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Historical DNA Places an Apparently Extinct Gladiator Frog in the Phylogeny of the *Boana pulchella* Group (Anura: Hylidae)

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Abstract: *Boana cymbalum* has not been collected for the past six decades and is one of the two species of Brazilian frogs formally considered to be extinct. It is also the only species of the *B. pulchella* group that has never been included in molecular phylogenetic analyses, because no fresh tissue is available. Using specialized historical DNA (hDNA) extraction protocols and high-throughput sequencing, we obtained mitochondrial and nuclear sequences from the 62-yr-old holotype of *B. cymbalum*. Our results place *B. cymbalum* as the sister taxon of the clade formed by *B. prasina*, *B. cordobae*, and *B. pulchella*, supporting its inclusion in the *B. prasina* clade. The scars on the dorsum of adult males suggest male–male combat. Microcomputed tomography revealed the presence of a large postarticular process and a distal prepollex directed laterad to pass ventral to Metacarpal II, two known synapomorphies of the *B. pulchella* group. On the basis of recordings from 1963, we provide an expanded description of the vocalization of *B. cymbalum*, consisting of a short, tonal note (Note A) and a longer, multipulsed note (Note B). Finally, we discuss the conservation status *of B. cymbalum* and the potential application of the newly generated hDNA sequences to screen water bodies near the type locality using environmental DNA in an effort to rediscover this species.

Key words: Archival DNA; Call description; Cophomantini; Extinction; Museomics; Taxonomy

The prospect of retrieving DNA sequences from extinct species has captivated researchers since the seminal paper of Higuchi et al. (1984), in which short fragments of an extinct quagga were recovered. Whereas ancient DNA (aDNA) is preserved under natural conditions (e.g., caves and permafrost deposits), historical DNA (hDNA) is derived from preserved specimens in natural history museums (Raxworthy and Smith 2021). Although natural history museums store billions of biological specimens (Wheeler et al. 2012), most were collected before tissue sampling for genetic studies was common practice (Wandeler et al. 2007; Bi et al. 2013; Holmes et al. 2016; Yeates et al. 2016; Lopez et al. 2020; Straube et al. 2021). As such, many species have not been included in molecular phylogenetic studies (e.g., Lyra et al. 2020; Nakamura et al. 2025). However, recent advances in museomics have enabled DNA sequences to be obtained from formalin-fixed museum specimens (e.g., Straube et al. 2021).

The genus *Boana* (Hylidae: Hylinae: Cophomantini) comprises approximately 100 species distributed from central Argentina in South America to Nicaragua and Hispaniola in Central America and the Caribbean (Frost 2024). Faivovich et al. (2005) recognized seven species groups, including the *B. albopunctata*, *B. benitezi*, *B. faber*, *B. pellucens*, *B. pulchella*, *B. punctata*, and *B. semilineata* groups. Recently, Lyra et al. (2020) successfully sequenced hDNA from museum larval specimens of the former *Bokermannohyla claresignata* group, which comprised two lost species, namely *Bok. claresignata* and *Bok. clepsydra.* Their phylogenetic results revealed it to be placed within *Boana* as the sister clade of the *B. pulchella* group, and hence expanded the number of *Boana* species groups from seven to eight.

The *Boana pulchella* group is the most speciose group in the genus, with 36 known species (Faivovich et al. 2021; Pinheiro et al. 2024). This group was recognized by Bokermann (1963) while describing *Hyla cymbalum*, but he used the name *Hyla raddiana* instead of *H. pulchella* (see Bokermann 1965; Barrio 1965; and Garcia et al. 2003 for nomenclatural history). Lutz (1973) proposed the *H. pulchella* cycle including five species, and Duellman et al. (1997) redefined the *H. pulchella* group to include 11 species. Faivovich et al. (2004) performed the first phylogenetic analysis of the group, expanding it from 15 to 26 species. Shortly thereafter, Faivovich et al. (2005) added nuclear sequences and performed an expanded phylogenetic analysis. Subsequent analyses recovered similar topologies (Wiens et al. 2005, 2006, 2010; Pyron and Wiens 2011; Pyron 2014; Duellman et al. 2016; Jetz and Pyron 2018; Pinheiro et al. 2019a; Lyra et al. 2020; Dubois et al. 2021; Vasconcellos et al. 2021; Portik et al. 2023). Recently, Faivovich et al. (2021) provided the most comprehensive phylogenetic analysis of the *B. pulchella* group and recognized five clades: the *B. balzani*, *B. polytaenia*, *B. prasina*, *B. riojana*, and *B. semiguttata* clades. The only species not included in the analysis of Faivovich et al. (2021) is the Campo Grande treefrog, *B. cymbalum*.

Bokermann (1963) described *Boana cymbalum* on the basis of three specimens from Campo Grande da Serra (Santo André, São Paulo, Brazil). Since then, *B. cymbalum* has been mentioned in the literature only a few times, with no studies providing new morphological or molecular data. For instance, Barrio

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(1965) noted acoustic similarities between *B. cymbalum*, *B. prasina*, and *B. pulchella*. Lutz (1973) suggested that *B. cymbalum* could be conspecific either with *B. semiguttata* or *B. pulchella*. Faivovich et al. (2021) indicated that this lost frog is acoustically and morphologically similar to *B. cordobae*, *B. prasina*, and *B. pulchella*, and Dena et al. (2024) described its vocalizations. Since 2022, *B. cymbalum* has been listed as extinct by the Brazilian Environmental Ministry (MMA 2022).

Here, we test the phylogenetic position of *Boana cymbalum* using hDNA sequences from formalin-fixed type material. On the basis of our results, we provide an updated systematic account of *B. cymbalum.* Specifically, we update the diagnostic comparisons with the currently recognized species of the *B. pulchella* group, because many taxonomic rearrangements have been made in the group (e.g., Faivovich et al. 2021) and new species have been described (e.g., Marinho et al. 2022; Pinheiro et al. 2024). Furthermore, although Bokermann (1963) adequately described the holotype of *B. cymbalum*, we augment the systematic account with intraspecific variation and morphological characteristics he did not report (e.g., relative digit length and webbing formula). Finally, although Dena et al. (2024) recently described vocalizations of *B. cymbalum*, we provide an expanded description and reinterpretation.

Materials and Methods

The following collection abbreviations are used throughout the text, following Sabaj (2020, 2023): KU (University of Kansas Biodiversity Institute, Lawrence, USA); MNRJ (Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil); MZUSP (Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil); UF (University of Florida, Florida Museum of Natural History, Gainesville, USA); WCAB (W. C. A. Bokermann collection, now mostly transferred to MZUSP).

Historical DNA Data

Following Nakamura et al. (2024), we sampled approximately 0.5 mm^3 of formalin-fixed muscle tissue from an unexposed region of the trunk via a short dorsolateral incision on the holotype of *Boana cymbalum* (MZUSP 74194, collected at Campo Grande da Serra, Santo André, São Paulo, Brazil by W.C.A. Bokermann, on 13 October 1962). Given the high degree of morphological preservation and tissue rigidity, the specimen was apparently fixed in formalin (unknown concentration) before being stored in 70% alcohol (alone in the jar). Except for changes in coloration (see details in Systematic Account), this specimen is in good condition. Fresh tissues of *B. cymbalum* are unknown, thus precluding polymerase chain reaction (PCR) + Sanger sequencing and requiring instead specialized protocols for museomics of wet collections (e.g., Straube et al. 2021).

To prevent contamination, we carried out hDNA protocols (e.g., decontamination of all materials, use of cleanroom clothing, specialized reagents, and negative controls; e.g., Llamas et al. 2017; Fulton and Shapiro 2019; Straube et al. 2021) in a dedicated cleanroom laboratory in the Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo. Prior to DNA extraction, we washed tissues with 1 mL phosphate buffer saline solution twice to decrease the amount of potential inhibitors (e.g., formaldehyde). For the hDNA extraction, we used the guanidine treatment (Dabney et al. 2013) and prepared the single-stranded DNA library using uracil-DNA glycosylase and endonuclease VIII for excision of uracil and abasic sites, and T4 DNA ligase (Gansauge et al. 2017). Finally, we shotgun-sequenced approximately 20 million reads using the Illumina Nextseq 500/550 sequencing platform (500/550 High Output v2.5; 75 cycles; single-end) at TUCF Genomics (Tufts University School of Medicine, Boston, MA).

We trimmed Illumina adapters using Cutadapt v1.16 (Martin 2011), with a minimum read length of 21 base pairs (bp), and removed PCR duplicates using Tally (Davis et al. 2013). We evaluated the quality of filtered reads using FASTQC (Andrew 2010). We filtered out contaminant reads using FastqScreen (Wingett and Andrews 2018). For that we indexed the contaminant references used by Straube et al. (2021), including human and bacterial genomes (e.g., *Escherichia coli* and *Paraburkholderia* sp.).

We used MITObim v1.8 to reconstruct mitochondrial sequences using baiting and iterative mapping (Hahn et al. 2013). Because of the absence of annotated *Boana* mitogenomes on GenBank (Sayers et al. 2019; the 27 available mitogenomes are not annotated and exhibit unverified, chimeric regions), we used single-gene mapping (see GenBank codes of seeds in Supplemental Material S1 and Supplemental Table S1, available online). We initially used different seeds and parameters to assess whether reference bias could impact our results (Supplemental Material S1; Supplemental Fig. S1; Supplemental Tables S2 and S3, available online), but as we found similar consensus sequences from different assemblies, we selected *B. pulchella* as the initial seed to maximize coverage. Following Westbury et al. (2017), we consolidated the sequences generated using different parameters of MITObim (k-bait = 15 and 19; mismatch = 1 and 3) by calling the majority consensus sequence with a minimum of 5× coverage (Lyra et al. 2020) and mapping reads back to it, which was polished using Pilon v.124 (Walker et al. 2014). We mapped reads to nuclear genes using BWA-aln (Li and Durbin 2009). To ensure hDNA authenticity, we visually checked .MAF files to search for suspect regions with high coverage depth due to contaminants and we checked their anuran identity using BLAST (Altschul et al. 1990); otherwise, we codified these regions as nucleotides of unknown identity (N).

Phylogenetic Analyses

Our ingroup included all species of the *Boana pulchella* group. If available, we included at least two specimens of each species to maximize gene sampling per species. Our final ingroup sample comprised 73 terminals representing all 36 currently described species of the *B. pulchella* group (Faivovich et al. 2021; Marinho et al. 2022; Pinheiro et al. 2024), plus two undescribed species (Faivovich et al. 2021). The identity of the terminals referred to as *Boana* sp. 1 and *Boana* sp. 2 was updated to *B. itajahy* and *B. quiriri*, respectively, following Pinheiro et al. (2024), and *Boana* sp. 3 was updated to *B. guarinimirim* following Marinho et al. (2022). As outgroup, we sampled 37 species of Cophomantini, including representatives of all genera and all species groups of *Boana.* The terminal referred to as *B. cinerascens* in Faivovich et al. (2021) was updated to *B. gracilis*, following

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Sturaro et al. (2020). Therefore, our outgroup sample was identical to that of Faivovich et al. (2021), except for the addition of *B. cinerascens* (sensu Sturaro et al. 2020).

Our character sample included four mitochondrial fragments encoding eight loci (12S-tRNA^{Val}-16S, 16S-tRNA^{Leu}-ND1-tRNA^{Ile}, cytochrome oxidase I [COI], and cytochrome b [CytB]) and six nuclear loci (seven in absentia homolog 1 [SIAH1], exon 1 of rhodopsin [RHO], exon1 of tyrosinase [TYR], a single exon of Recombination Activating Gene 1 [RAG-1], exon 2 of CXCR4, and 28S rRNA). GenBank accession numbers, voucher numbers, and locality data are available in Supplemental Material S2 (available online).

We conducted phylogenetic analysis using maximum parsimony (MP) and maximum likelihood (ML). We performed the MP analyses in POY v5.1.1 (Wheeler et al. 2015), in which unaligned sequences are submitted to direct optimization (Wheeler 1996). We considered equal weights for cost regimes and added partition breaks following the criteria of Grant et al. (2006) to maximize data inclusion. We conducted heuristic searches using the command *search*, which implements a driven search composed of random addition sequence Wagner builds (RAS; Farris 1970), subtree pruning and regrafting (SPR), tree bisection and reconnection (TBR) branch swapping (Goloboff 1996, 1999), parsimony ratcheting (Nixon 1999), and tree fusing (Goloboff 1999). We ran three independent 48-h driven searches using 512 CPUs (= 24,576 CPUh), followed by exact iterative pass optimization (Wheeler 2003a). To verify the cost reported by POY v5.1.1 and search for additional most parsimonious trees (MPTs), we used the implied alignment (Wheeler 2003b) from the optimal tree found by POY as input for a final heuristic search with the *xmult = level 10 chklevel 5 consense 5* command in TNT v1.5 (Goloboff et al. 2008; Goloboff and Catalano 2016). Following Grant and Kluge (2008), we computed Goodman–Bremer (GB; Goodman et al. 1982; Bremer 1988) as a direct measure of support using the *bremem.run* macro; we also calculated parsimony jackknife absolute frequencies (JK) via 1000 pseudoreplicates hitting the best score five times under level 1.

We performed the ML analysis in IQ-TREE v2 (Minh et al. 2020). We aligned sequences using the online version of MAFFT v7 (Katoh et al. 2019) under the strategies E-INS-i for the 12S-tRNA^{Val}-16S fragment and L-INS-i for other fragments (Faivovich et al. 2021), with default gap opening and extension parameters. We treated second and third codon positions for each protein-coding gene as separate partitions for model selection. Based on the corrected Akaike information criterion (AICc), we estimated best-fitting models for each partition using IQ-TREE v2 with the greedy algorithm (Lanfear et al. 2012). We computed approximate likelihood ratio tests (aLRT; argument = –alrt 1000; Guindon et al. 2010) and nonparametric bootstrap (1000 pseudoreplicates). Additionally, we calculated uncorrected p distances in MEGA X (Kumar et al. 2018) for the 16S rRNA gene fragment delimited by the primers 16Sar-L and 16S-Wilk2 (Kessing et al. 1989; Wilkinson et al. 1996).

Phenotypic Data

We examined the holotype (MZUSP 74194), paratype (MZUSP 73697), and three topotypes (KU 92010 and MZUSP 106980, 160852). Additional specimens examined and taxonomic authorities are listed in the Appendix and Supplemental Material 3 (available online), respectively.

Except for the dorsal outline of the snout, which follows Heyer et al. (1990), and finger numbering, which follows Fabrezi and Alberch (1996), terminology for external morphology follows Duellman (1970). We identified sex by the presence of vocal slits and development of prepollex in males. Females were determined by a less developed prepollex, slender arms (Pinheiro et al. 2022), presence of oocytes, and absence of both vocal sac and vocal slits. Color in life is based on the literature. We measured specimens with digital calipers to 0.1 mm under a ZEISS SteREO Discovery.V8 stereomicroscope. We followed Duellman's (1970) definitions of snout–vent length (SVL), head length (HL), head width (HW), eye diameter (ED), eye–nostril distance (END), internarial distance (IND), interorbital distance (IOD), tympanum diameter (TD), tibia length (TL), tarsal length (TAL), and foot length (FL). We also measured snout length (SL; Cei 1980), thigh length (THL; Heyer et al. 1990), Finger IV disc diameter (4FD), and Toe IV disc diameter (4TD; Napoli and Caramaschi 1999). We report statistical summaries as the sample mean ($\bar{x}$), standard error (SE), and range of values.

To visualize prepollex morphology, we scanned MZUSP 106980 using a high-resolution nano-CT scanning tomography (Phoenix V, TOME, XS 240) at Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo (FFCLRP-USP). Images were reconstructed, segmented, and rendered with Avizo v9.4.0 (Visualization Sciences Group, Thermo Fisher Scientific Inc., Agawam, MA, USA).

Vocalizations were recorded by Werner C.A. Bokermann at the type locality on 21 December 1963 and the files are available at Fonoteca Neotropical Jacques Vielliard (accession number FNJV 31866). The air temperature was 16°C; the distance between the microphone and the calling individuals is unknown. A single recording lasting 83 s was made with a Uher 4000 Report IC (microphone unknown) at a tape velocity of 9.5 cm/s. Recordings were digitized with a MOTU Ultra Lite Mk3. Following the terminology defined by Köhler et al. (2017), we measured temporal parameters from waveforms and spectral parameters from spectrograms and power spectra (Windows Type Hann, Time Grid with 75% overlap, 512-point fast Fourier transformation resolution, and hop size of 128 samples). We used Raven Pro v1.6.4 sound analysis software (Cornell Lab of Ornithology, Ithaca, NY, USA) to score the following variables: call duration (s), notes per call, note duration (ms), peak frequency (Hz), minimum frequency at 5% of energy (using Raven's Frequency 5% tool), maximum frequency at 95% of energy (using Raven's Frequency 95% tool), number and frequency of harmonics (Hz), and bandwidth 90% (−10 dB; using Raven's Bandwidth 90% tool).

RESULTS

Historical DNA Sequences

We obtained a total of 20,322,035 reads. After trimming and excluding duplicate fragments, we retained 14,543,875 reads. The average fragment length was 38 bp, average quality sequence was 32, and GC content was 41%. The FastqScreen analysis revealed contaminant reads mapped to *Escherichia coli*, *Homo sapiens*, and *Paraburkholderia* sp.

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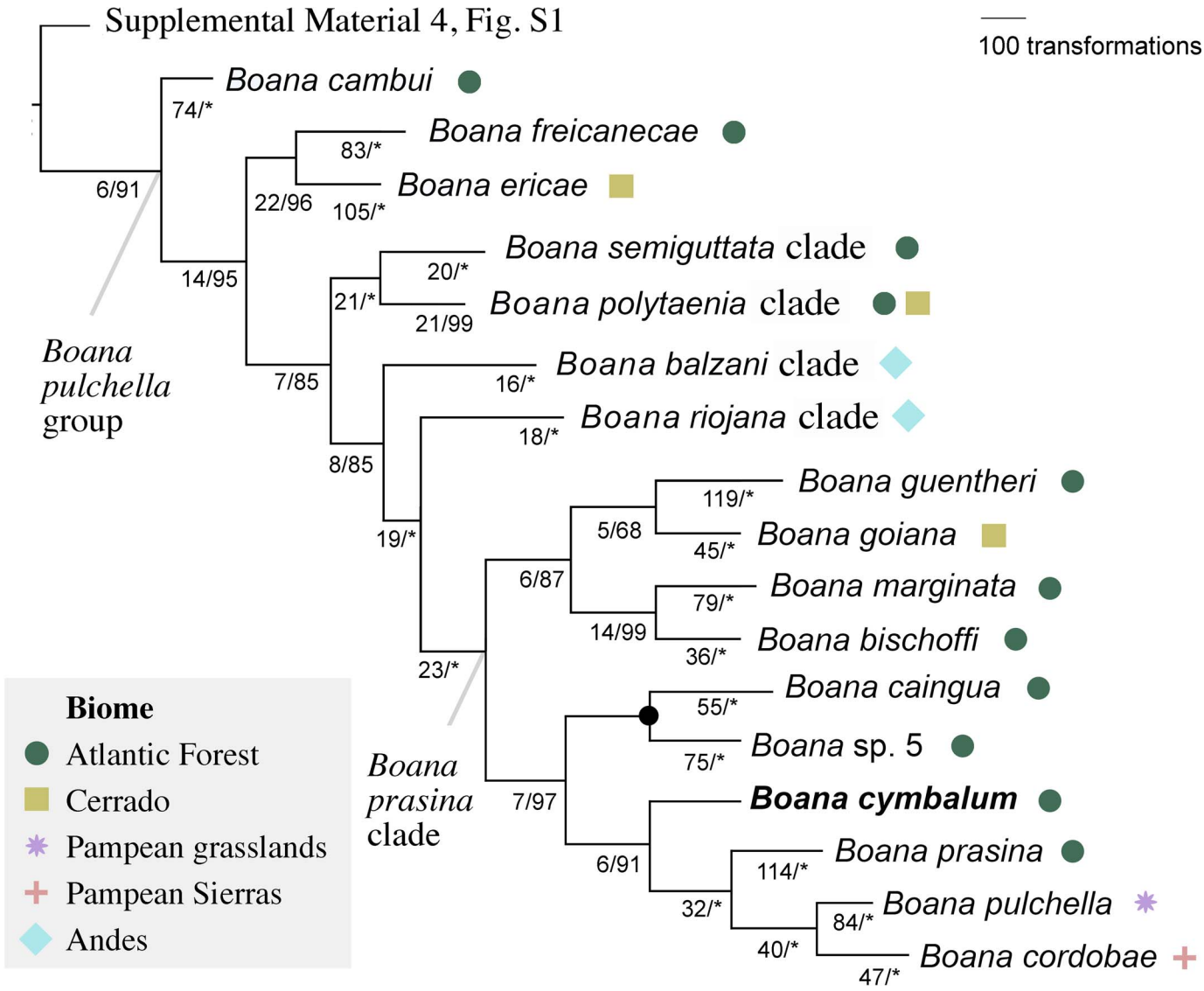

FIG. 1.—Phylogenetic relationships of the *Boana pulchella* group. One of the three most parsimonious trees obtained in the parsimony analysis under dynamic homology (20,383 steps). Most genera of Cophomantini, species groups of *Boana*, and clades of the *Boana pulchella* group are condensed. Numbers around nodes are Goodman–Bremer support/absolute jackknife frequencies. Asterisks indicate 100% jackknife values. Black circles indicate nodes that collapse in the strict consensus. See the complete topology for MP and ML analyses in Supplemental Material 4, Supplemental Figs. S1 and S2, respectively. A color version of this figure is available online.

(Supplemental Material S1; Supplemental Table S4, available online), which were removed, resulting in 14,480,638 reads. Our MITObim analyses successfully assembled ~3,968 bp from four mitochondrial fragments (12S-tRNA^{Val}-16S, 16S-tRNA^{Leu}-ND1-tRNA^{Ile}, CytB, and COI; Supplemental Material S1; Supplemental Tables S2 and S3). Regarding nuclear genes, we successfully assembled ~596 bp from 28S rRNA using BWA.

Phylogenetic Results

The driven searches from parsimony analyses under dynamic homology performed 34,766 RAS + swapping, 258,147 rounds of Tree Fusing, and 17,337 rounds of Ratcheting. Our optimal topologies using direct optimization recovered four MPTs of 20,444 steps (Fig. 1). Iterative pass reduced the tree cost to 20,387 steps. Additional heuristic searches of the implied alignment in TNT found three MPTs of 20,383 steps.

Overall, the strict consensus was highly resolved, with only two conflicts among MPTs: (1) relationships among *B. quiriri*, *B. caipora*, and *B. semiguttata*, and (2) relationships between *Boana* sp. 5 and *B. caingua* (Supplemental Material S4, available online; Supplemental Fig. S1). Among outgroup taxa, our data support the monophyly of all genera and species groups of *Boana*. Within the ingroup, the early diverging *B. cambui* is the sister taxon of a clade with all other species of the *B. pulchella* group (GB = 14, JK = 95%). *Boana ericae* and *B. freicanecae* form a well-supported group (GB = 22; JK = 96%). Our data support the monophyly of all clades, namely, *B. balzani* (GB = 16; JK = 100%), *B. polytaenia* (GB = 21; JK = 99%), *B. prasina* (GB = 23; JK = 100%), *B. riojana* (GB = 18; JK = 100%), and *B. semiguttata* clades (GB = 20; JK = 100%).

Boana cymbalum is nested within the *B. prasina* clade as the sister of a clade composed of *B. cordobae*, *B. prasina*, and *B. pulchella* (Fig. 1). This position is consistent in both

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TABLE 1.—Some measurements in millimeters ($\bar{x} \pm$ SE, minimum–maximum) of four individuals of *Boana cymbalum* (MZUSP 73697, 74194, 106980, and 160852). See text for measurement definitions.

Variable	$\bar{x} \pm$ SE (minimum–maximum)	MZUSP 73697	MZUSP 74194	MZUSP 106980	MZUSP 160852
SVL	44.5 ± 2.1 (41.1–46.0)	46.0	45.1	43.9	41.1
HL	14.6 ± 0.5 (14.3–15.5)	14.4	15.5	14.8	14.3
HW	13.0 ± 0.6 (12.7–14.0)	12.7	14.0	12.9	13.1
ED	5.3 ± 0.4 (5.1–6.0)	6.0	5.1	5.4	5.2
END	4.4 ± 0.7 (3.4–5.1)	4.6	3.4	5.1	4.3
IND	4.1 ± 0.2 (3.8–4.2)	4.2	3.8	4.2	4.1
IOD	6.7 ± 0.8 (6.6–8.2)	6.7	6.6	6.6	8.2
TD	2.9 ± 0.4 (2.2–3.2)	3.2	2.8	2.2	3.1
TL	22.4 ± 0.7 (22.2–23.7)	23.7	22.3	22.2	22.4
TAL	14.8 ± 0.8 (13.6–15.5)	15.5	14.5	15.2	13.6
FL	18.7 ± 1.9 (15.7–20.2)	20.2	19.1	15.7	18.2
SL	5.7 ± 0.2 (5.5–5.9)	5.8	5.5	5.5	5.9
THL	22.9 ± 0.7 (22.1–23.7)	23.7	23.0	22.1	22.7
4FD	1.8 ± 0.2 (1.6–2.1)	2.1	1.8	1.6	1.8
4TD	1.6 ± 0.3 (1.5–2.1)	2.1	1.5	1.7	1.5

the MP and ML analyses (Supplemental Material S4; Supplemental Figs. S1 and S2; see best-fitting models of ML analysis in Supplemental Table S1). The uncorrected pairwise distances between *B. cymbalum* and other species of the *B. prasina* clade is 4.5–6.7% (Supplemental Material S4; Supplemental Table S2).

SYSTEMATIC ACCOUNT

Boana cymbalum (Bokermann 1963)
(Table 1; Figs. 2–4, 6)

Hyla cymbalum Bokermann 1963: Bokermann (1966), comment on type locality; Lutz (1973), species account and comment on possible conspecificity with *Hyla pulchella* or *Hyla semiguttata*; Caramaschi in Frost (1985), comment on possible conspecificity with *Hyla pulchella*; Faivovich et al. (2004), comment on phylogenetic relationships.

Hypsiboas cymbalum (Bokermann 1963): Faivovich et al. (2005), first combination with *Hypsiboas*.

Boana cymbalum (Bokermann 1963): Dubois et al. (2017), first combination with *Boana*; Faivovich et al. (2021), comment on phylogenetic relationships; Dena et al. (2024), call description.

Holotype.—MZUSP 74194 (ex-WCAB 9153), an adult male collected in Campo Grande da Serra, Santo André, São Paulo, Brazil by W.C.A. Bokermann, 13 October 1962.

Paratype.—MZUSP 73697 (ex-WCAB 9154), an adult male collected together with the holotype.

Referred specimens.—KU 92010 (ex-WCAB 14317, 15 January 1964), MZUSP 106980 (ex-WCAB 14074, 21 December 1963), and MZUSP 160852 (ex-WCAB 14075), all topotypic adult males collected by W.C.A. Bokermann.

Diagnosis.—A species of the *Boana pulchella* group, as indicated by the presence of a long postarticular process of the distal prepollex and curved spine-shaped distal prepollex directed laterad to pass ventral to Metacarpal II (Fig. 2). *Boana cymbalum* is distinguished by the following combination of characters: (1) male SVL of 41.1–46.0 ($n = 4$; Table 1; Fig. 3); (2) presence of dark spots on a white background on flanks and (3) concealed surfaces of thighs (in preservative, dark brown spots on a cream background; Fig. 4A); (4) presence of a white supralabial stripe ventral to the tympanum and extending posteriad; (5) absence of a large calcar; (6) absence of a triangle on the dorsal surface between the orbits and the tip of the snout; and (7) absence of cream lines bordered by a thin dark line on the dorsolateral region, forearms, and shank.

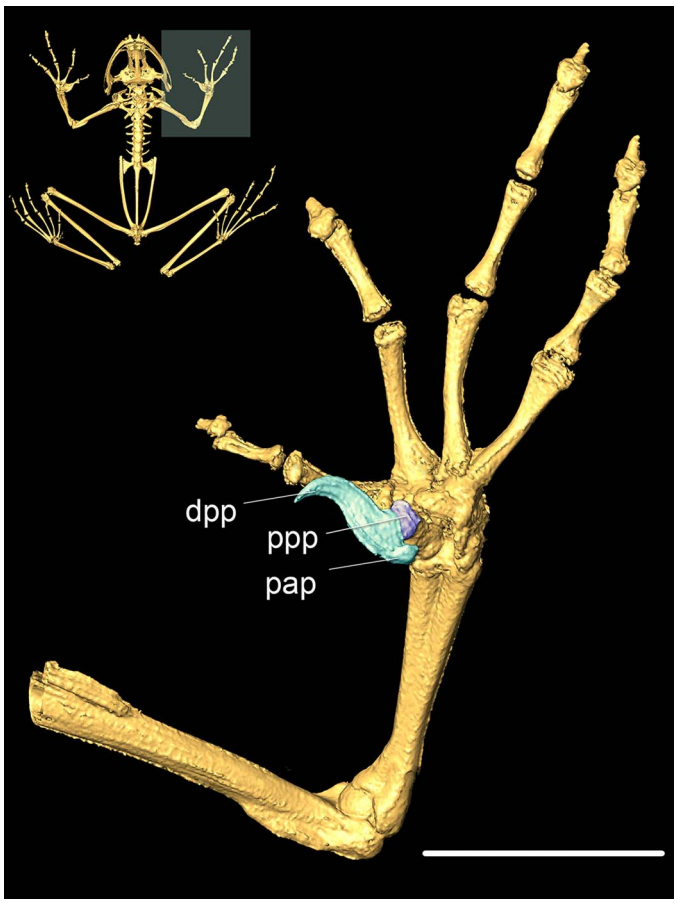

FIG. 2.—Left hand of *Boana cymbalum* (MZUSP 106980) in ventral view, highlighting prepollex structures: dpp = distal prepollex; pap = postarticular process of the distal prepollex; ppp = proximal prepollex. Scale bar = 5 mm. A color version of this figure is available online.

Comparisons.—Within the *Boana pulchella* group, male SVL differentiates *B. cymbalum* (41.1–46.0 mm; $n = 4$) from *B. botumirim* (25.9–31.8 mm; $n = 20$), *B. buriti* (22.2–31.9 mm; $n = 3$), *B. callipleura* (32.0–38.7 mm; $n = 14$), *B. cambui* (26.3–32.8 mm; $n = 16$), *B. caingua* (32.9–39.8 mm; $n = 25$), *B. caipora* (29.7–37.5 mm; $n = 18$), *B. cipoensis* (26.0–29.0 mm; $n = 3$), *B. ericae* (29.2–34.0 mm; $n = 20$), *B. goiana* (29.0–33.0 mm; $n = 4$), *B. guarinimirim* (24.1–28.1 mm; $n = 15$), *B. guentheri* (33.0–40.0 mm; $n = 34$), *B. jaguariaivensis* (25.2–29.9 mm; $n = 17$), *B. leptolineata* (27.2–31.6 mm; $n = 36$), *B. polytaenia* (27.3–32.6 mm; $n = 10$), *B. riojana* (48.0–56.0 mm; $n = 14$), and *B. stenocephala* (26.0–30.4 mm; $n = 17$; comparative measurements obtained from Barrio 1965; Lutz 1968, 1973; Braun and Braun 1977; Heyer et al. 1990; Reynolds and Foster 1992; Caramaschi and Cruz 1999, 2000; Garcia et al. 2007; Antunes et al. 2008; Caramaschi et al. 2009, 2010; Campos 2015; Pinheiro et al. 2016; Marinho et al. 2022; see also Supplemental Material S5, available online; Supplemental Table S1).

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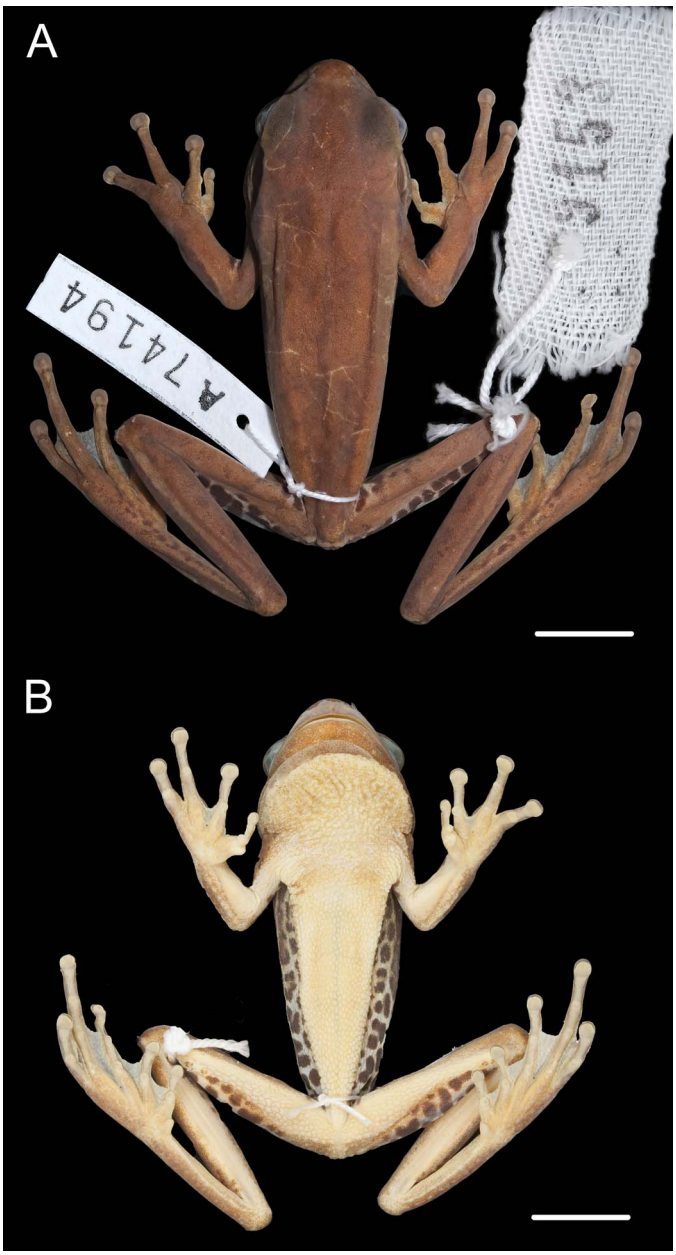

FIG. 3.—Holotype of *Boana cymbalum* (MZUSP 74194) in (A) dorsal and (B) ventral views. Scale bars = 5 mm. A color version of this figure is available online.

The presence of dark spots on a white background on the flanks differentiates *Boana cymbalum* from *B. botumirim*, *B. buriti*, *B. cambui*, *B. cipoensis*, *B. freicanecae*, *B. goiana*, *B. guarinimirim*, *B. jaguariaivensis*, *B. leptolineata*, *B. marginata*, *B. palaestes*, *B. polytaenia*, and *B. stenocephala* (spots on flanks absent in these species; Cope 1870; Boulenger 1887; Barrio 1965; Lutz 1968; Braun and Braun 1977; Duellman et al. 1997; Cruz and Caramaschi 1998; Caramaschi and Cruz 1999; Carnaval and Peixoto 2004; Caramaschi et al. 2009, 2010; Pinheiro et al. 2016; Marinho et al. 2022) and *B. aguilari*, *B. balzani*, *B. caipora*, *B. callipleura*, *B. curupi*, *B. ericae*, *B. gladiator*, *B. itajahy*, *B. joaquini*, *B. marianitae*, *B. melanopleura*, *B. quiriri, B. riojana*, *B. semiguttata*, and *B. stellae* (white, cream, or yellow spots on flanks in these species; Lutz 1973; Carrizo 1992; Duellman et al. 1997; Caramaschi and Cruz 2000; Garcia et al. 2003, 2007; Antunes et al. 2008; Kwet 2008; Köhler et al. 2010; Lehr et al. 2010; Pinheiro et al. 2024; see Fig. 5, Supplemental Material S5; Supplemental Table S2).

The presence of dark spots on a white background on the concealed surface of the thighs distinguishes *Boana cymbalum* from *B. aguilari*, *B. botumirim*, *B. buriti*, *B. caipora*, *B. cambui*, *B. cipoensis*, *B. ericae*, *B. freicanecae*, *B. goiana*, *B. guarinimirim*, *B. itajahy, B. jaguariaivensis*, *B. joaquini*, *B. leptolineata*, *B. marginata*, *B. melanopleura*, *B. palaestes*, *B. poaju*, *B. polytaenia*, *B. quiriri, B. semiguttata*, and *B. stenocephala* (spots on concealed surface of thighs absent in these species; Boulenger 1887, 1912; Cope 1870; Lutz 1968; Braun and Braun 1977; Duellman et al. 1997; Cruz and Caramaschi 1998; Caramaschi and Cruz 1999, 2000; Carnaval and Peixoto 2004; Antunes et al. 2008; Garcia et al. 2008; Caramaschi et al. 2009, 2010; Lehr et al. 2010; Pinheiro et al. 2016; Marinho et al. 2022; Pinheiro et al. 2024), and also from *B. balzani*, *B. callipleura*, *B. gladiator*, *B. guentheri*, *B. marianitae*, and *B. stellae* (white, cream, or yellow spots on concealed surface of thighs in these species; Boulenger 1886; Lutz 1973; Carrizo 1992; Duellman et al. 1997; Kwet 2008; Köhler et al. 2010; Kwet et al. 2010).

The presence of a white supralabial stripe ventral to the tympanum and extending posteriad differentiates *Boana cymbalum* from *B. balzani*, *B. cordobae*, *B. gladiator*, and *B. palaestes* (supralabial stripe absent in these species; Barrio 1965; Duellman et al. 1997; Köhler et al. 2010). The absence of a large calcar distinguishes *B. cymbalum* from *B. bischoffi*, *B. freicanecae*, *B. guarinimirim*, and *B. polytaenia* (present in these species; Marinho et al. 2022). The absence of a dorsal triangle on the dorsal surface anterior to the orbits distinguishes *B. cymbalum* from *B. cambui* and *B. freicanecae* (present in these species; Carnaval and Peixoto 2004; Pinheiro et al. 2016).

The absence of cream lines bordered below by a thin dark line on the dorsolateral region, forearms, and shanks distinguishes *Boana cymbalum* from *B. prasina* (present in this species; Bokermann 1963; Barrio 1965; material examined herein). In *B. pulchella*, these cream dorsolateral lines are mostly similar to those from *B. prasina* (Supplemental Material 5; Supplemental Fig. S1). However, in a few populations the dorsolateral line has irregular, nonstraight margins (Barrio 1965: Fig 1.5), or there are several dark blotches overlapping it (Barrio 1965; Fig. 1.6; both absent in *B. cymbalum*). Uncorrected pairwise distances of 16S further corroborate the distinctiveness of *B. cymbalum* and *B. cordobae* (5.33–6.63%), *B. prasina* (4.51–4.65%), and *B. pulchella* (5.63–5.77%).

Variation.—The description that follows is based on five adult males (KU 92010, MZUSP 73697, 74194, 106980, and 160852; Fig. 6); measurements and proportions are derived from all available adult males (Table 1). Females are unknown.

Body slender. Head wider than body, slightly longer than wide (HW/HL = 0.87–0.91); snout rounded (KU 92010, MZUSP 74194, and 160852) or truncated (MZUSP 73697 and 106980) in dorsal view, rounded in profile; END shorter than ED (END/ED = 0.67–0.94); canthus rostralis distinct, curved in dorsal view; loreal region slightly concave; lips thin, not flared; internarial region slightly depressed; nostrils weakly protuberant, directed dorsolaterad; nares protuberant, oval, directed anterolaterad (MZUSP 73697, 74194,

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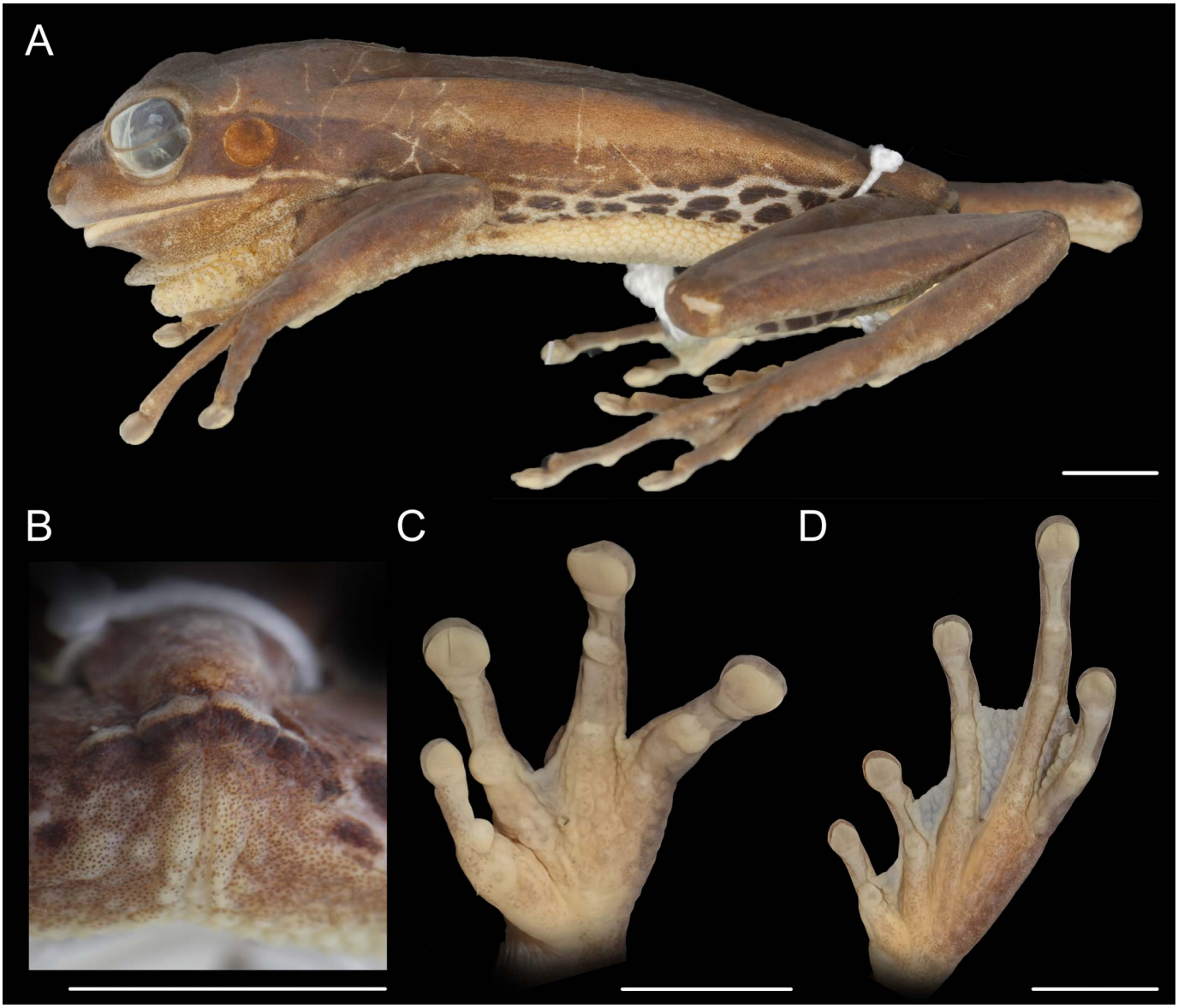

FIG. 4.—Details of the external morphology of *Boana cymbalum* (MZUSP 74194; holotype). (A) Lateral view; (B) cloacal surface; (C) left hand, ventral view; and (D) left foot, ventral view. Scale bars = 5 mm. A color version of this figure is available online.

106980, and 160852) or dorsolaterad (KU 92010); interorbital area flat, larger than ED (IOD/ED = 1.12–1.59), approximately half the size of HW (IOD/HW = 0.47–0.63). Eyes large, protuberant (ED/HL = 0.33–0.42; ED/HW = 0.36–0.48), directed slightly anteriorad; palpebral membrane translucent, without reticulation. Supratympanic fold evident above of the tympanic ring. Tympanum moderately large (TD/ED = 0.40–0.56; TD/HL = 0.15–0.22), distinct, directed dorsolaterad.

Upper arms slender, lacking an axillary membrane; forearms thicker than upper arms; row of small and juxtaposed ulnar tubercles extending from the proximal limit of hand to the elbow. Fingers long, bearing round discs; disc diameter on Finger IV narrower than tympanum (TD/4FD = 1.35–1.78); relative finger lengths II < III < V < IV; webbing formula II—III 2^{+}—$3^{1/2}$ IV 3^{+}—$2^{1/2}$ V; lateral fringes present on fingers. Subarticular tubercles on hands distinct, rounded in ventral view, simple (MZUSP 73697, 106980, and 160852) or bifid on distal portion of fingers III, IV, and V (MZUSP 74194); supernumerary tubercles present on fingers and palm; outer and inner metacarpal tubercles flat, almost indiscernible. Nuptial pad absent. Prepollex osseous, spine-shaped, protruding through the medial outline of Finger II.

Hind limbs long, slender (THL/SVL = 0.50–0.55; TL/SVL = 049–0.52); tarsal fold present, extending from inner metatarsal tubercle to heel; foot longer than tarsus (FL/TAL = 1.03–1.33), smaller than tibia (FL/TL = 0.71–0.86). Calcars absent. Toes long, bearing large discs, diameter of the fourth toe disc subequal to the fourth finger disc (4TD/4FD = 0.80–1.07); relative toe lengths I < II < III = V < IV; webbing formula I 2^{-}—2^{+} II 1^{+}—3^{-} III $1^{1/2}$—3^{+} IV 3^{+}—$1^{1/2}$ V; granules on webbing surface present; lateral fringes on toes present. Subarticular tubercles large, round in ventral view, slightly conical in profile; outer metatarsal tubercle absent; inner metatarsal tubercle distinct, flat, and elliptical in ventral view.

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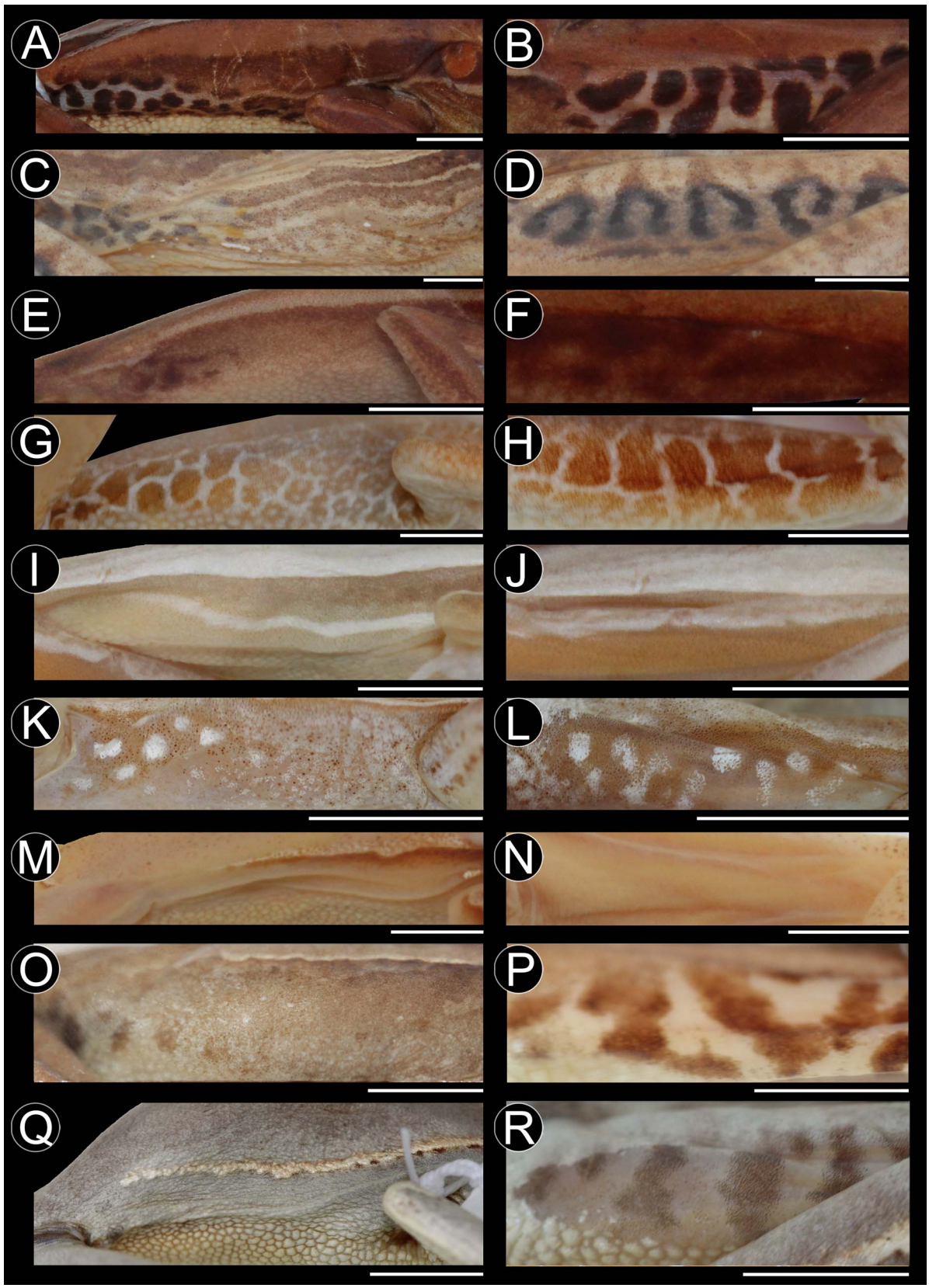

FIG. 5.—Comparison of markings on flanks (left column) and concealed surfaces of thighs (right column) in *Boana cymbalum* MZUSP 74194 (A and B), *B. bischoffi* MZUSP 157432 (C and D), *B. caingua* MZUSP 60858 (E and F), *B. cordobae* MZUSP 73689 (G and H), *B. goiana* MZUSP 146253 (I and J), *B. guentheri* MZUSP 106714 (K and L), *B. marginata* MZUSP 35394 (M and N), *B. prasina* MZUSP 145388 (O and P), and *B. pulchella* MZUSP 109309 (Q and R). Scale bars = 0.5 cm. See Appendix for locality data of each voucher. A color version of this figure is available online.

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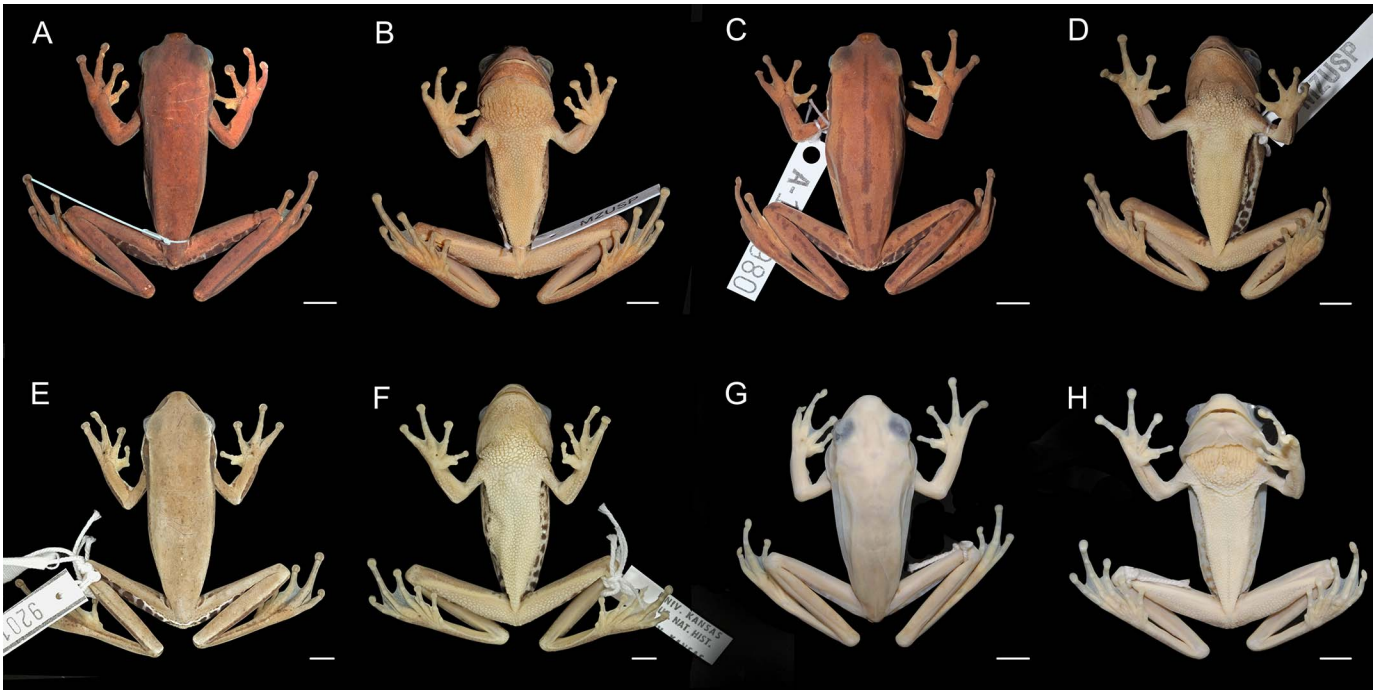

FIG. 6.—Variation in *Boana cymbalum*. (A) Dorsal and (B) ventral views of MZUSP 73697. (C) Dorsal and (D) ventral views of MZUSP 106980. (E) Dorsal and (F) ventral views of KU 92010. (G) Dorsal and (H) ventral views of MZUSP 160852. Scale bars = 0.5 cm. A color version of this figure is available online.

Skin smooth except ventral surfaces of thighs and gular, pectoral and abdominal areas, where it is granular. Pectoral fold absent. Cloacal opening directed posteroventrad at upper level of thighs; cloacal sheath absent; well-developed, cream, supracloacal crest present; cloacal tubercles present; subcloacal dermal fold absent, cloacal melanophores present, scattered below and around the cloacal opening.

Tongue ovoid, barely free posteriorly; dentigerous processes of vomers in the palatine region absent (MZUSP 73697) or well defined, distributed in two close, transverse series between and behind choanae, each bearing five teeth (MZUSP 74194), five and six on the left and right processes, respectively, in MZUSP 106980, or four and six on the left and right processes, respectively, in MZUSP 160852. Choanae large, rounded to triangular. Vocal slits moderately long. Vocal sac well developed, single, median, subgular, with several longitudinal folds, except in KU 92010 and MZUSP 106980, wherein vocal sacs are smaller.

Coloration.—According to Bokermann (1963) description in life, dorsal surfaces with dark green coloration; lower and upper lips whitish, with the latter continuing in a stripe passing above the arm insertion until the reticulation on flanks; black spots on a white background on flanks and concealed surfaces of thighs; white supracloacal crest; melanophores scattered on throat; two halves of the iris with different coloration (upper half lighter according to Lutz 1973, but see Discussion).

After 62 yr in preservative, bodies have become olivaceous brown dorsally and cream ventrally, respectively. Dorsum with dark, discontinuous, longitudinal stripes is present in MZUSP 106980 (absent in others). Dark brown dorsolateral stripe extending from anterior portion of upper eyelid almost to snout, then from posterior portion of eyes to hind limb insertion, passing dorsad to the dark spots on flanks. Upper and lower lips, white background on flanks and concealed surfaces of thighs, supracloacal crest, and scars became pale cream. Two weakly defined circular blotches posteriorly between the eyes are either present (MZUSP 106980) or absent (MZUSP 73697 and 74194). The tympanum became orange brown. Melanophores scattered on the throat are densely concentrated on the anterior portion. The two halves of the iris became silvery. The coloration of MZUSP 160852 is almost entirely faded, except for a few dark spots on the flanks and concealed surfaces of thighs.

Vocalization.—The vocalizations of *Boana cymbalum* comprises two types of notes, here referred to as Note A ($n = 54$; Fig. 7A) and B ($n = 13$; Fig. 7B). Note A is tonal and shorter in duration, whereas Note B is multipulsed and longer.

Calls with Note A are composed of 5.0 ± 1.3 tonal notes (3–8 notes), with call duration of 0.34 ± 0.11 s (0.14–0.86 s) and note duration of 26.5 ± 47.1 ms (14.5–47.4 ms). Decreasing amplitude is usually present in each note. The peak frequency lies within the fundamental band at 1875 ± 104 Hz (1,687–2,062 Hz). The minimum frequency at 5% energy is 1500 ± 200 Hz (1312–1687 Hz), the maximum frequency at 95% energy is 2062 ± 67 Hz (1875–2250 Hz), and 90% bandwidth is 562 ± 128 Hz (375–750 Hz). Most calls present six harmonic bands in addition to the fundamental frequency: the second band has peaks at 3562 ($n = 20$), 3750 ($n = 23$), and 3937 Hz ($n = 2$); the third one has peaks at 5437 ($n = 24$), 5625 ($n = 11$), 6000 Hz ($n = 10$); the fourth one has peaks at 7312 ($n = 23$), 7500 ($n = 9$), 7687 ($n = 8$), and 8062 Hz ($n = 7$); the fifth one has peaks at 8812 ($n = 6$), 9000 ($n = 12$), 9312 ($n = 5$), 9562 ($n = 14$), and 9750 Hz ($n = 12$); the sixth one has peaks at 10,875 ($n = 27$) and 11,250 Hz ($n = 16$); and the seventh has peaks at 12,375 ($n = 9$), 12,562 ($n = 6$), 12,937 ($n = 5$), and 13,687 Hz ($n = 5$).

Calls with Note B are composed of a single, long, multipulsed note of 0.88 ± 0.33–s (0.27–1.46 s) duration and pulse duration of 10 ± 4 s (4–20 ms). The peak frequency lies within

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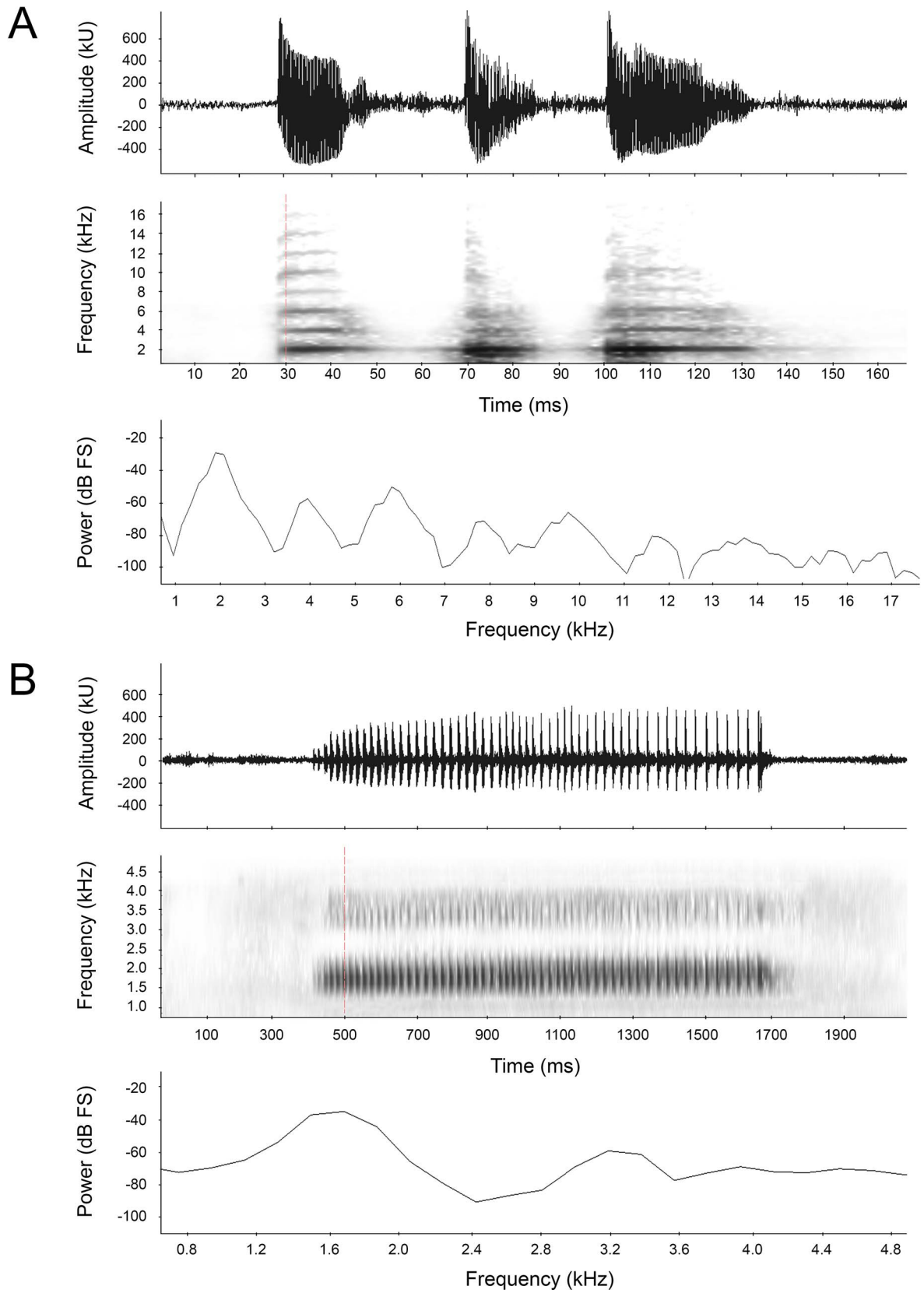

FIG. 7.—Oscillogram, audiospectrogram, and power spectrum (spectrogram slice used to build the power spectrum is indicated by the red vertical bar) of vocalizations of *Boana cymbalum* (16°C air temperature). (A) Type A with three short tonal notes. (B) Type B with a long multipulsed note. A color version of this figure is available online.

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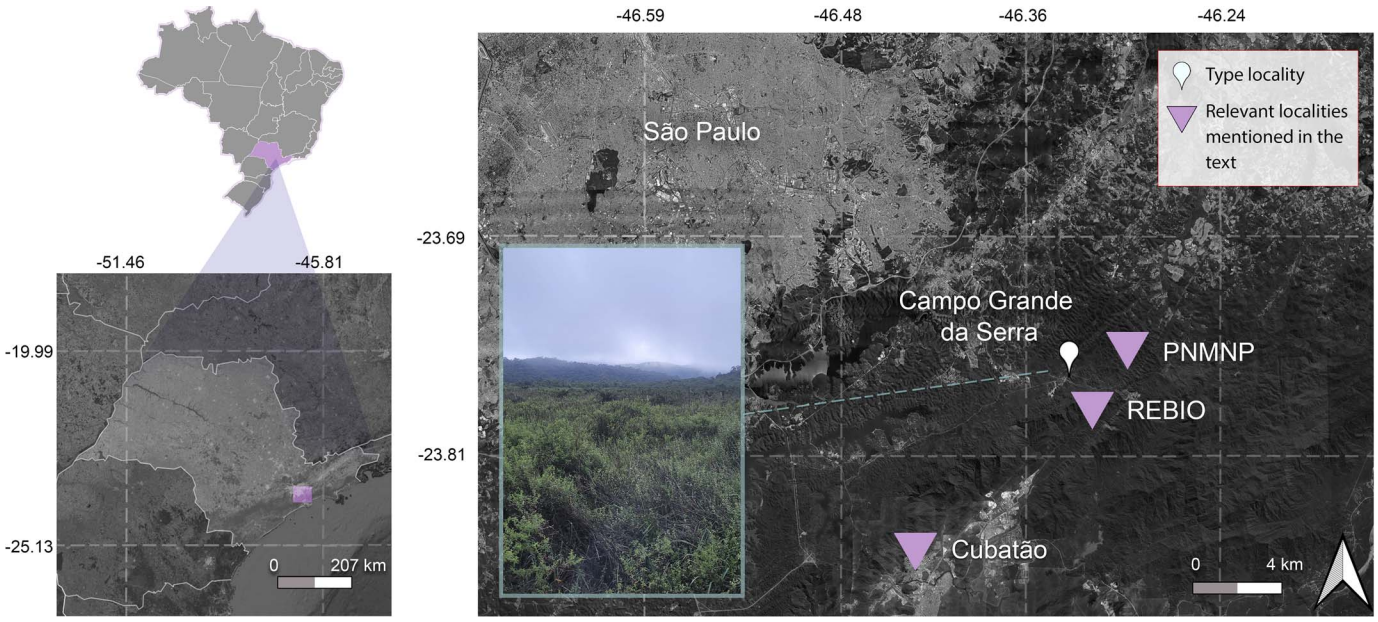

FIG. 8.—Region of the type locality of *Boana cymbalum*. Dark and light areas indicate presently forested and deforested areas, respectively. The inset shows the current vegetation at the type locality (17 March 2023). A color version of this figure is available online.

the fundamental band at 1687 ± 156 Hz (1500–2060 Hz). The minimum frequency at 5% energy is 1312 ± 52 Hz (1312–1500 Hz), the maximum frequency at 95% energy is 1875 ± 135 Hz (1875–2062 Hz), and 90% bandwidth is 562 ± 329 Hz (375–750 Hz). Most calls present a second harmonic band with peaks at 3187 ($n = 7$), 3375 ($n = 2$), and 3562 Hz ($n = 2$).

Larva.—Unknown.

Distribution.—*Boana cymbalum* is known exclusively from the type locality of Campo Grande da Serra (Fig. 8) near the "ABC Paulista" industrial complex, which comprises the municipalities of Santo André, São Bernardo, and São Caetano in São Paulo state. Individuals from the type series were found in a forest near the Campo Grande station of the Santos–Jundiaí railway line.

Natural history.—Bokermann (1963) found some individuals on shrubs next to a slow-flowing creek at the type locality; he stated that *Boana cymbalum* behaves similarly to *B. pulchella* in captivity, with no additional details. Although direct observations of male–male combat have not been reported, the presence of multiple scars on the dorsum of some individuals in preservative suggests combat using their spine-shaped prepollex (Shine 1979; Candaten et al. 2020; Pinheiro et al. 2022). Scars on the dorsum of males are absent in MZUSP 106908 and 160852, scattered in KU 92010 and MZUSP 74194, and concentrated in the suprascapular region in MZUSP 73697.

Etymology.—The specific epithet is derived from the Latin word *cymbalum* (from the Ancient Greek κύμβαλον, whereby κύμβη means "hollow of a vessel" and the suffix –αλον means "rattle"). A cymbal refers to a flat round instrument that produces a metallic sound when hit. Despite the choice of name by Bokermann (1963), Lutz (1973) commented that vocalizations of *B. cymbalum* are not similar to cymbal sounds. We agree and instead describe them either as tonal (Note A) or pulsed (Note B) calls.

Remarks.—In addition to the five known specimens of *Boana cymbalum*, two other specimens were misidentified as this species in herpetological collections (Supplemental Material S5; Supplemental Fig. S2). MNRJ 23778 was collected by A. Pinheiro and C. Dias in Vila Nova Manchester in March 1946. This specimen has a robust body (slender in *B. cymbalum*; variable in *B. prasina* and *B. pulchella*) and lacks the dark band dorsal to the black spots on flanks (present in *B. cymbalum*; variable in *B. prasina* and *B. pulchella*). Given the morphological evidence against the determination of MNRJ 23778 as *B. cymbalum* and the previous occurrence of *B. prasina* in Vila Nova Manchester (e.g., MNRJ 23939–23943), we consider MNRJ 23778 to be *B. prasina*. UF 100604 was collected in Brazil on 25 April 1963 and identified as *B. cymbalum*, with no additional details about locality or determiner except a collection remark stating "R. Curtis to L. D. Ober" (presumably, collected by R. Curtis and sent to L.D. Ober). UF 100604 has a robust body (slender in *B. cymbalum*), lacks the dark band dorsal to the black spots on flanks (present in *B. cymbalum*), and presents dark lines on the dorsolateral region, forearms, and shanks (absent in *B. cymbalum*; Bokermann 1963), leading us to conclude that it is not *B. cymbalum*. However, without acoustic, locality, and/or molecular data, UF 100604 can be associated either with *B. prasina* or *B. pulchella*.

DISCUSSION

Historical DNA

With the newly generated hDNA sequences of *Boana cymbalum*, all the currently recognized species of the *B. pulchella* group have been included in molecular phylogenetic analyses. Our results are congruent with Straube et al. (2021), who found a high number of copies of the mitochondrial region 12S-tRNA^{Val}-16S, whereas coverage of coding genes (e.g., CytB and COI) was shallower. Although most previous studies in anuran museomics did not include nuclear sequences (e.g., only 12S-tRNA^{Val}-16S was recovered by Lyra et al. 2020; 16S and COI

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by Scherz et al. 2020; whole mitogenomes by Reyes-Velasco et al. 2021 and Goutte et al. 2022), we successfully mapped reads to the 28S rRNA gene. This finding is not surprising, because 28S presents several copies tandemly arrayed in the nucleolus organizer region (Wellauer et al. 1976; King et al. 1990), which might overcome the high degree of DNA degradation in liquid-preserved specimens.

Congruence with Previous Phylogenetic Hypotheses

Outgroup relationships among the genera of Cophomantini are congruent with previous studies (e.g., Faivovich et al. 2005, 2013, 2021; Wiens et al. 2010; Duellman et al. 2016; Pinheiro et al. 2019a; Lyra et al. 2020). However, our MP results for *Hyloscirtus* differ from those of Rojas-Runjaic et al. (2018) in that we found the *H. larinopygion* group to be paraphyletic with respect to the *H. bogotensis* group (represented by *H. palmeri*), whereas Rojas-Runjaic et al. (2018) found the *H. bogotensis* group to be sister to the clade of *H. armatus* group + *H. larynopigion* group. Our MP results corroborate the monophyly of the *B. punctata* group (as tentatively proposed by Faivovich et al. 2005) with GB = 14 and JK = 99%, whereas our ML tree rejects it (consistent with recent studies; e.g., Pinheiro et al. 2019a; Lyra et al. 2020; Sturaro et al. 2020). Inasmuch as our outgroup sampling was not designed to test the relationships within *Hyloscirtus* or the *B. punctata* group, we do not consider those groups to be refuted.

Ingroup relationships are largely congruent with the topology of Faivovich et al. (2021). The position of the *B. balzani* clade varies between MP and ML trees, being poorly supported in both Faivovich et al. (2021) and the present study. Only two differences are observed in the *B. prasina* clade: (1) *B. guentheri* is poorly supported (GB = 5; JK = 68%) as the sister taxon of *B. goiana* in this study, whereas *B. guentheri* is poorly supported (JK < 50%) as sister taxon of all other species of the *B. prasina* clade in Faivovich et al. (2021); and (2) among our three MPTs, *Boana* sp. 5 (an undescribed species similar to *B. prasina*) is the sister taxon of either *B. caingua* or the clade comprising *B. cymbalum*, *B. prasina*, *B. cordobae*, and *B. pulchella*, whereas Faivovich et al. (2021) found *Boana* sp. 5 and *B. caingua* as sister species forming a poorly supported clade (JK = 62%). These differences could be due to different taxon sampling and alignment (i.e., dynamic vs static). Finally, our results in both parsimony and maximum likelihood analyses, as those of Faivovich et al. (2021), support the monophyly of the *B. polytaenia* clade (GB = 21; JK = 99%), unlike those of Vasconcellos et al. (2021).

Boana cymbalum: Phylogenetic Position, Morphology, and Calls

The phylogenetic position of *Boana cymbalum* has not been tested previously because of the lack of molecular data. Nevertheless, Bokermann (1963), Barrio (1965), and Faivovich et al. (2021) associated *B. cymbalum* with the *B. pulchella* group, and Faivovich et al. (2021) proposed a close relationship to species of the *B. prasina* clade on the basis of the overall similarity of its morphology and call with those of *B. cordobae*, *B. prasina*, and *B. pulchella*, which is corroborated by our results. In contrast, Lutz's (1973) hypothesis that *B. cymbalum* could be conspecific either with *B. semiguttata* or *B. pulchella* is rejected, as is Caramaschi's hypothesis (in Frost 1985) that *B. cymbalum* could be conspecific with *B. pulchella*.

As expected, the hand of *Boana cymbalum* possesses a curved distal prepollex directed laterad to pass ventral to the Metacarpal II and a long postarticular process of the distal prepollex. Both character states are two known synapomorphies of the *B. pulchella* group (Pinheiro et al. 2022; see also Garcia and Haddad 2008).

The most remarkable characteristic in the external morphology of *Boana cymbalum* is the presence of dark spots on a white background on the flanks and concealed surfaces of thighs (Bokermann 1963). The function of coloration is unknown for the *B. pulchella* group, although the marks on flanks and thighs have been associated with visual communication during female choice in some hylids (e.g., *Agalychnis callidryas* and *Hyla arborea*; Gomez et al. 2009; Robertson et al. 2022). These markings are polymorphic in *B. cordobae*, *B. prasina*, and *B. pulchella* (Pinheiro et al. 2019b; Supplemental Material S5; Supplemental Table S2). Notably, Barrio (1965) described *B. cordobae* as lacking markings on the flanks, but we observed some specimens with reticulation on this region, including a topotype (MZUSP 73689; Fig. 5G). The reticulation is present in all examined specimens of *B. cymbalum* (Bokermann 1963), but a larger sample size would be necessary to determine if this character is polymorphic, as observed in other related species.

Faivovich et al. (2004, 2005, 2021) suggested the presence of a striped dorsal pattern as a synapomorphy of the *Boana polytaenia* clade (with instances of homoplasy in *B. bischoffi*, *B. caingua*, and *B. goiana*). We found at least one specimen of *B. cymbalum* with discontinuous, irregularly shaped dorsal stripes (MZUSP 106980) that resemble those of the dorsal-striped species of the *B. prasina* clade. However, whereas the polymorphism in *B. bischoffi* varies geographically (dorsal stripes are present in populations from São Paulo state but absent in those from Paraná to Rio Grande do Sul states; Marcelino et al. 2009), the variation in *B. cymbalum* does not seem geographically structured, as dorsal striped and nonstriped individuals from the same locality are known. However, this hypothesis could only be tested with a larger sample size.

According to Lutz (1973), the presence of the upper half of the iris lighter than the lower is shared by *Boana cymbalum* and *B. semiguttata*. However, this was not mentioned by Bokermann (1963) and it is unclear if Lutz (1973) inspected living specimens while describing iris coloration. Further, the presence of a bicolored iris has not been reported in the recent literature of the *B. pulchella* group, and even in some photographs from living specimens, the difference in color between the two halves of the iris is not as contrasting as in other hylids (e.g., *Aplastodiscus perviridis* group, *Cruziohyla*, *Phrynomedusa*, *Julianus*, *Osteocephalus castaneicola*, *O. leoniae*, *Scinax danae* and *S. funereus* groups; Garcia et al. 2001; Jungfer and Lehr 2001; Faivovich et al. 2005; Baêta et al. 2016; Araujo-Vieira et al. 2023).

Although Dena et al. (2024) characterized both types of notes as advertisement calls, we suggest Note A resembles the advertisement calls of related species (characterized by multiple short notes; e.g., Grenat et al. 2023), whereas the structure of Note B is similar to that of aggressive calls

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(characterized by a long trilled, pulsed call; Batista et al. 2015). However, given that calls are known to vary according to the social context in anurans (Toledo et al. 2015; Köhler et al. 2017), we refrain from including the calls in the species diagnosis because the social context of the recorded male is unknown. Assuming that the advertisement call is composed of series of Notes A, its dominant frequency distinguishes *Boana cymbalum* (1687–2062 Hz) from *B. caingua* (3234–3843 Hz; Batista et al. 2015), *B. callipleura* (1140–1320; Köhler et al. 2010), *B. cambui* (3382–5076 Hz; Pinheiro et al. 2016), *B. ericae* (2030–3600 Hz; Garcia and Haddad 2008), *B. goiana* (2295–3280 Hz; Menin et al. 2004), *B. guentheri* (2627–2799 Hz; Forti et al. 2019), *B. marianitae* (398–924 Hz; Köhler et al. 2010), *B. poaju* (2473–2885 Hz; Garcia et al. 2008), and all species of the *B. balzani* and *B. polytaenia* clades (Supplemental Material S5; Supplemental Table S3, available online). The tonal quality of Note A differentiates *B. cymbalum* from *B. prasina* (pulsed note; Barrio 1965; Reynolds and Foster 1992; Delgado and Haddad 2015), as well as all species of the *B. semiguttata* clade (Pinheiro et al. 2024). The absence of an advertisement call composed of one A note followed by a series of B notes distinguishes *B. cymbalum* from *B. itajahy* and *B. marginata* (Pinheiro et al. 2024).

Conservation Status of *Boana cymbalum*

Almost 90% of the original expanse of the Brazilian Atlantic Forest has been deforested, and more than half of its remnants are restricted to the Serra do Mar, surrounded by human settlements or adjacent to industrial centers (Galindo-Leal and Câmara 2005; Ribeiro et al. 2009). Specifically, the Reserva Biológica do Alto da Serra de Paranapiacaba (REBIO) is located between the metropolitan area of São Paulo and the coast (Lopes and Kirizawa 2009), with Campo Grande da Serra—the type locality of *B. cymbalum*—being close to REBIO and ca. 14 km from Cubatão. During the 1970s and 1980s, heavy industrial activity from the petrochemical pole of Cubatão deposited a large amount of pollution in several areas of the Atlantic Forest (Mayer et al. 2000; Verdade et al. 2009; Trevine et al. 2014), making it "the most polluted forest ecosystem with respect to sulfur, nitrogen, and fluorine" (Mayer et al. 2000). The pollution from Cubatão appears to have affected the original phytophysiognomy of REBIO, as evidenced by the absence of large trees and tussocks and low abundance of bromeliads (Domingos et al. 2009; Verdade et al. 2009). Moreover, the pollution from Cubatão is associated with anatomical and physiological changes in toads of species *Rhinella ornata*, such as hypertrophied organs related to detoxification (e.g., kidney and liver) or immune response (e.g., spleen; Santana et al. 2021). It is unknown if similar changes are present in frogs of the *Boana pulchella* group exposed to the pollution from Cubatão.

Among species of the *Boana pulchella* group, *B. bischoffi*, *B. cymbalum*, *B. polytaenia*, and *B. prasina* were historically reported in this region (Verdade et al. 2009; Trevine et al. 2014). Verdade et al. (2009) suggested that the montane areas of REBIO are possibly more affected by the winds blowing from Cubatão, whereas the Parque Natural Municipal Nascentes de Paranapiacaba (PNMNP) is more protected from them, which could explain why the anurofauna in the PNMNP is more abundant than in the REBIO. Although a slow-flowing creek close to the Campo Grande railway station—as was described by Bokermann (1963)—is still present, the area of the type locality has been disturbed by multiple wildfires and is currently covered by secondary vegetation, with only grass and a few large trees (DYMN and PDPP, personal observations). It is unknown whether the pollution of Cubatão affected the type locality of *B. cymbalum*, but intense vegetation clearing for charcoal production was reported in Campo Grande da Serra near the railway station even before the description of this species (I. Grantsau, personal communication), which suggests that this species was already threatened in the 1960s.

Since 2023, *Boana cymbalum* has been classified as extinct according to the IUCN Red List of Threatened Species because "extensive searches in the appropriate habitat, during the appropriate season within the known range, since 1964 have failed to locate this species" (IUCN 2023). In addition to the Red List, the Brazilian Environmental Ministry (MMA) frequently updates conservation status of species through reports. This species was first considered threatened in the Instrução Normativa MMA No. 3 (MMA 2003). Subsequently, Portaria MMA No. 444 classified this species as critically endangered (possibly extinct; MMA 2014). Recently, its conservation status was officially listed as extinct according to Portaria MMA No. 148, expanding the list of extinct amphibian species in Brazil from one to two, namely *B. cymbalum* and *Phrynomedusa fimbriata* (MMA 2022). Luedtke et al. (2023) also listed *B. cymbalum* as extinct due to disease, although we are unaware of any disease reported in this species.

W.C.A. Bokermann collected *B. cymbalum* on at least three different field trips (October 1962, December 1963, and January 1964); subsequently, fieldwork by Verdade et al. (2009), Trevine et al. (2014), and us (DYMN and PDPP, 17 March 2023) failed to detect the species. Despite being listed as extinct, absence of evidence is not evidence of absence, and the causes of its presumed extinction are speculative. More fieldwork is required to assess whether local populations of *B. cymbalum* remain near Campo Grande da Serra, especially in several lentic water bodies in forested areas southeast of the ABC Paulista towards Paranapiacaba (DYMN and PDPP, personal observations). Previous studies have demonstrated the importance of continuous fieldwork and metagenomic efforts to rediscover lost species. For instance, Moraes et al. (2022) rediscovered *Phrynomedusa appendiculata* after extensive fieldwork. In addition, using metagenomics, Lopes et al. (2021) revealed that environmental DNA (eDNA) from water samples could detect remaining populations of threatened species. In the case of *B. cymbalum*, DNA in water samples from areas near the type locality could be sequenced to map eDNA reads to our newly generated sequences, thereby integrating eDNA and hDNA in an effort to rediscover this lost species.

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Supplemental Material

Supplemental material associated with this article can be found online at https://doi.org/10.1655/Herpetologica-D-24-00036.S1; https://doi.org/10.1655/Herpetologica-D-24-00036.S2; https://doi.org/10.1655/Herpetologica-D-24-00036.S3; https://doi.org/10.1655/Herpetologica-D-24-00036.S4; https://doi.org/10.1655/Herpetologica-D-24-00036.S5.

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APPENDIX

Additional Specimens Examined

Museum and field series abreviations: KU = University of Kansas Biodiversity Institute, Lawrence, USA; MZUSP = Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil; MNRJ = Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; TG = T. Grant field series.

Boana bischoffi.—BRAZIL: RIO DE JANEIRO: Teresópolis: MZUSP 10556; SÃO PAULO: Bororé: MZUSP 129843–129844; São Paulo: Parque Estadual da Cantareira: MZUSP 60902, 112767–112779; Eldorado: Parque Estadual de Jacupiranga: Núcleo Caverna do Diabo: MZUSP 135467, 135469; Juquitiba: MZUSP 134711–134713; Perus: MZUSP 157432; Pirituba: MZUSP 145705; Ribeirão Grande: Reserva Florestal do Morro: MZUSP 142169; Salesópolis: Estação Biológica de Boracéia: TG 4020; Santo Amaro: Cocaia: MZUSP 10029; Santo André: Parque Natural Municipal Nascentes de Paranapiacaba: MZUSP 153776; SANTA CATARINA: Campo Alegre: MZUSP 55869–55870.

Boana caingua.—BRAZIL: SÃO PAULO: Águas de Santa Bárbara: MZUSP 151010–151011; Botucatu: MZUSP 2960, 54374, 134110; Itapeva: MZUSP 60856–60858, 139395.

Boana cordobae.—ARGENTINA: CÓRDOBA: Santa Rosa de Calamuchita: MZUSP 73689 (paratype); Pampa de Achala: MZUSP 55728; Yacanto de Calamuchita: MZUSP 95040–95041.

Boana cymbalum.—BRAZIL: SÃO PAULO: Santo André: Campo Grande: KU 92010, MZUSP 74194 (WCAB 9153; holotype), 73697 (WCAB 9154; paratopotype), 106980 (WCAB 14074), 160852 (WCAB 14075).

Boana goiana.—BRAZIL: MINAS GERAIS: Patrocínio: MZUSP 145253, 146253.

Boana guentheri.—BRAZIL: SANTA CATARINA: Joinville: MZUSP 20660–20661, 106711–106714, 106715–106720.

Boana marginata.—BRAZIL: SANTA CATARINA: Novo Horizonte: MZUSP 35386–35396.

Boana prasina.—BRAZIL: PARANÁ: Pinhal: MZUSP 127717–127721; Wenceslau Braz: 128573–128582; SANTA CATARINA: Ibirama: MZUSP 31139; Rio Vermelho: MZUSP 109314; São Bento do Sul: MZUSP 64694; 109320–109322; SÃO PAULO: Buri: MZUSP 128494–128495; Campos do Jordão: MZUSP 129733–129734, 133970–133979; Cotia: MZUSP 134383; Embu das Artes: MZUSP 34431, 71713; Itapecerica: MZUSP 34430; Jundiaí: MZUSP 147438; Juquitiba: MZUSP 134700; Vila Nova Manchester: MNRJ 23778, MNRJ 23939–23943; Paranapiacaba: MZUSP 145388–145389; Piraju: MZUSP 72403; Ribeirão Grande: MZUSP 88022, 87586–87588; 87657–87659; 142163; Ribeirão Grande da Serra: MZUSP 93790, 93794–93799; Salesópolis: Estação Biológica de Boracéia: MZUSP 3857; 31556–31560; Santana do Parnaíba: MZUSP 139388; Santo André: Parque do Pedroso: MZUSP 135094; São Paulo: MZUSP 34511, 34567, 60019.

Boana pulchella.—ARGENTINA: BUENOS AIRES: Mar del Plata: 16283–16285; San Isidro: 95068–95073. BRAZIL: RIO GRANDE DO SUL: São Leopoldo: MZUSP 36075–36076; Taim: MZUSP 70778–70780; Tramandaí: MZUSP 36083–36084; SANTA CATARINA: Lagoa: MZUSP 36012. URUGUAY: CANELONES: Parque del Plata: 95074–95077.

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