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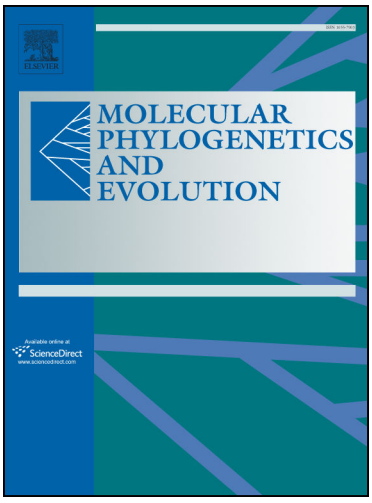

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Phylogenetic relationships of the *Boana pulchella* Group (Anura: Hylidae)

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ABSTRACT

In this paper we present a phylogenetic analysis of the treefrogs of the *Boana pulchella* Group with the goals of (1) providing a rigorous test its monophyly; (2) providing a test of relationships supported in previous studies; and (3) exploring the relationships of the several species not included in previous analyses. The analyses included more than 300 specimens of 37 of the 38 species currently included in the group, plus 36 outgroups, exemplars of the diversity of *Boana* and the other genera of the hylid tribe Cophomantini. The dataset included eight mitochondrial genes (12S, 16S, *CytB*, *COI*, *ND1*, and the $tRNA^{Ile}$, $tRNA^{Leu}$, and $tRNA^{Val}$) and five nuclear genes (*RHO*, *TYR*, *RAG-1*, *CXCR4*,

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SIAH 1). The phylogenetic analyses recover the monophyly of the *B. pulchella* Group with lower support than previous analyses, as a result of the inclusion of the *B. claresignata* Group, which is recovered as its sister taxon. Within the *B. pulchella* Group, the inclusion of almost all species of the group had little impact on previous notions of its phylogeny, except for the rejection of the hypothesized *B. polytaenia* Clade (*B. goiana* and *B. phaeopleura* are nested in the clade here called the *B. prasina* Clade), which is redefined. Phylogenetic support is strong for five major clades, which collectively include all but three of the species sampled: the *B. balzani* Clade (*B. aguilari*, *B. balzani*, *B. gladiator*, *B. melanopleura*, *B. palaestes*), the redefined *B. polytaenia* Clade (*B. botumirim*, *B. buriti*, *B. cipoensis*, *B. jaguariaivensis*, *B. leptolineata*, *B. polytaenia*, *B. stenocephala*, and two undescribed species), the *B. prasina* Clade (*B. bischoffi*, *B. caingua*, *B. cordobae*, *B. goiana*, *B. guentheri*, *B. marginata*, *B. phaeopleura*, *B. prasina*, *B. pulchella*, and one undescribed species), the *B. riojana* Clade (*B. callipleura*, *B. marianitae*, *B. riojana*), the *B. semiguttata* Clade (*B. caipora*, *B. curupi*, *B. joaquini*, *B. poaju*, *B. semiguttata*, *B. stellae,* and two undescribed species). The monophyly of the *B. prasina* + *B. riojana* clades, and that of *B. polytaenia* + *B. semiguttata* are well-supported. The relationships among these two clades, the *B. balzani* Clade, *B. ericae* + *B. freicanecae*, and *B. cambui* (representing the deepest phylogenetic splits within the *B. pulchella* Group) are recovered with weak support. We discuss the phenotypic evidence supporting the monophyly of the *B. pulchella* Group, and the taxonomy of several species, identifying three new synonyms of *Boana polytaenia*, one new synonym of *Boana goiana*, and one new synonym of *B. riojana*.

Keywords: Hylidae, Hylinae, Cophomantini, systematics, taxonomy, Neotropics

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"The call of Hyla polytaenia*...is one of the most common and cheerful hylid calls in the mountain resorts of the state of Rio de Janeiro. At night it resounds in gardens, hedges, and roadsides, at the edges of ponds and marshes, and is as charming as the appearance of the songster, one of the prettiest of the lesser species of Brazilian tree frogs"*
Bertha Lutz, 1973: 114.

1. Introduction

The hylid genus *Boana* currently comprises 97 species distributed from central Argentina to Nicaragua and Hispaniola, in the West Indies (Frost, 2020; Lyra et al., in press). Its species were historically associated with some species groups formerly included in *Hyla* that Faivovich et al. (2005) rearranged and redefined in seven species groups, the *B. albopunctata*, *B. benitezi*, *B. faber*, *B. pellucens*, *B. pulchella*, *B. punctata*, and *B. semilineata* groups. This rearrangement was subsequently corroborated by several authors (Wiens et al. 2006, 2010; Pyron and Wiens, 2011, Pyron, 2014: supp. data; Duellman et al., 2016; Jetz & Pyron, 2018) and refined in terms of the contents of the *B. benitezi*, *B. punctata*, and *B. semilineata* groups by Faivovich et al. (2006; 2013), Pinheiro et al. (2019), and Sturaro et al. (2020). More recently, Lyra et al. (in press) added an eighth species group, the *B. claresignata* Group. Of these eight groups, the *B. pulchella* Group is the most species-rich, having today 38 known species. Its monophyly has been corroborated by molecular phylogenetic analyses with high support values in multiple studies (Faivovich et al., 2004, 2005, 2013; Wiens et al., 2006, 2010; Pyron and Wiens, 2011; Pyron, 2014: supp. data; Duellman et al., 2016; Jetz and Pyron, 2018; Pinheiro et al., 2019a).

Lyra et al. (in press) transferred the former *Bokermannohyla claresignata* Group to *Boana*, as they obtained the nominal species of that group as the sister taxon of the *B.*

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pulchella Group. Lyra et al. (in press) obtained a monophyletic *B. pulchella* Group, albeit with a notable decrease in the support of its monophyly: the 100% (analysis with gaps as fifth state) or 99% (gaps as missing data) jackknife support in the results of Pinheiro et al. (2019a) decreased to 80% (gaps as fifth state) or 86% (gaps as missing data). Lyra et al. (in press) noticed that several species of the *B. pulchella* Group were still unavailable for phylogenetic studies, stressed the need of additional testing of the monophyly of the group, and discussed some putative phenotypic synapomorphies shared by the *B. claresignata* and *B. pulchella* groups.

Our knowledge of the diversity in the *B. pulchella* Group has grown remarkably, as, by 2003, it only contained 15 species. This growth was due to a combination of increased phylogenetic knowledge of hylids in general, the description of several new species, and the rediscovery of others. Faivovich et al. (2004) reviewed the taxonomic history of the then *Hyla pulchella* Group and presented the first phylogenetic analysis. It included 10 of the then 15 species in the group plus several outgroups, including species that had tentatively been associated with it in the past. Their results solved taxonomic problems associated with the recognition of several subspecies in the past (Barrio, 1965a), and presented a new framework that included a leap in the diversity of the group: the former *Hyla polytaenia* Group was nested in the *H. pulchella* Group. The addition of the seven species included by that time in the former *H. polytaenia* Group, plus the other four species added as a result of recognition of species status to former subspecies, and the inclusion of other species previously unassigned to any group, expanded the group from 15 to 26 species. The topology of this redefined *H. pulchella* Group was recovered as well by Faivovich et al. (2005), who added nuclear sequences for the same terminals. Subsequent analyses by Wiens et al. (2006, 2010), Pyron and Wiens (2011), and Pyron (2014: supp. data) recovered a similar topology, or differing in details (Duellman et al.,

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2016; Sturaro et al., 2020; Caminer and Ron, 2020) without adding new sequences for the group.

Since the analysis of Faivovich et al. (2004), 13 species were added to the group, 11 of which were newly described species (*B. aguilari*, *B. bandeirantes*, *B. botumirim*, *B. caipora*, *B. cambui*, *B. curupi*, *B. freicanecae*, *B. gladiator*, *B. jaguariaivensis*, *B. poaju*, and *B. stellae*), one of which was resurrected from the synonymy of *B. balzani* (*B. callipleura*; Köhler et al., 2010), and the other one transferred from the former *Hyla geographica* Group (*B. secedens*). Furthermore, *B. melanopleura* was rediscovered by Lehr and von May (2004). Of these, *B. curupi* was included as an undescribed species by Faivovich et al. (2004, 2005); *B. aguilari*, *B. caipora*, *B. gladiator*, and *B. melanopleura* were included in a phylogenetic framework (Antunes et al., 2008; Köhler et al., 2010; Lehr et al., 2010), and more recently in analyses of most species of *Boana* (Sturaro et al., 2020; Caminer and Ron, 2020) or Cophomantini with sequences available in GenBank (Faivovich et al., 2013; Pinheiro et al., 2019a). Caramaschi et al. (2004) implicitly included *B. secedens* in the *B. pulchella* Group by their tentative association with *B. bischoffi* based on its color pattern. More recently, Peloso et al. (2018) demonstrated that it is indeed a species of the *Boana semilineata* Group. In all, 15 species of the group have not been included in previous analyses. These are *B. albonigra*, *B. bandeirantes*, *B. beckeri*, *B. botumirim*, *B. buriti*, *B. cambui*, *B. cipoensis*, *B. cymbalum*, *B. freicanecae*, *B. goiana*, *B. jaguariaivensis*, *B. phaeopleura*, *B. poaju*, *B. stellae*, and *B. stenocephala*.

In this study, we examined a nearly complete sample of known species in the *B. pulchella* Group, including 14 of the 15 species of this group unavailable in previous phylogenetic studies, and multiple specimens of several species. We further extended our character sampling to the mitochondrial genes cytochrome c oxidase I (*COI*) and NADH dehydrogenase 1 (*ND1*), and the nuclear gene C-X-C motif chemokine receptor 4

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(*CXCR4*), in addition to the genes used in earlier studies, with the goals of (1) providing a rigorous test of the monophyly of the *B. pulchella* Group; (2) providing a rigorous test of relationships supported in previous phylogenetic analyses; and (3) exploring the relationships of the several species not included in previous analyses.

2. Material and Methods

2.1 Taxonomic sampling

We included all species of the *Boana pulchella* Group with the exception of *B. cymbalum*. In most cases, we included multiple specimens from each species. Our sampling included as well two undescribed species (*Boana* sp. 1 and *Boana* sp. 2), and topotypes of 22 of the 38 species in the group. As outgroups we included 36 species, exemplars of all the currently recognized species groups of *Boana*, with an emphasis on those most closely related to the *B. pulchella* Group (the *B. faber* Group and the only available species of the *B. claresignata* Group), a species representing each species group of the other genera of Cophomantini: *Aplastodiscus*, *Bokermannohyla*, *Hyloscirtus*, *Myersiohyla*, and the only species of *Nesorohyla* (Faivovich et al., 2005, 2013; Wiens et al., 2010; Pyron and Wiens, 2011; Pinheiro et al., 2019a). The tree was rooted with *Myersiohyla neblinaria*, one of the earlier diverging clades of Cophomantini (Pinheiro et al., 2019a). See Supp. Data 1 for locality data of all voucher specimens of the sequences included in this study. Collection codes are those of Sabaj (2019), with the exceptions noticed in Supp. Data 1.

2.2 Character sampling

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The four mitochondrial gene sequences produced for this project include portions of the genes encoding cytochrome *b* (*CytB)*, COI, 12S, $tRNA^{Val}$, 16S, and a fragment including the complete upstream section of 16S, $tRNA^{Leu}$, NADH dehydrogenase subunit 1 (*ND1*), and $tRNA^{Ile}$, which was first incorporated by Wiens et al. (2005). The nuclear gene sequences produced include portions of seven in absentia homolog 1 (*SIAH1*, mistakenly called Seventh in Absentia by Faivovich et al., 2005), exon 1 of rhodopsin (*RHO*), exon 1 of tyrosinase (*TYR*), single exon of Recombination Activating 1(*RAG-1*), and exon 2 of *CXCR4*. All the primers employed are the same as those employed by Faivovich et al. (2005), with the addition of 16S-frog and tMet-frog (fragment of 16S + $tRNA^{Leu}$ + *ND1* + $tRNA^{Ile}$; Wiens et al., 2005), T3-AnF1 and T7-AnR1 (COI; Lyra et al. 2017), and CXCR4-C and CXCR4-G (Biju and Bossuyt, 2003). We also included GenBank sequences produced by Darst and Cannatella (2004), Faivovich et al. (2004, 2005, 2010, 2013), Wiens *et al.* (2005), Antunes *et al.* (2008), Koscinsky et al. (2008), Köhler *et al.* (2010), Lehr *et al.* (2010), Coloma *et al.* (2012), Berneck *et al.* (2016), Orrico *et al.* (2017), Rojas-Runjaic et al. (2018), and Pinheiro et al. (2016, 2019a). See Supp. Data 1 for GenBank accession numbers of the sequences employed in this study.

2.3. DNA isolation and sequencing

Whole cellular DNA was extracted from ethanol-preserved tissues with the DNeasy (QIAGEN, Valencia, CA) isolation kit or standards high-salt protocol. Amplification was performed in a 25-µl-volume reaction using TAQ DNA polymerase and reagents (Thermo Fisher Scientific, Massachusetts, USA) or Taq DNA Polymerase master mix (Ampliqon A/S, Denmark). For all the amplifications, the PCR program included an initial denaturing step of 30 seconds at 94°C, followed by 35 (mitochondrial gene fragments) or 45 (nuclear gene fragments) cycles of amplification (94 °C for 30

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seconds; 48–64°C for 30 seconds; 60–72°C for 60 seconds), with a final extension step at 72°C for 6 minutes. Polymerase chain reaction (PCR) amplification products were cleaned using Exonuclease I and Alkaline phosphatase (Thermo Fisher Scientific, Massachusetts, USA) and sequenced using fluorescent-dye labels terminators (ABI Prism Big Dye Terminators v. 1.1 cycle sequencing kits; Applied Biosystems, Foster City, CA) with an ABI 3730XL (Applied Biosystems, Foster City, CA); all samples were sequenced in both directions to check for potential errors. Chromatograms obtained from the automated sequencer were read and contigs made using the sequence editing software Sequencher 5.0 (Gene Codes, Ann Arbor, MI). Sequences were aligned using the online software MAFFT v7 (Katoh and Toh, 2008; Katoh et al., 2019) under the strategy E-INS-i (for the 12S-tRNAVal-16S fragment) and L-INS-i (for remaining fragments), with default parameters for gap opening and extension. These alignments were used for both phylogenetic analyses and clade support estimations. Complete sequences were edited with BioEdit (Hall, 1999).

2.4 Phylogenetic analysis

Numerous authors have discussed the rationale for using the parsimony optimality criterion (Farris, 1983; Goloboff, 2003; Goloboff and Pol, 2005; Kluge and Grant, 2006; Grant and Kluge, 2009). The phylogenetic analyses were 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) percentages 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

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the original data matrix showed that minimum length is hit with this search strategy. Analyses were performed considering gaps 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 GTRCAT model and treating gaps as missing data in all analyses (the only option in this software). 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). Uncorrected p-distances were calculated in PAUP* (Swofford, 2002) for the 16S rRNA gene fragment delimited by the primers 16Sar-L and 16S-Wilk2. Fouquet et al. (2007) established a threshold of 3% uncorrected p-distances to establish candidate species in the absence of our data. Although we did not employ this approach in this paper, we consider that p-distances < 3% are relatively low, and in the absence of phylogenetic (e.g., non-monophyly) or phenotypic evidence (e.g., differences in external morphology, advertisement calls) we do not question specific status.

3. Results

The length of the aligned sequences (including gaps) was 2605 bp for 12S-tRNA^{Val}-16S, 1337 bp for 16S- tRNA^{Leu}-*ND1*- tRNA^{Ile}, 385 bp for *CytB*, 651 bp for *COI*,

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675 bp for *CXCR4*, 427 bp for *RAG-1*, 316 bp for *RHO*, 397 bp for *SIAH1*, and 532 bp for *TYR*. Taxon occupancy of each gene (ie, the percentage of taxa where that gene was retrieved; in parenthesis the value considering only the ingroup species) was 100% (100%) for 12S- $tRNA^{Val}$-16S, 76% (94.87%) for 16S- $tRNA^{Leu}$-*ND1*- $tRNA^{Ile}$, 77.33% (82.05%) for *CytB*, 61.33% (89.74%) for *COI*, 46.67% (48.72%) for *CXCR4*, 54.67% (66.67%) for *RAG-1*, 86.67% (97.44%) for *RHO*, 68% (71.8%) for *SIAH1*, and 60.67% (48.72%) for *TYR*.

The parsimony analysis considering gaps as a fifth state recovered 5295 most parsimonious trees (MPT; 23,689 steps), and the analysis with gaps treated as missing data recovered 2228 MPTs of 22,750 steps. In both cases, subsequent rounds of TBR branch swapping revealed that there were more MPT (>15,000). However, successive strict consensus converged on the same topologies as that obtained with the initial MPTs, and so we considered that further efforts to find more equally parsimonious trees were unnecessary (Goloboff, 1999). Most conflict among MPTs in both analyses involves intraspecific relationships, with the exception of a few outgroups in the *B. faber* Group, *Boana semiguttata* and *Boana* sp. 2, and two clades in the *B. polytaenia* Clade (Fig. 1; Supp. Data 2).

The results of these and the ML analysis are largely congruent in terms of the well-supported groups (Figs. 1, 3–7; Supp. Data 2, 3) unless otherwise stated. Among outgroups, the exemplars of genera *Nesorohyla*, *Hyloscirtus*, *Bokermannohyla*, and *Aplastodiscus* form a pectinated series within which *Boana* is nested (Fig. 1). The relationships among the species groups of *Boana* are poorly supported. The *B. claresignata* group is the sister taxon of the *B. pulchella* group, and the *B. faber* Group is the sister taxon of these two groups (Fig. 1).

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The *B. pulchella* Group is monophyletic with 82% jackknife support in the analysis with gaps considered as a fifth state, and 72% in the analysis with gaps considered as missing data, and 73% bootstrap in the ML results (Fig. 1; Supp. Data 2, 3). The results described below are congruent among the analyses; unless otherwise stated, the informed support values are those of the analysis considering gaps as a fifth state (Fig. 1).

The deepest split in the *B. pulchella* Group separates *B. cambui* from a poorly supported clade (62% jackknife) that includes *B. ericae* + *B. freicanecae* (72% jackknife), and a poorly supported clade (<50% jackknife) with all other species of the group. These are included in five well supported clades (95–100 % jackknife). We recognize these (Fig. 1) as (1) the redefined *B. polytaenia* Clade (including *B. bandeirantes*, *B. beckeri*, *B. buriti*, *B. botumirim*, *B. cipoensis*, *B. jaguariaivensis*, *B. latistriata*, *B. leptolineata*, *B. polytaenia*, *B. stenocephala*, and additional undescribed species); (2) the *B. semiguttata* Clade, composed of *B. poaju* and all species that are morphologically similar to *B. semiguttata* (including *B. caipora*, *B. curupi*, *B. joaquini*, *B. stellae*, and two undescribed species); (3) the *B. balzani* Clade, which includes several Andean species (*B. aguilari*, *B. balzani*, *B. gladiator*, *B. melanopleura*, and *B. palaestes*); (4) the *B. riojana* Clade, which includes the remaining Andean species of the *B. pulchella* Group (*B. albonigra*, *B. callipleura*, *B. marianitae*, and *B. riojana*); and (5) the *B. prasina* Clade, which includes Atlantic Forest species (*B. bischoffi*, *B. caingua*, *B. guentheri*, *B. marginata*, and *B. prasina*) among which are nested a clade (100% jackknife) of Cerrado inhabiting species that had been included in the *B. polytaenia* Clade (*B. goiana* and *B. phaeopleura*), and a clade (100% jackknife) of the southernmost distributed species (*B. cordobae* and *B. pulchella*).

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It is unclear to which degree the incomplete gene coverage in our study has an impact in the support values. In particular, the gene coverage is quite complete at the level of most species of the ingroup; preliminary analyses using a single terminal per species and maximizing gene coverage did not result in a notable increase in support values for the deeper splits within the *B. pulchella* Group (data not shown).

4. Discussion

4.1 Outgroup topology

The recovered relationships among genera of Cophomantini are congruent with all previous analyses (e.g., Faivovich et al., 2005, 2013; Wiens et al., 2010; Duellman et al., 2016; Pinheiro et al., 2019a; Lyra et al., in press). Our parsimony results for *Hyloscirtus*, however (Fig. 1), differ from those of Rojas-Runjaic et al. (2018) in that *H. jahni* is recovered as the sister taxon of our only exemplar of the *H. bogotensis* Group, instead of sister taxon of all species of *Hyloscirtus*. Our taxon sampling differs fundamentally from that of Rojas-Runjaic et al. (2018), and as such it does not constitute a valid test of their results. Relationships among most species groups of *Boana* are poorly supported (Faivovich et al., 2013; Pinheiro et al., 2019a; Sturaro et al., 2020; Caminer and Ron, 2020; Lyra et al., in press) and this is observed in our results. Like the recent results of Lyra et al. (in press), the only available exemplar of the *B. claresignata* Group (*B. claresignata*) is recovered as the sister taxon of the *B. pulchella* Group.

4.2 The Boana pulchella *Group*

The monophyly of the *B. pulchella* Group is corroborated as in previous tests, with 82% jackknife in the parsimony analysis considering gaps as fifth state; 72% with gaps

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considered as missing data, and 73% bootstrap in the ML analysis. As noticed by Lyra et al. (in press), the inclusion of the *B. claresignata* Group and its recovery as the sister taxon of the *B. pulchella* Group resulted in a decrease in the support of this last group from previous studies (e.g., Faivovich et al., 2004, 2005, 2013; Wiens et al., 2010; Duellman et al., 2016; Pinheiro et al., 2019a; Sturaro et al., 2020; Caminer and Ron, in press). It is unclear whether this is due to the limited available sequence data for *B. claresignata* (only 1580 bp of 12S-16S; Lyra et al., in press) or to actual character conflict. In any case, we consider that the monophyly of the *B. pulchella* Group will require further testing.

The recently discovered position of the *B. claresignata* Group introduces several uncertainties regarding the putative phenotypic synapomorphies of the *B. pulchella* Group. Lyra et al. (in press) reported that species of the *B. claresignata* Group share with the *B. pulchella* Group the only putative morphological synapomorphy that has so far been reported for this group, the absence of the slip of the m. *depressor mandibulae* that originates on the dorsal fascia at the level of the m. *dorsalis scapularis* (Faivovich et al., 2005).

Pinheiro et al. (2018) noticed that the absence of the anterolateral process of the hyoid plate is so far known only in the *B. pulchella* Group. Lyra et al. (in press) showed that an anterolateral process is absent as well in *B. clepsydra* but present in *B. claresignata*. The taxonomic distribution of this character state is still unknown in some species of the *B. pulchella* Group (*B. aguilari*, *B. balzani*, *B. cambui*, *B. melanopleura*, and *B. palaestes*), and the anterolateral process is present in *B. freicanecae* (P.D. Pinheiro pers. obs.); hence, its polarity is still unclear.

The posterolateral process of the hyoid plate 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). It is absent as well

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in the two species of the *B. claresignata* Group (Lyra et al., in press). Although it could be another putative synapomorphy supporting the monophyly of the *B. claresignata* and the *B. pulchella* groups, its taxonomic distribution requires additional study.

While known egg clutches of species in most groups of *Boana* are laid as adherent films on the water surface (see Brunetti et al., 2014, for a list of *Boana* species known to have this egg clutch structure), so far most known species in the *B. pulchella* Group have been reported to lay submerged egg clutches adhered to vegetation (Fernández, 1926; Gallardo, 1961; Lutz, 1973; Eterovick et al., 2002; Menin et al., 2004; Carnaval and Peixoto, 2004; Garcia et al., 2007; Kwet, 2008; Kwet et al., 2010; Guerra et al., 2017; R.A. Brandão pers. obs.). The clutches have generally been described as gelatinous masses (e.g. Cochran, 1955; Carnaval and Peixoto, 2004; Menin et al., 2004), clumps (Kwet, 2008) or compact masses (e.g. Kwet et al., 2010), although there seems to be some variation (see Eterovick et al., 2002: fig. 2) that should be surveyed. Knowledge on oviposition in the *B. claresignata* Group is restricted to the occurrence of relatively large, unpigmented ova (Lyra et al., in press). While the clutch laid as a compact egg mass (as opposed to the plesiomorphic adherent films on the water surface) could be a putative synapomorphy of the *B. pulchella* Group, the fact that this is unknown in the *B. claresignata* group precludes an unambiguous polarization of this character state.

Ferro et al. (2018) proposed that the occurrence of a conspicuous interstitial heterochromatic C-band in chromosome pair 11 probably represents a synapomorphy for the *B. pulchella* Group, or a less inclusive clade pending the characterization of chromosomes of *B. cambui*, *B. ericae*, and *B. freicanecae*. Chromosome morphology should also be assessed in the *B. claresignata* Group to infer the node where the C-band in pair 11 evolved.

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Several authors noticed that species of the *B. pulchella* Group release a peculiar smell when handled, which has been likened to crushed plants (Faivovich et al., 2013) or skunk-like or fox-like (Gallardo, 1958; Langone, 1995). This smell has been so far reported for *B. aguilari*, *B. bischoffi*, *B. caipora*, *B. cipoensis*, *B. curupi*, *B. cordobae*, *B. gladiator*, *B. prasina*, *B. pulchella*, *B. polytaenia* (populations from outskirts of Belo Horizonte, Minas Gerais; Teresópolis, Rio de Janeiro; and São José do Barreiro, São Paulo), *B. riojana*, *B. semiguttata*, *B. stellae*, and *B. stenocephala* (Gallardo, 1958, 1961; Barrio, 1962, 1965a; Garcia et al., 2007; Antunes et al., 2008; Faivovich et al., 2013; D. Baêta, J.C. Chaparro, and P.D Pinheiro pers. obs.; T.L. Pezzuti pers. com.). To our knowledge, anecdotal data of smell in other species groups of *Boana* are limited to *B. claresignata*, *B. faber*, *B. geographica*, and *B. semilineata* (Lutz and Orton, 1946; Lutz, 1973; Azevedo-Ramos, 1995; A.E. Brunetti, pers. com; D. Baêta, pers. obs); strong smells had also been reported in species of *Bokermannohyla*, *Hyloscirtus*, *Myersiohyla* (see Faivovich et al., 2013 for a review), and at least one species of *Aplastodiscus*, *A. musicus* (Lutz, 1973). More recently Brunetti et al. (2015) analyzed volatile secretions of *B. pulchella* and *B. riojana*, identifying 35 compounds in common for these two species. Brunetti et al. (2016) further described some differences in the distribution of the ordinary serous glands, those inferred to be the source of volatiles, on the dorsum among some species in the *B. pulchella* Group and *B. punctata*, a species without perceptible volatile secretions. More recently, Brunetti et al. (2019) suggested that symbiotic skin bacteria could also be the source of odorous compounds in this species group. Increased knowledge on the taxonomic distribution and chemistry of volatile secretions in *Boana* would reveal whether if they could be considered a synapomorphy of the *B. pulchella* Group or a more inclusive clade (like the *B. claresignata* + *B. pulchella* groups).

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Overall, the internal relationships of the *B. pulchella* group are congruent with the most recent analysis with the densest sampling of Cophomantini (Pinheiro et al., 2019a; Lyra et al., in press), with topological differences associated to terminals included for the first time (e.g., *B. cambui*, *B. freicanecae*, and *B. goiana*) or clades consistently recovered in positions with low support (e.g., the position of the clade including central Andean species; see below).

4.3 Boana cambui*,* B. ericae*, and* B. freicanecae

All previous phylogenetic analyses and reanalyses of the *B. pulchella* Group with a relevant sampling consistently recovered *B. ericae* as the sister taxon to the remainder of the group (Faivovich et al., 2004, 2005, 2013; Pinheiro et al., 2019a; Lyra et al., in press). However, the present study, with its expanded taxon sampling, weakly supports (62% jackknife) in the parsimony analyses the recently described *B. cambui* as the sister taxon of all other species of the *B. pulchella* Group. *Boana ericae*, instead, is recovered as the sister taxon of *B. freicanecae* (Fig. 1, Supp. Data 2, 3). In the ML analysis, the deepest split separates this clade from the rest of the *B. pulchella* Group, within which *B. cambui* is the sister taxon to all remaining species (60% bootstrap).

As noted by Pinheiro et al. (2016), *B. cambui* is morphologically most similar to *B. freicanecae*, with which it shares a dorsal coloration pattern unique in the group. Interestingly, despite this unique pattern, our results indicate that these two species are not closely related. While *B. cambui* is known only from a single locality in the Atlantic Forest along the Mantiqueira mountain range, *B. freicanecae* is known from two localities within Atlantic Forest remnants in Alagoas and Pernambuco (Fig. 2; Carnaval and Peixoto, 2004; Cardoso et al., 2006), northeastern Brazil, and is the northern-most distributed species of the *B. pulchella* group.

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The sister-group relation of *B. ericae* and *B. freicanecae* is supported with a 72% jackknife value (Fig. 1). *Boana ericae* occurs in the highland Cerrado formations of Chapada dos Veadeiros (State of Goiás) in Central Brazil, about 1300 km SW from the closer locality where *B. freicanecae* occurs (Fig. 2). Garcia and Haddad (2008) characterized *B. ericae*, pointing out some osteological differences with other species of the group. The polarity of these characters is still unknown.

4.4 The rejection of the previously hypothesized Boana polytaenia *Clade*

Faivovich et al. (2004) commented that the monophyly of the *Hyla polytaenia* Group was quite likely based on the absence of any marking (they mistakenly used the term "pattern") on the covered surfaces of thighs and the mostly striped dorsal pattern, and for that reason they recognized the *B. polytaenia* Clade in the *B. pulchella* Group. Faivovich et al. (2005) considered the striped dorsal pattern a putative morphological synapomorphy and stressed that it was homoplastic with *B. bischoffi*, where it occurs in some populations. Although not stated by the authors, they considered the absence of markings on the thighs a plesiomorphy at that level (as it also occurs in *B. curupi*, *B. ericae*, *B. joaquini*, and *B. semiguttata*). Faivovich et al. (2005) included only *B. latistriata*, *B. leptolineata*, and *B. polytaenia* as exemplars of their hypothesized *B. polytaenia* Clade. Recent analyses including these same exemplars in the context of the addition of some newly described species either failed to recover these species as monophyletic (Antunes et al., 2008) or did so with variable support (Lehr et al., 2010; Faivovich et al., 2013; Pinheiro et al., 2019a; Sturaro et al., 2020; Caminer and Ron, 2020; Lyra et al., in press). The present analysis, having included multiple exemplars of all described species, in addition to some still undescribed taxa, and additional sequence data, supersedes all previous tests of relationships in the *B. pulchella* Group, and rejects the

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previously hypothesized *B. polytaenia* Clade. Our results reject grouping *B. goiana* + *B. phaeopleura* with *B. polytaenia* and its closest relatives, and recovers them instead in the phylogenetically distant *B. prasina* Clade (Fig. 7), as the sister taxon of *B. guentheri*, a species from the Atlantic slopes of the Serra do Mar in the states of Rio Grande do Sul and Santa Catarina (Supp. Data 5). For that reason, we exclude those two species from our redefined *B. polytaenia* Clade, which now is restricted to the described species *B. bandeirantes*, *B. beckeri*, *B. botumirim*, *B. buriti*, *B. cipoensis*, *B. jaguariaivensis*, *B. latistriata*, *B. leptolineata*, *B. polytaenia*, and *B. stenocephala* (but see below).

4.5 The redefined Boana polytaenia *Clade*

Species of the now redefined *B. polytaenia* Clade have a broad distribution in the Atlantic Forest of southeastern Brazil from the State of Rio Grande do Sul to Espírito Santo, and westwards in forested and non-forested regions reaching the central plateaus in the states of Goiás, Minas Gerais, and the Distrito Federal (Fig. 2, Supp. Data 5). The relationships within this clade reveal a number of taxonomic problems involving the presence of highly divergent lineages in some species and very shallow divergence among others.

Boana leptolineata, the southern-most distributed member of the clade, is the sister taxon of *B. jaguariaivensis* (100% jackknife), and together they are the sister taxon of all other species of the *B. polytaenia* Clade (Figs. 1, 3), whose grouping is weakly supported (60% jackknife). The recently described *B. jaguariaivensis* shares the presence of thin light brown lines on a dark brown background with *B. leptolineata*. The samples of *B. leptolineata* include individuals (Supp. Data 5) from municípios of São Domingos and Campo Belo do Sul, State of Santa Catarina, and from Cambará do Sul, State of Rio Grande do Sul, Brazil (type locality). The 16S p-distances between samples from both

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areas are 1.4–2.8% (Tab S4.1). Our study of the vouchers and other material so far fails to show any significant morphological difference. A clade comprising *B. botumirim* and *B. cipoensis* (98% jackknife), from the Serra do Espinhaço mountain range in Minas Gerais, Brazil (Supp. Data 5), represents a deep and poorly supported (from < 50% to 60% jackknife) split within the *B. polytaenia* Clade.

Populations attributed to *B. polytaenia* occur at multiple, phylogenetically disparate places within the *B. polytaenia* Clade (Fig. 3). One clade includes sister taxa showing species-level divergence from the outskirts of Belo Horizonte, Minas Gerais (Belo Horizonte, Bom Jesus de Amparo, Congonhas do Campo, Muriaé, Ouro Branco, Santana do Riacho), and the other one from Espírito Santo (Domingos Martins, Pedra Menina) and adjacent Minas Gerais (Caratinga; see Supp. Data 5). All these populations have been considered *B. polytaenia* in the literature (e.g., Lutz, 1973; Cruz and Caramaschi, 1998; Montesinos et al., 2012; Pinheiro et al., 2012). The 16S p-distances between the lineages are 2.31–3.57% (Tab. S4.2). The remaining samples of *B. polytaenia* do not form a monophyletic group, but they are part of a well-supported clade (96% Jackknife) that also includes *B. bandeirantes, B. becker*i, and *B. latistrata*. This clade comprises sister taxa from Serra da Mantiqueira and Serra do Mar (Fig. 3; Supp. Data 5). The 16S p-distances between this latter clade and the clade of the *B. polytaenia* populations described above are 2.50–4.46% (Tab. S4.3). The fact that the clade with specimens historically identified as *B. polytaenia* from the Serra da Mantiqueira and the Serra do Mar is only distantly related to the remaining *B. polytaenia* (Fig. 3) indicates that this name is not applicable to a single species distributed from Rio de Janeiro westwards to Belo Horizonte, as assumed by Cruz and Caramaschi (1998), but instead that it has been applied to a non-monophyletic assemblage (Fig. 3; see discussion below). The Cerrado inhabitants *B. buriti* and *B. stenocephala* form a clade with the combined specimens from

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Serra da Mantiqueira and Serra do Mar (jackknife 99%), but then monophyly of the latter and *B. stenocephala* is poorly supported (59% jackknife). All these topological results are congruent in the parsimony and ML analyses, but in the latter the bootstrap value is lower for the Serra do Mar + Serra da Mantiqueira clades (80% BS).

The Serra do Mar Clade (98% jackknife) is composed of all specimens from the Serra do Mar mountain range, from SW São Paulo to NE Rio de Janeiro (Fig. 3; Supp. Data 5). This clade includes the topotypes of the recently described *B. bandeirantes*—for which Caramaschi and Cruz (2013) only employed material from the State of São Paulo—and topotypes of *Hyla striata* Peters, 1872 (type locality "Neu-Friburg in Brasilien", Nova Friburgo, Rio de Janeiro, Brazil), currently a junior synonym of *B. polytaenia* (Boulenger, 1882; Cochran, 1955). The populations from the Serra do Mar in Rio de Janeiro (Teresópolis, Petrópolis, and Nova Friburgo) were previously considered to be *B. polytaenia* by Cruz and Caramaschi (1998), and implicitly by Caramaschi and Cruz (2013).

The Serra da Mantiqueira Clade (99% jackknife) includes all exemplars of the *B. polytaenia* Clade from the Serra da Mantiqueira mountain range and associated formations in the states of São Paulo, Minas Gerais, and Rio de Janeiro (Fig. 3; Supp. Data 5). It includes topotypes of *B. beckeri*, *B. latistriata*, and multiple exemplars of the populations that have been considered *B. polytaenia* in Serra da Mantiqueira. The 16S p-distances are low within each of these clades (0.00–1.96% in the Serra do Mar Clade; 0.00–0.71% in the Serra da Mantiqueira Clade), and genetic differentiation between the two clades (0.07–1.96%) largely overlaps values found within each clade (Tab. S4.3).

Although the topotypic samples of *B. beckeri* and *B. latistriata* are each monophyletic (even if topotypes of the latter and the population from Monteverde, Minas Gerais are not closely related), in general the level of sequence differentiation and support

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is low (maximum of 1.96 and 1.78% respectively in 16S; Tab. S4.3) and they are nested within the specimens of *B. polytaenia* from the Serra da Mantiqueira Clade (Fig. 3). The most notable differences among topotypic samples of *B. beckeri* and *B. latistriata* involve snout-vent length (SVL): Caramaschi and Cruz (2004) reported an SVL of 34.9–40.6 mm in males and 40.9–51.6 mm in females of *B. latistriata*, whereas for the type series of *B. beckeri* they reported 24.2–29.0 for males (n=12) and 32.0–33.9 mm (n=3) for females (SVL of topotypic vouchers studied by us for both species fall within the ranges informed by Caramaschi and Cruz, 2004). Interestingly, among the vouchers of *B. polytaenia* there is significant SVL variation, 23.8–37.0 (males; n=75) and 39.1–48.5 (females; n=8), suggesting that the large, characteristic SVL of the topotypic populations of *B. latistriata* or the small size of *B. beckeri* are not unique, and that there are notable differences in SVL within this clade.

The internal composition and the close relationship between the Serra do Mar and the Serra da Mantiqueira clades raises taxonomic and nomenclatorial problems: How many species are actually represented? What name or names should be applied to them? The first problem is an obvious consequence of the reduced internal 16S p-distances among all specimens of the Serra da Mantiqueira Clade, and, in turn, the relatively reduced p-distances between this and the Serra do Mar Clade. Moreover, there is no evident genetic differentiation between clades with respect to the internal p-distances observed within each clade. This problem is magnified by the complex taxonomy of species included in the *B. polytaenia* Clade and the ambiguity of most characters employed to differentiate *B. bandeirantes*, *B. beckeri*, *B. latistriata*, and *B. polytaenia*. The study of material of these species, including specimens of the last three that became available in collections in the last decade and a half, provides a more thorough perspective on their variation, reducing the diagnostic value of most if not all characters that have

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been used in the taxonomy of these four species (see Supp. Data 6). These data in combination with low 16S p-distances, suggest that *B. bandeirantes* (Caramaschi and Cruz, 2013), *B. beckeri* (Caramaschi and Cruz, 2004), *B. latistriata* (Caramaschi and Cruz, 2004), *Hyla striata* Peters, 1872, and all populations from the Serra do Mar and Serra da Mantiqueira and associated geological formations historically considered *B. polytaenia* correspond to a single species, with genetically structured populations along its geographical distribution. We are aware that the lower bootstrap support obtained in the ML analysis for this clade (80% BS) weakens its recognition as a single species. However, considering all the other evidence, at this point we find it to be the most reasonable course of action.

Cruz and Caramaschi (1998), in their taxonomic revision of the then *Hyla polytaenia* Cope, 1870 and some related species, established that the type locality of this species, only expressed as "Brazil" by Cope (1870), should be somewhere between Rio de Janeiro and Belo Horizonte, the area within the known distribution of the species that was included in the itinerary of the Thayer Expedition. The authors did not study the syntypes of the species but stated that it was the only species known to occur in that area. Our results indicate that populations from two distant clades (Fig. 3), representing at least two species are present in that area: those from the clade from the outskirts of Belo Horizonte, and those from the clade including all populations from the Serra do Mar, and Serra da Mantiqueira and associated formations. Therefore, the lineage to which the name *B. polytaenia* should be applied requires a careful reassessment.

José Rosado (MCZ, Harvard University) kindly provided photographs and information from the two syntypes of *B. polytaenia*. Both syntypes are relatively well preserved (Supp. Data 6: Fig. S1A–B); MCZ A 128772 (SVL 27.1 mm) seems to be an immature female and MCZ A 1544 (SVL 30.9 mm) is a male as revealed by the slightly

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expanded vocal sac. Unfortunately, the diagnostic morphological characters separating the clade from the Serra do Mar and Serra da Mantiqueira and associated formations, from the clade of the outskirts of Belo Horizonte, are very limited when considering the individual variation observed in the large number of specimens accumulated in collections in the last 20 years (see Supp. Data 6). However, a survey of snout-vent length of 265 adult males indicates that the male syntype is larger than the SVL range from all surveyed specimens from populations included in the clade of the outskirts of Belo Horizonte (22.0–28.6 mm; $n = 101$), and falls instead in the large range of SVL recorded for males of the populations from Serra do Mar and Serra da Mantiqueira and associated formations (23.8–40.4; $n = 164$).

Although there are detailed collecting points of the Thayer Expedition (e.g., Dick, 1977), the records for several amphibian specimens is notably incomplete. The collector of the syntypes of *B. polytaenia*, George Sceva, spent his time in Brazil traveling only in the states of Rio de Janeiro and Minas Gerais. Some indirect evidence (collection dates for insects in the online data base of the Museum of Comparative Zoology, Harvard University) suggests that he might have spent a considerable amount of time in Cantagalo, State of Rio de Janeiro, including the complete austral Spring-Summer season of 1865–1866. In the absence of any other relevant information regarding the point where the syntypes were collected, our inability to diagnose the two distant clades that had been associated with the name *B. polytaenia*, the fact that the snout-vent length of the adult male syntype does fall within the range of lengths recorded for males of the populations from Serra do Mar and Serra da Mantiqueira and associated formations, and considering that most literature associated with this name has been related to specimens from the states of Rio de Janeiro and São Paulo, we associate the name with the clade that includes the populations from Serra do Mar, Serra da Mantiqueira and associated formations. Further

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taxonomic knowledge and/or access to DNA sequences of the syntypes would allow testing this hypothesis. In this context, *B. bandeirantes* (Caramaschi and Cruz, 2013), *B. beckeri* (Caramaschi and Cruz, 2004), and *B. latistriata* (Caramaschi and Cruz, 2004), are here considered junior synonyms of *B. polytaenia* (Cope, 1870). The status of *Hyla striata* Peters, 1872 as another junior synonym of *B. polytaenia*, as suggested by Boulenger (1882) and Cochran (1955) is also corroborated (Fig. 3; Supp. Data 6: Figs. S1C–D). The association of the name *B. polytaenia* with these populations indicates that the lineage including populations from Espírito Santo and adjacent Minas Gerais, and the one from the outskirts of Belo Horizonte, Minas Gerais, should be recognized as different, still undescribed species, which we call *Boana* sp. 3 and *Boana* sp. 4, respectively (Fig. 3).

There are no phenotypic synapomorphies known for the *B. polytaenia* Clade. Putative morphological synapomorphies could include the conspicuous striped pattern, which is homoplastic, as it occurs in some populations of *B. bischoffi* (e.g., Heyer et al., 1990; Marcelino et al., 2009), in *B. goiana* and *B. phaeopleura* (Lutz, 1968; Cruz and Caramaschi, 1998; Caramaschi and Cruz, 2000), and in *B. caingua* (Carrizo, 1991). Another putative synapomorphy could be the reduction from four to three posterior labial tooth rows in the larval oral disc. Larvae of *Boana* have a diversity of labial tooth row formulae (see Kolenc et al., 2008, for a review). The taxonomic distribution of the presence of a fourth posterior labial tooth row in larvae of the *B. faber* Group (Kolenc et al., 2008) as well as its presence in *B. cambui*, *B. ericae*, and *B. freicanecae* (Carnaval and Peixoto, 2004; Pinheiro et al., 2016; T.L. Pezzuti and R.A. Brandão, pers. comm.), all known larvae of the *B. balzani*, *B. riojana*, and *B. semiguttata* clades (Duellman et al., 1997; Lötters et al., 1999; Lehr et al., 2011; Faivovich, 1996; Garcia et al., 2003, 2007, 2008; Antunes et al., 2008; Widholzer and Castroviejo 2018; polymorphic in *B. marginata* and *B. riojana*, Lavilla, 1984; Garcia et al., 2001; Kolenc et al., 2008), indicates that it is

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plesiomorphic for the *B. pulchella* Group. All known larvae of the *B. polytaenia* Clade have only three posterior labial tooth rows (Heyer et al., 1990; Eterovick et al., 2002; Both et al., 2007; Orrico et al., 2007; Pinheiro et al., 2012). The loss of the fourth posterior tooth row has occurred homoplastically in the group, as it is also absent in larvae of the *B. prasina* Clade (Heyer et al., 1990; Kolenc et al., 2008; Almeida-Silva et al., 2016).

Boana botumirim, *B. buriti*, *B. cipoensis*, and *B. jaguariaivensis* are Cerrado inhabitants (Caramaschi et al., 2009, 2010; Braga et al., 2010; Supp. Data 5), *B. stenocephala* is known to occur both in Cerrado and Atlantic Forest environments (Haddad et al., 1988 [as *Hyla cipoensis*]; Tolledo Santos et al., 2009), and *B. leptolineata*, *B. polytaenia*, *Boana* sp. 3 and *Boana* sp. 4 are Atlantic Forest inhabitants (Lutz, 1973; Kwet et al., 2010; Supp. Data 5). Available information on the biology of the *B. polytaenia* Clade indicates that the species from the Cerrado and Atlantic Forest use similar reproductive habitats (Lutz, 1973; Menin et al., 2004; Tolledo Santos et al., 2009; Kwet et al., 2010).

4.6 The Boana semiguttata *Clade*

Faivovich et al. (2004) identified a clade then composed of *B. curupi* (as *Hyla* sp. 1; Garcia et al., 2007), *B. joaquini*, and *B. semiguttata*, with which *B. caipora* was associated in a subsequent phylogenetic analysis by Antunes et al. (2008). This clade contains a number of species inhabiting streams in the forest and open areas in the southern sector of the Atlantic Forest, from southern State of São Paulo to northern Rio Grande do Sul and westwards to Misiones, Argentina and extreme eastern Paraguay (Brusquetti and Lavilla, 2006; Garcia et al., 2007; Antunes et al., 2008; Fig. 2; Supp. Data 5).

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Our analyses included multiple specimens of the already available species, as well as from *B. stellae*, which has been related to species of this clade by Kwet (2008), and two undescribed species from Santa Catarina, Brazil (*Boana* sp. 1 and *Boana* sp. 2). Our results (Figs. 1, 4) are congruent with previous hypotheses (Faivovich et al., 2004, 2005, 2013; Antunes et al., 2008; Lehr et al., 2010) and further indicate that *B. stellae* is the sister taxon of *B. joaquini* + *B. curupi*, and that *Boana* sp. 1, is the sister taxon a clade that includes a polytomy with *B. caipora*, *B. semiguttata*, and *Boana* sp. 2. All resolved relationships among these species are well-supported (87–100% jackknife), and all of them form together the sister taxon of *B. poaju* (100% jackknife).

Garcia et al. (2007), based on variation in SVL and color pattern, expressed doubts regarding the taxonomic status of populations of *B. joaquini* from Cambará do Sul, Canela, Bom Jesus, and São José dos Ausentes (State of Rio Grande do Sul), and the ones from São Francisco de Paula (State of Rio Grande do Sul). Specimens sampled by us from Cambará do Sul and some from São Francisco de Paula are recovered nested in the same clade together with topotypes of *B. joaquini* from Urubici (Fig. 4), suggesting that they are conspecific. *Boana joaquini* shows significant phenotypic variation, and a considerable amount of genetic diversity (p-distances 0.00–1.29%; Tab. S4.4).

The smaller size and polymorphic color pattern led Garcia et al. (2007) to consider the populations from Ponta Grossa and Palmeira (State of Paraná) as *Hypsiboas* sp. (aff. *semiguttatus*). The sample that we included from Palmeira is recovered with 100% jackknife as the sister taxon of all remaining specimens of *B. semiguttata* (Fig. 4). The 16S p-distances between this and all other specimens of *B. semiguttata* are 0.89–1.42% (Tab. S4.4), and we consider that they are the same species. Interestingly, the specimens from Piraquara (State of Paraná, ca. 100 km straight line from Palmeira) are more closely related to populations from Santa Catarina and Rio Grande do Sul (Fig. 4; Supp. Data 5).

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The taxonomy of the species in the *B. semiguttata* Clade is quite complex, and the high genetic diversity, coupled with the problems in delimiting species, indicate that we are still far from understanding its diversity.

The information on chromosome morphology of species in the *B. pulchella* Group is scarce (Barale et al., 1991; Ananias et al., 2004; Raber et al., 2004; Baraquet et al., 2013; Ferro et al. 2018). However, these data indicate that *B. curupi*, *B. joaquini*, *B. semiguttata*, and *B. stellae* share a Nucleolar Organizer Region (NOR) on the telomere of chromosome pair 1 (Ananias et al., 2004; Ferro et al., 2018). This character state is shared with *B. cipoensis*, the only species of the *B. polytaenia* Clade cytogenetically studied so far (Ferro et al., 2018). The other species of the *B. pulchella* Group with known karyotype have a NOR on chromosome pair 11 (*B. bischoffi*, *B. cordobae*, *B. guentheri*, *B. marginata*, *B. marianitae*, *B. prasina*, and *B. riojana*; Ananias et al., 2004; Raber et al., 2004; Ferro et al., 2018) or pair 12 (*B. caingua*, *B. prasina*, and *B. pulchella*; Ananias et al., 2004; Ferro et al., 2018). Among outgroups, NORs have been reported mostly on pair 11 (see Mattos et al., 2014, and Ferro et al., 2018 for recent reviews). Ferro et al. (2018) noted that NOR on pair 11 is plesiomorphic in the *B. pulchella* Group, whereas their presence on pair 1p is a putative synapomorphy of the *B. polytaenia* + *B. semiguttata* clades (although no data are available for the *B. balzani* Clade, *B. ericae*, *B. freicanecae*, and *B. cambui*). The NOR on pair 12q is a synapomorphy of the clade including *B. caingua*, *B. cordobae*, *B. prasina*, and *B. pulchella*, with a reversal to NOR on pair 11 in *B. cordobae* (Ferro et al., 2018).

An unexpected result is the highly supported sister-taxon relationship of *B. poaju* with all species from this clade, and that species being only distantly related to *B. marginata* (Figs. 1, 4). The morphological similarity of these two species has been noticed by Garcia et al. (2008), who also showed that Mertens (1952) even confused them. Garcia

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et al. (2008) further compared *B. poaju* to the species with which *B. marginata* has been related in previous analyses (Faivovich et al., 2004, 2005; Antunes et al., 2008: *B. bischoffi* and *B. guentheri*). The similarities among *B. marginata* and *B. poaju* include the transparent green coloration due to impregnation with biliverdin (Barrio, 1965b) and the white membranes on the parietal peritonea visible through the transparent flank. These character states occur as well in *B. guentheri*, which differs in having a more slender body.

4.7 Central Andean Clade: the Boana balzani *Clade*

Faivovich et al. (2004) identified a clade that included all their exemplar species with Andean distribution, *B. callipleura* (as *H. balzani*), *B. marianitae*, and *B. riojana*. Based on their similarities and distributions, they tentatively considered related to these, the other Andean species that were not available at that time, *B. albonigra*, *B. melanopleura*, and *B. palaestes*. Including all the relevant data from Faivovich et al. (2004, 2005), Lehr et al. (2010) rejected the hypothesized monophyly of the Andean species, as they identified a second clade including *B. aguilari*, *B. melanopleura*, and, tentatively, *B. palaestes*. Almost simultaneously, Köhler et al. (2010), described a new Andean species, *B. gladiator*, and established the correct identity of *B. balzani*. They further added sequences of these species, *B. palaestes*, and multiple terminals of most other Andean species (with the exception of *B. albonigra*, *B. aguilari*, and *B. melanopleura*, but see below), and performed a phylogenetic analysis using a fraction of the sequences of Faivovich et al. (2004).

Our results are congruent with those of Lehr et al. (2010), Faivovich et al. (2013), Pinheiro et al. (2019a), and Lyra et al. (in press) and indicate that *B. aguilari*, *B. balzani*, *B. gladiator*, *B. melanopleura*, and *B. palaestes* form another well-supported Andean clade, which we call the *B. balzani* Clade (Figs. 1, 2, 5). The nominal species and *B.*

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melanopleura form a clade (100% jackknife), which is the sister taxon of the remaining species (100% jackknife), with *B. aguilari* + *B. palaestes* being the sister taxon of *B. gladiator* (96% jackknife; Fig. 5).

Its species occur in the central Andes from NW Bolivia and SE Peru to Central Peru, all between 400–2200 a.s.l. (Fig. 2; Supp. Data 5). Their taxonomy is complex, as shown by the fact that thev distantly related *B. callipleura* until recently has been considered a junior synonym of *B. balzani*. Köhler et al. (2010) could only advance vocalization structure and sequence data as convincing diagnostic evidence between both species. *Boana balzani* remains without a diagnosis in the context of the other species included in the *B. balzani* Clade. Our results show low p-distances between *B. aguilari* and *B. palaestes* (1.74%), and in general the level of variation in these species and *B. gladiator*, is much lower than in the sister taxon, *B. balzani* + *B. melanopleura* (Tab. S4.5).

Boana aguilari and *B. gladiator* were described almost simultaneously and only their tadpoles have been compared in the literature (Köhler et al., 2010; Lehr et al., 2010, 2011). When described, both species were diagnosed particularly from *B. melanopleura* and *B. palaestes*. In comparisons with the former, Köhler et al. (2010) primarily advanced characters related to coloration pattern differentiating it from *B. gladiator*, which they explicitly considered tentative. Lehr et al. (2010), besides color pattern, added the occurrence of dorsolateral folds, and the shape of the prepollical spine as differences between *B. melanopleura* and *B. aguilari*. Although they had available advertisement calls recorded in captivity from both species, these did not show significant differences. Lehr et al. (2011) mentioned some differences in tadpole coloration between *B. aguilari* and *B. palaestes*, and noticed differences in labial tooth row formulae (LTRF) between these [2 (2)/4 (1)] and *B. gladiator*. For the latter they extracted the information from the

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description provided by Duellman et al. (1997, as *Hyla balzani*), informing LTRFs 3 (1,3) /4 (1) and 2 (2)/3 (1). However, Duellman et al. (1997) also inform LTRF 2 (2)/4 (1) as occurring in some individuals in developmental stages comparable to those described for *B. aguilari*.

Adults of *B. aguilari* and *B. gladiator* were diagnosed from *B. palaestes* based on minor differences in color pattern, but mostly on the remarkably different advertisement call described for this species by Duellman et al. (1997). As discussed in Supp. Data 6, the described call actually belongs to a centrolenid frog, explaining the remarkable differences that were pointed out in the diagnoses of *B. aguilari* and *B. gladiator*. The actual call of *B. palaestes* (Supp. Data 6 and fig. S3) is certainly more reminiscent of those of other species in the *B. balzani* Clade in terms of frequency spectra and pulsed structure. While in general there is lack of knowledge on variation of calls in these species and precise social context of the recordings, the information available on the calls of *B. gladiator* indicates clear differences in number of pulses per note and note duration in comparison with *B. palaestes*. The differences in call structure with the calls of *B. aguilari* are less clear. The call briefly described by Lehr et al. (2010) is from a specimen in captivity. Although the pulses are poorly defined in *B. aguilari* (Lehr et al., 2010: fig. 4), the number of pulses (15–26 in *B. aguilari*; 7–32 in *B. palaestes*) and frequency parameters overlap (dominant frquency 944–1086 Hz in *B. aguilari*; 861–1550 Hz in *B. palaestes*); the pulse rate is continuous (50–60 pulses/sec in *B. aguilari*; 20–49 pulses/sec in *B. palaestes*, see Supp. Data 6). The audiospectrograms, however, look quite different (compare Lehr et al., 2010: fig. 4 with Supp. Data 6: Fig. S3). It would be necessary to have calls of *B. aguilari* recorded in the field to produce a proper comparison with those of *B. palaestes*. From our perspective this would be the only evidence preventing recognizing *B. aguilari* as a junior synonym of *B. palaestes*. Considering that *B. aguilari*, *B. gladiator*, and *B. palaestes* are

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distributed along the eastern slope of the Andes, from Central Peru to Southeast Peru (Supp. Darta 5), spanning nearly 600 km (straight line) between extreme known localities, we find that more sampling effort and studies are necessary to evaluate their variation and to reassess their taxonomic status.

4.8 Southern Andean Clade: the Boana riojana *Clade*

We recognize as the *B. riojana* Clade the Andean clade of Faivovich et al. (2004, 2005), which includes species distributed from Northwest Bolivia to the Andes in the Province of La Rioja, in Argentina (Figs. 1, 2, 5). Faivovich et al. (2004) included on their Andean clade *B. callipleura* (as *H. balzani*), *B. marianitae*, *B. riojana* (and its junior synonym *Hyla andina*), and tentatively, because they lacked samples, *B. albonigra* and the species that were subsequently shown to belong to the *B. balzani* Clade. Relationships in the *B. riojana* Clade are congruent with those supported by the analyses of Faivovich et al. (2004, 2005) and subsequent reanalyses (Wiens et al., 2010; Faivovich et al., 2013), with the forest-dwelling *B. callipleura* and *B. marianitae* being sister taxa, and in turn the sister taxon of *B. riojana* (Fig. 6). Our results show *B. albonigra* nested within this clade (Fig. 6).

Koscinsky et al. (2008) presented a phylogeographic analysis of the Argentinean populations of the former *B. andina* based on the mitochondrial control region of 257 samples and, for a subset of 33 samples, a fragment of *CytB*. In their results, 26 specimens identified by them as *B. andina*, apparently solely based on previous assertions regarding its geographic distribution (Koscinsky et al., 2008), form a monophyletic group with their only sample identified as *B. riojana*. Problems in distinguishing both taxa had already been mentioned by Faivovich et al. (2004) and Koscinsky et al. (2008). These latter authors and Köhler et al. (2010) further considered that the only distinguishing feature

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tentatively advanced by Faivovich et al. (2004), the absence of the dashed white or cream dorsolateral stripes that begin behind the eyes, was a poor taxonomic character and in the absence of other data considered *Hyla andina* Müller, 1924 as a junior synonym of *H. riojana* Koslowsky, 1895.

Our results recover two well-supported lineages including terminals of *B. riojana*; the "central lineage" that includes specimens from Catamarca, Argentina (including a topotype of *Hyla andina*), northwards to southern Bolivia; and the "austral lineage" includes specimens from the provinces of Catamarca, and La Rioja, Argentina (Fig. 6; Supp. Data 5). These two lineages largely correspond to those identified by Koscinsky et al. (2008) and Köhler et al. (2010). The austral lineage also includes some populations from Tucumán as shown by Koscinsky et al. (2008) employing mitochondrial control region (D-loop) sequences. The 16S p-distances within this lineage range from 0.00 to 0.78% (Tab. S4.6). Internal p-distances in the central lineage range from 0.00 to 1.06% (Tab. S4.6). The p-distances between these lineages are 0.36–1.75%, overlapping with the internal distances and indicating no noticeable genetic differentiation between them (Tab. S4.6).

The austral and central lineages are the well-supported sister taxon of a clade including specimens tentatively identified as *B. riojana* by Köhler (2000) and Köhler et al. (2010) from Cochabamba and Santa Cruz de la Sierra, Bolivia, with the available specimens of *B. albonigra* nested among them and not monophyletic (Fig. 6). The study of some vouchers of the specimens tentatively identified as *B. riojana* (MNCN 42732, 42571, 42572, 42573) and the available photos (De la Riva et al., 2000; Köhler, 2000: pl. II H; Köhler et al. 2010: fig. 3p–r) indicate that their external morphology and color pattern are within the known, extreme variation of *B. riojana*. *Boana albonigra* is a poorly known species from a few localities in the highlands of southern Bolivia and extreme

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Northwest Argentina (Duellman et al., 1997; Ferro et al., 2018; Supp. Data 5). This species was diagnosed from *B. riojana* (as *Hyla andina*) for having flanks with dark vertical bars (small dark spots in *B. riojana*), unmarked ventral surface of the shank (small dark spots in *B. riojana*), width of disc on Finger III equal to tympanum diameter (smaller in *B. riojana*), an elevated tarsal fold, skin on dorsum coarsely granular, and the absence of a white supracloacal stripe (Duellman et al., 1997). Our study of multiple specimens of *B. riojana* (see Pinheiro et al., 2019b: supp. data) indicates that from the list of diagnostic characters of *B. albonigra*, only the occurrence of dark vertical bars on flanks seems to be indeed unique for the populations identified as this species. All other characters occur polymorphically in the studied populations of *B. riojana*. The internal genetic distances (Tab. S4.6) in this clade are 0.00–1.89%, while the distances between specimens originally identified as *B. albonigra* and *B. riojana* are 0.18–1.34%, suggesting that dark vertical bars on flanks could be considered a polymorphic color pattern.

While the association of the two lineages originally identified as *B. riojana* with this species is not controversial, the identity of the other lineage requires some discussion. As reported above, the voucher specimens of the terminals included in this lineage fall within the important phenotypic variation of *B. riojana*, which is also evident in the other two lineages at the level of adult morphology (Duellman et al., 1997; Köhler et al., 2010; Pinheiro et al., 2019b), larval morphology (Lavilla, 1984), and vocalizations (Köhler et al., 2010). The 16S p-distances between this lineage and the other two lineages are 1.42–2.97% (Tab. S4.6). The internal genetic variation in that lineage reaches 1.89%, well above the maximum 1.06% in the other lineages (Tab. S4.6). While it is tempting to apply the name *B. albonigra* to this lineage, as discussed above, we find that there is no phenotypic evidence supporting its status as a distinct species, and the genetic distances do not provide any conclusive support either. Recognizing that these are highly variable

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frogs, for the time being, we associate this lineage to *B. riojana*, and we consider *Hyla albonigra* Nieden, 1923 as a junior synonym of *Hyla riojana* Koslowsky, 1895.

Faivovich et al. (2004, 2005) first noticed that their Andean Clade was nested in a clade of mostly Atlantic Forest species, a pattern at that time unknown in other vertebrate groups. Percequillo et al. (2011) noticed a similar pattern in sigmodontine rodents of the genera *Drymoreomys* and *Eremoryzomys*, and pointed to phylogenetic studies of the genera *Akodon* (Smith and Patton, 2007) and *Rhagomys* (Luna and Patterson, 2003) where there are sister-group relations between Andean and Atlantic Forest clades. A similar pattern was recovered in phylogeographic studies of passerine birds (Trujillo-Arias et al., 2017, 2018; Cabanne et al., 2019). The results of Lehr et al. (2010) revealed an intriguing pattern where there are actually two Andean clades, called here the *B. balzani* and the *B. riojana* clades. In the context of our results, where the relationships of the *B. balzani* Clade with other major clades of the *B. pulchella* Group are poorly supported, it is unclear if the *B. balzani* and *B. riojana* clades represent a single Andean radiation, or two independent contacts between the Andean and the Atlantic Forest biotas. Species from the *B. balzani* and *B. riojana* clades are mainly forest inhabitants (Duellman et al., 1997; Köhler et al., 2010; Lehr et al., 2010). The exception is *B. riojana*, which besides forests also inhabits drier areas, particularly on the Southern limits of its distribution, and dry, highland areas (up to 4500 m.a.s.l.) in Southwest Bolivia and extreme Northwest Argentina, being among the highest elevation records for hylids.

4.9 The Boana prasina *Clade*

We recognize as the *B. prasina* Clade the well-supported clade including *B. bischoffi*, *B. caingua*, *B. cordobae*, *B. goiana*, *B. guentheri*, *B. marginata*, *B. phaeopleura*, *B. prasina*, and *B. pulchella* (Fig. 1, 7). Relationships within this clade are in general

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congruent with previous tests, and our analysis introduces a number of novel results involving relationships, taxonomy, and suggesting more diversity than previously thought. In terms of relationships, the most notable addition (Fig. 1, 7) is the clade composed of *B. goiana* + *B. phaeopleura*, former members of the *B. polytaenia* Clade (see above), which is the sister taxon of *B. guentheri*, but this relationship is poorly supported (< 50% jackknife), as is the monophyly of this clade with *B. bischoffi* + *B. marginata* (< 50% jackknife). That result implies a clade of Cerrado inhabitant species (Supp. Data 5) nested in a larger clade whose other members are from the distant Atlantic Forest clade (at least 800 km straight line). At the same time, it raises interesting questions regarding the origin of the striped pattern, which in the past supported the association of these species with the *B. polytaenia* Clade. Notably, *B. guentheri* was considered related to the former *Hyla polytaenia* Group by B. Lutz (1973) and Braun and Braun (1977) based on the vivid coloration on hidden surfaces of the thigh and possibly the striped dorsal pattern (all species treated by Lutz [1973] as "elongate species with red flash colors" have a striped dorsal pattern). Remarkably, there are some "green phases" of *B. guentheri* (Kwet et al., 2010) that lack any dorsal stripe and show the same coloration as *B. marginata* and *B. poaju*.

Boana phaeopleura was considered more closely related to *B. goiana* than to other species of the former *B. polytaenia* Group by Caramaschi and Cruz (2000), based on the absence of a supracloacal crest, absence of a calcar tubercle, large discs, and dorsal color pattern. They differentiated the new species from the type series of *B. goiana* using details of color pattern in the dorsum and limbs. Although the polarity of most characters employed to associate both species is unclear, our results indicate that they are monophyletic, and that 16S p-distances among topotypes of the two species are quite reduced (0.72%; Tab. S4.7). Samples of *B. phaeopleura*, including topotypes, form a clade

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nested within *B. goiana*, being more closely related to a specimen from São João D'Aliança in Goiás (type locality), than to a clade of *B. goiana* including specimens from southern localities in the states of Goiás and Minas Gerais (the 16S p-distances between both clades is 1.43–2.68%; Tab. S4.7). A study of some specimens indicates that the diagnostic characters employed to differentiate these species are quite variable (see Supp. Data 6). The available information on advertisement calls of both species indicates no substantial differences (Guimarães et al., 2001; Menin et al., 2004; Pinheiro et al., 2012), to the point that Pinheiro et al. (2012) suggested that a taxonomic reassessment was required. These facts, coupled to the relatively reduced 16S p-distances suggest that both names actually correspond to a single species; hence *B. phaeopleura* (Caramaschi and Cruz, 2000) is here considered a junior synonym of *B. goiana* (B. Lutz, 1968).

Previous analyses recovered *B. caingua* in different positions in this clade: as the sister taxon of the other included species of this clade (*B. bischoffi*, *B. cordobae*, *B. guentheri*, *B. marginata*, *B. prasina*, and *B. pulchella*; Faivovich et al., 2004), and in a basal polytomy with *B. bischoffi* + *B. guentheri* + *B. marginata* and *B. cordobae* + *B. prasina* + *B. pulchella* (Pinheiro et al., 2019a). Our results (Figs. 1, 7) differ in that *B. caingua* is recovered as the poorly supported sister taxon (61% jackknife) of a new species morphologically similar to *B. prasina* (see next paragraph), and together they are the sister taxon of the clade composed of *B. cordobae*, *B. prasina*, and *B. pulchella*. Most remarkably, in our results *B. caingua* is composed of two well supported clades, one including terminals from São Paulo and Paraná (Brazil), and a point in central Misiones, Argentina, and another clade that includes three samples from southwestern Misiones (Fig. 7). The 16S p-distances between these clades are 1.68–2.14% (Tab. S4.8).

Boana prasina has a broad distribution; from Rio de Janeiro to Rio Grande do Sul, and westwards to Minas Gerais, Brazil. Our sampling included specimens from Minas

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Gerais, Santa Catarina, and São Paulo (Supp. Data 5). Our results show a clade that includes a topotype from Nova Friburgo, Rio de Janeiro, and the samples from northern Santa Catarina, São Paulo, and Minas Gerais (Fig. 7). However, the samples from southern Santa Catarina (Bom Jardim da Serra and Campo Belo do Sul) are recovered as the sister taxon of *B. caingua* (Fig. 7). Our preliminary study of external morphology of the voucher specimens failed to show significant differences between them and *B. prasina*. These populations require further study; in the meantime, we call them *Boana* sp. 5.

Boana bischoffi is characterized by a notable geographic variation regarding its dorsal pattern, with longitudinal stripes, or ranging from disorganized and discontinuous stripes to light gray (Marcelino et al., 2009). This variation has been considered as intraspecific variation (Haddad and Sazima, 1992), or has induced the recognition of these population groups as different subspecies (Lutz, 1973), or even as different species (*Hyla multilineata* Lutz and Lutz, 1939; Heyer et al., 1990). Marcelino et al. (2009) presented an analysis of variation in dorsal pattern, indicating a sharp transition zone between both patterns in southern São Paulo and northern Paraná, Brazil, where they report what they consider intermediate patterns, and failed to find evidence supporting the existence of two different species. Our sampling of *B. bischoffi* includes samples from both the striped (four localities from São Paulo) and unstriped pattern (two localities from Santa Catarina; see Supp. Data 5). Our results (Fig. 7) indicate that the unstriped populations are not monophyletic and are nested within the striped ones.

Boana cordobae is a montane, stream-dwelling species that occurs in the slopes and summits of central Pampean Sierras of the provinces of Córdoba and San Luís, Argentina. We included samples from the eastern and western slopes of the Comechingones, and from the Sierra de San Luís (Supp. Data 5). The 16S p-distances

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among all samples are quite low (0.00–0.18%; Tab. S4.9). Some aspects of the biology of this species have been recently reported (e.g., Baraquet et al., 2015, 2018; Otero et al., 2017).

Boana pulchella has a broad distribution in the Pampean grasslands of Argentina, Uruguay and southern Brazil, and is among the few non-montane species of the group, with the exception of a few populations from the relatively low (< 1000 m a.s.l.) Ventania mountain range in southern Buenos Aires, Argentina (Supp. Data 5). This species is likely among the better studied of the *B. pulchella* Group in terms of its biology. We included 14 exemplars from throughout its distribution and overall our results (Fig. 7) indicate that the only available sample from Santa Catarina, Brazil is the sister taxon of a well-supported clade including all other specimens, having 16S p-distances of 0.89–1.24% with these (Tab. S4.10). These are further divided in a clade that includes the two samples from Uruguay and a clade including samples from Argentina and Brazil.

4.10 The missing species

The only known species of the *B. pulchella* Group that is missing from our analysis is *B. cymbalum*. This species was described by Bokermann (1963) based on three specimens (only two of which were included in the type series; WCAB 9153–9154; currently MZUSP 73697 and 74194) from Campo Grande, Município of Santo André, São Paulo, Brazil. Subsequently a topotype specimen (WCAB 14074; currently MZUSP 106980) was referred by Lutz (1973), and another (WCAB 14075) was apparently given on loan by W.C.A. Bokermann to Avelino Barrio, found in the collection of the Museo Argentino de Ciencias Naturales, and is being returned to MZUSP. Furthermore, there is another specimen from the neighborhood of Nova Manchester, in the city of São Paulo in the MNRJ collection (MNRJ 23778). Lutz (1973) suggested that *B. cymbalum* could be

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conspecific with *B. semiguttata* or *B. pulchella*, two species that are distantly related, which bracket a large fraction of the phylogenetic and morphological diversity of the *B. pulchella* group. Our study of the available specimens of *B. cymbalum* and of the only available recording made by W.C.A. Bokermann on December, 1963 (FNJV 31866) indicates an evident similarity with *B. prasina* and *B. pulchella*, as noticed by Barrio (1965a), but also with *B. cordobae*. The taxonomic status of *B. cymbalum* requires additional studies.

5. Conclusions

The present study complements previous analyses and nearly exhausts the taxonomic sampling of the *B. pulchella* Group, allowing us to recognize five major clades (the *B. balzani*, *B. polytaenia*, *B. pulchella*, *B. riojana*, *B. semiguttata* clades). However, far from being definitive, our results point to a number of weak areas in our knowledge of the phylogenetic relationships and taxonomy of the group. This is exemplified by the lack of support for relationships at various levels, such as some of the major clades or among species in the *B. polytaenia* Clade. As a result of our study, five new junior synonyms are recognized: *B. bandeirantes* (Caramaschi and Cruz, 2013), *B. beckeri* (Caramaschi and Cruz, 2004), and *B. latistriata* (Caramaschi and Cruz, 2004), are junior synonyms of *B. polytaenia* (Cope, 1870); *B. phaeopleura* (Caramaschi and Cruz, 2000) is a junior synonym of *B. goiana* (B. Lutz, 1968); and *B albonigra* (Nieden, 1923) is a junior synonym of *B. riojana* (Koslowsky, 1895). These synonymies reduce the number of valid species included in the group from 38 to 33. However, at least other five species remain to be described. The taxonomy of some species, such as those included in the *B. balzani* and *B. polytaenia* clades, remains challenging. Furthermore, the recent discovery of *B. cambui* (Pinheiro et al., 2016), the new species included in this study, and those revealed by our

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results, indicate that the areas encompassed by the broad distribution of this species group are still far from adequately sampled.

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FIGURE LEGENDS

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FIG. 1. Phylogenetic relationships of the *Boana pulchella* Group. One of the > 15000 most parsimonious trees obtained in the parsimony analysis (23,689 steps; considering gaps as fifth state). The presentation is condensed, omitting multiple samples of the outgroup genera of Cophomantini, species groups of *Boana*, and multiple terminals within each species of the *B. pulchella* Group. The numbers in parentheses indicate number of species included for each outgroup genus and species group. See Supp. Data 2 for outgroup relationships and details of *B. cambui*, *B. ericae*, and *B. freicanecae*, and Figs. 2, 3, 4, 5, and 6 for complete results in the major clades of the *B. pulchella* Group as indicated in the right. The nodes that collapse in the strict consensus have a black circle. Numbers around nodes are jackknife percentages with gaps considered as a fifth state / as missing data. The asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. See Supp. Data 2 for the complete topology.

Fig. 2. Map with the distribution of the samples of the *B. pulchella* Group employed in this study, including *B. cambui*, *B. ericae*, *B. freicanecae*, and all other species condensed as the five clades recognized here: the *B. balzani*, *B. polytaenia*, *B. prasina*, *B. riojana*, and *B. semiguttata* clades. The inset (bottom left) shows South America with a black rectangle delimiting the area of the main map. Abbreviations are: AR: Argentina. BO: Bolivia. BR: Brazil. CL: Chile. PE: Peru. PY: Paraguay. UY: Uruguay. For the coordinates of each sample see Supp. Data 1; for the distribution of each species, see maps on Supp. Data 5.

FIG. 3. Phylogenetic relationships of the *Boana pulchella* Group. Part 1: the *B. polytaenia* Clade. One of the > 15000 most parsimonious trees obtained in the parsimony analysis

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(23,689 steps; considering gaps as fifth state). The nodes that collapse in the strict consensus have a black circle. Numbers around nodes are jackknife percentages with gaps considered as fifth state / as missing data. The asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. The specimens followed by an asterisk (*) are topotypes. See Fig. 1 for a general perspective and Supp. Data 2 for the complete topology. The localities are shortened; see Supp. Data 1 for complete locality data. Abbreviations: Countries: AR: Argentina. BO: Bolivia. BR: Brazil. PE: Peru. UY: Uruguay. Other political units: AL: Alagoas. BA: Buenos Aires. CA: Catamarca. CB: Córdoba. CHQ: Chuquisaca. CO: Cochabamba. CR: Corrientes. CU: Cusco. DF: Distrito Federal. ER: Entre Ríos. ES: Espírito Santo. GO: Goiás. JU: Junín. JY: Jujuy. LP: La Paz. LR: La Rioja. MD: Madre de Dios. MI: Misiones. MG: Minas Gerais. MS: Mato Grosso do Sul. PA: Pasco. PER: Pernambuco. PR: Paraná. PU: Puno. RJ: Rio de Janeiro. RO: Rocha. RS: Rio Grande do Sul. SA: Salta. SC: Santa Catarina. SCZ: Santa Cruz de la Sierra. SJ: San José. SL: San Luis. SP: São Paulo. TA: Tarija. TU: Tucumán. The frog pictures are not to scale.

FIG. 4. Phylogenetic relationships of the *Boana pulchella* Group. Part 2: the *B. semiguttata* Clade. One of the > 15000 most parsimonious trees obtained in the parsimony analysis (23,689 steps; considering gaps as fifth state). The nodes that collapse in the strict consensus have a black circle. Numbers below nodes are jackknife percentages with gaps considered as fifth state / as missing data. The asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. The specimens followed by an asterisk (*) are topotypes. See Fig. 1 for a general perspective and Supp. Data 2 for the complete topology. The localities are shortened; see Supp. Data 1 for complete locality data. See legend of Fig. 3 for abbreviations. The frog pictures are not to scale.

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FIG. 5. Phylogenetic relationships of the *Boana pulchella* Group. Part 3: the *B. balzani* Clade. One of the > 15000 most parsimonious trees obtained in the parsimony analysis (23,689 steps; considering gaps as fifth state). The nodes that collapse in the strict consensus have a black circle. Numbers around nodes are jackknife percentages with gaps considered as fifth state / as missing data. The asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. The specimens followed by an asterisk (*) are topotypes. See Fig. 1 for a general perspective and Supp. Data 2 for the complete topology. The localities are shortened; see Supp. Data 1 for complete locality data. See legend of Fig. 3 for abbreviations. The frog pictures are not to scale.

FIG. 6. Phylogenetic relationships of the *Boana pulchella* Group. Part 4: the *B. riojana* Clade. One of the > 15000 most parsimonious trees obtained in the parsimony analysis (23,689 steps; considering gaps as fifth state). The nodes that collapse in the strict consensus have a black circle. Numbers around nodes are jackknife percentages with gaps considered as fifth state / as missing data. The asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. The specimens followed by an asterisk (*) are topotypes. See Fig. 1 for a general perspective and Supp. Data 2 for the complete topology. The localities are shortened; see Supp. Data 1 for complete locality data. See legend of Fig. 3 for abbreviations. The frog pictures are not to scale.

FIG. 7. Phylogenetic relationships of the *Boana pulchella* Group. Part 5: the *B. prasina* Clade. One of the > 15000 most parsimonious trees obtained in the parsimony analysis (23,689 steps; considering gaps as fifth state). The nodes that collapse in the strict consensus have a black circle. Numbers around nodes are jackknife percentages with gaps

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considered as fifth state / as missing data. The asterisk (*) indicates 100% jackknife. Nodes without values have jackknife < 50%. The specimens followed by an asterisk (*) are topotypes. See Fig. 1 for a general perspective and Supp. Data 2 for the complete topology. The localities are shortened; see Supp. Data 1 for complete locality data. See legend of Fig. 3 for abbreviations. The frog pictures are not to scale.

SUPPLEMENTARY DATA

SUPP. DATA 1. GenBank accession numbers for the sequences employed in this study and locality data of all voucher specimens.

SUPP. DATA 2. Complete topology of the phylogenetic hypothesis of Figs. 1–6.

SUPP. DATA 3. Maximum likelihood tree and results of the Partition Finder analysis.

SUPP. DATA 4. Uncorrected p-distances between the 16S gene fragment sequences of some species discussed in the text.

SUPP. DATA 5. Maps with the distribution of the samples of the *B. pulchella* Group employed in this study, for species of each of the five named clades recognized within the *B. pulchella* Group.

SUPP. DATA 6. Comments on the taxonomy of some species of the *Boana pulchella* Group.

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- The monophyly of the *Boana pulchella* Group is corroborated
- Five well-supported major clades are recognized
- The relationships among the early diverging species are weakly supported
- Five currently recognized species are considered synonyms of other species

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