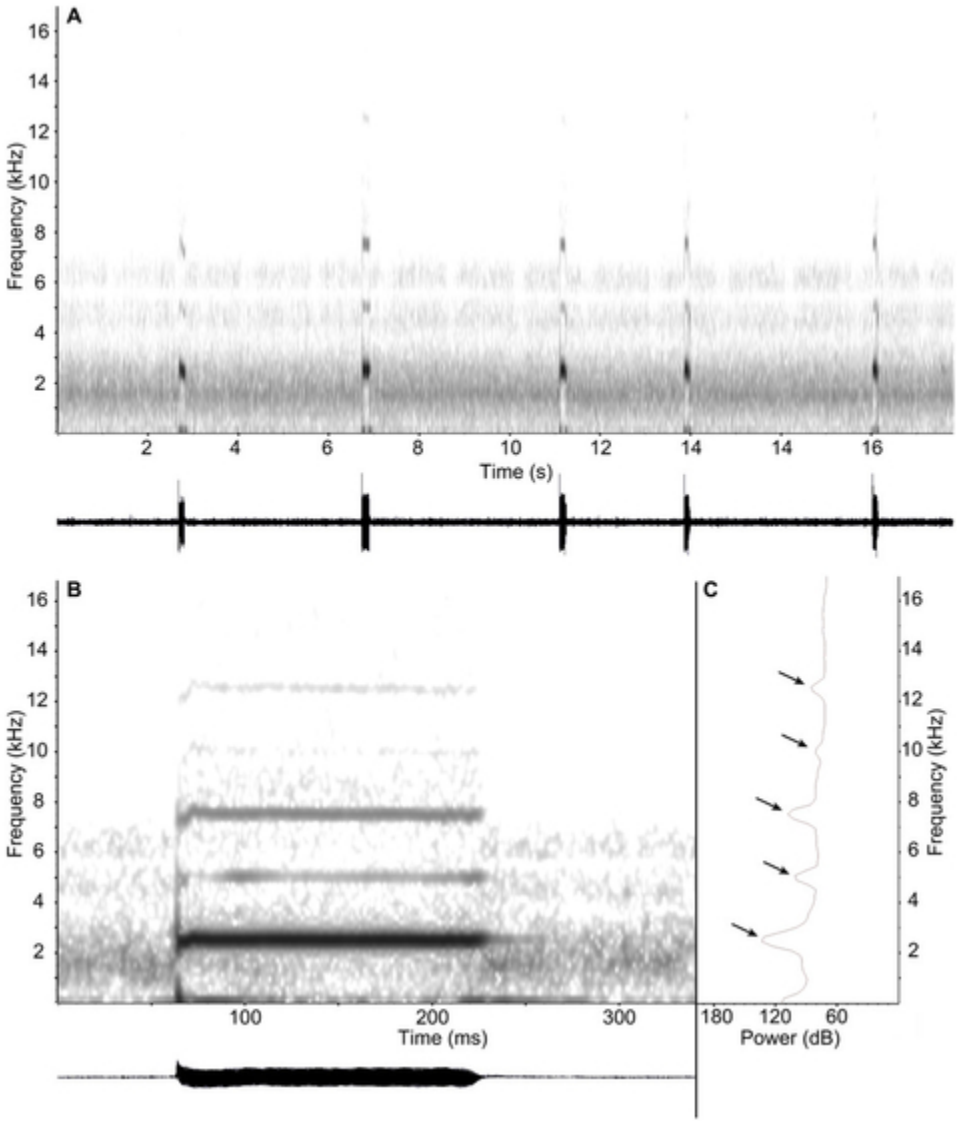

Fig. 2. Advertisement call of an unvouchered male of Boana clespsydra.

20x24mm (600 x 600 DPI)