The Tadpole of *Proceratophrys bigibbosa* (Peters, 1872) (Anura: Odontophrynidae), with a Description of Its Buccopharyngeal Morphology and Proposal of Novel Synapomorphies for the *P. bigibbosa* Species Group

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The Tadpole of *Proceratophrys bigibbosa* (Peters, 1872) (Anura: Odontophrynidae), with a Description of Its Buccopharyngeal Morphology and Proposal of Novel Synapomorphies for the *P. bigibbosa* Species Group

Pedro H. S. Dias[1]

The *Proceratophrys bigibbosa* species group comprises four species and is the only group likely to be monophyletic within the genus. I describe the tadpole of *P. bigibbosa* and its buccopharyngeal morphology. Comparisons among *Proceratophrys* larvae in the light of a phylogenetic tree suggest that a conical vent tube, tail fin originating in the body/tail junction, large A2 gap, and trapezoid median ridge may represent putative synapomorphies for the *P. bigibbosa* group.

THE widely distributed genus *Proceratophrys* currently comprises 40 species of small to medium-sized frogs that inhabit several ecosystems, such as the Amazon and Atlantic forests, savannas, and Chaco, occurring in Argentina, Brazil, and Paraguay (Frost, 2017). The genus was divided into phenetic groups mainly based on aspects of adult external morphology (Lynch, 1971; Izecksohn et al., 1998; Kwet and Faivovich, 2001; Prado and Pombal, 2008). However, recent hypotheses of phylogenetic relationships based on mitochondrial and nuclear DNA sequences suggested the non-monophyly of most of these groups (Amaro et al., 2009; Pyron and Wiens, 2011; Teixeira Jr. et al., 2012; Dias et al., 2013a; Pyron, 2014). The only group recovered as monophyletic was the *Proceratophrys bigibbosa* group (Amaro et al., 2009; Pyron and Wiens, 2011; Teixeira Jr. et al., 2012; Dias et al., 2013a; Pyron, 2014). Species belonging to this group are found in south and southeastern Brazil, Argentina, and Paraguay. They are characterized by a blunt and short snout, post-ocular swellings, and a large marginal row of tubercles on the eyelids (Kwet and Faivovich, 2001). Currently, four species are assigned to the group: *P. avelinoi*, *P. bigibbosa*, *P. brauni*, and *P. palustris*.

Proceratophrys bigibbosa is the largest member of the group. It can be promptly diagnosed by the presence of two well-developed bulbous bony post-ocular swellings and well-developed toe webbing (Kwet and Faivovich, 2001). The species inhabits woodlands of the Araucaria plateau and mountains slopes with subtropical rain forests at altitudes of 300–1200 meters; its distribution ranges from southern Brazil, in the states of Santa Catarina and Rio Grande do Sul, to northeastern Argentina, in the province of Misiones (Kwet and Faivovich, 2001; Santos et al., 2009). Adult males call in shallow streams, during night and occasionally during the day, from September to November (Kwet and Faivovich, 2001).

Despite this species' importance for the taxonomy and systematics of the genus—*Proceratophrys bigibbosa* is the type species of *Proceratophrys* by monotypy—data on its morphology are extremely scarce and no tadpole description has been published. Herein I describe the tadpole of *Proceratophrys bigibbosa* and its buccopharyngeal anatomy.

MATERIALS AND METHODS

Morphological assessment.—Acronyms are in accordance with Sabaj (2016). The description is based on 15 tadpoles in Gosner (1960) stages 30–37. External morphology characters are in accordance with the terms of Altig and McDiarmid (1999) and Altig (2007). Tadpoles were measured to a 0.1 mm accuracy with a digital caliper, following Altig and McDiarmid (1999) and Altig (2007): total length (TL), body length (BL), tail length (TAL), body width (BW), body height (BH), tail height (TH), nostril to snout distance (NSD), eye to snout distance (ESD), interorbital distance (IOD), eye to nostril distance (END), internarial distance (IND), oral disc width (ODW), and eye diameter (ED). Lateral line system terminology follows Schlosser (2002).

One specimen (Gosner stage 27) was used for describing the buccopharyngeal cavity; it was dissected according to procedures in Wassersug (1976) and submitted to the protocol of Alcalde and Blotto (2006) for Scanning Electron Microscopy (SEM) analysis; terminology follows Wassersug (1976, 1980).

DNA extraction and analysis.—Total genomic DNA was extracted from ethanol-preserved tissue samples of two individuals (CFBH 21295, 21296—CFBHt 9664, 9659, respectively) using the Qiagen DNeasy kit following the manufacturer's guidelines. PCR amplification was carried out in 25 μl reactions using the PCR Master Mix (Thermo Scientific). The PCR protocol consisted of an initial denaturing step of 3 min at 96°C followed by 35 cycles of 30 s at 96°C, 30 s at 45°C, and 1 min at 72°C; the process ended with an extra cycle of 72°C for 3 min. The amplified product of the PCR was purified with Agencourt AMPure XP (Beckman Coulter). Cycle-sequencing using BigDye Terminators (Applied Biosystems) was run in 10 μl reactions. Sequencing was done on an ABI 3170 automated DNA sequencer (Applied Biosystems).

Mitochondrial gene 16S rDNA (Table 1) was targeted. Chromatograms were read and contigs assembled and edited using the software Sequencher 5.3 (Gene Codes). Obtained sequences were compared with those of *Proceratophrys avelinoi* and *P. bigibbosa* available on GenBank (see Material Examined for voucher accession numbers). Comparisons with sequences of *P. brauni* were done with an unpublished sequence generated by Dr. R. C. Amaro. The sequence DQ283038 (voucher JF1948) was not employed in the comparative analysis once it is partially contaminated with DNA sequences of *Odontophrynus* (see Faivovich et al., 2014;

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Table 1. Primers employed in this study.

Gene region	Primer name	Primer sequence (5′ to 3′)	Reference
16S rDNA	16SAR	CGCCTGTTTATCAAAAACAT	Palumbi et al., 1991
	16SBR	CCGGTCTGAACTCAGATCACGT	Palumbi et al., 1991
	16SH10	TGCTTACGCTACCTTTGCACGGT	Hedges, 1994
	16SL2A	CCAAACGAGCCTAGTGATAGCTGGTT	Hedges, 1994

Table 2). Genetic distances were calculated in the software Geneious 6.1.6 (Kearse et al., 2012).

Species assignment.—Species identification was based on two criteria: 1) morphological traits observed in a specimen at an advanced developmental stage (*sensu* Gosner, 1960) compared with the conditions observed in adults; and 2) analysis of mtDNA sequences. The tadpole in advanced developmental stage (stage 45 of Gosner, 1960) has some morphological traits that can be associated with the adult morphology of *Proceratophrys bigibbosa*, but also distinguish it from *P. brauni* and *P. avelinoi* (see Kwet and Faivovich, 2001). Among these traits are: 1) a rounded snout in dorsal view (with a pointed tip in *P. brauni*); 2) poorly developed subarticular tubercles (more developed in *P. avelinoi* and in *P. brauni*) and palpebral tubercles (more developed in *P. brauni*); and 3) well-developed toe webbing (poorly developed in *P. avelinoi* and *P. brauni*). Nevertheless, some of these traits may be subject to ontogenetic variation.

The sequenced individuals are 99.8% similar to each other regarding the 16S sequence (Table 3). Compared to other individuals, they are more similar to *Proceratophrys bigibbosa* from Misiones, Argentina (FJ685692), with 99.1% of similarity, followed by two individuals of *P. avelinoi*, also from Misiones (DQ283039 and FJ685691), and *P. brauni* from Santa Catarina State, Brazil, with 96.2% and 95.5% of similarity, respectively.

RESULTS

Tadpole description.—In dorsal view (Fig. 1B), body oval, wider posteriorly, snout rounded. In lateral view (Fig. 1A), body oval, depressed ($BH/BL = 0.2$). Eyes dorsal, large ($ED/BW = 0.13$; $ED/BL = 0.09$), directed anterolaterally. Nares (Fig. 1D) reniform, located dorsally, directed anterolaterally; nares with marginal rim and small triangular fleshy projection on sagittal margin (Fig. 1D). Interobital distance three times eye diameter; $IOD/IND = 0.46$. Eye equidistant to eye and snout; $ESD/BL = 0.3$.

Mouth anteroventral, laterally emarginated, bordered by single row of conical, alternating marginal papillae (Fig. 2); upper lip with large diastema; submarginal papillae absent; paired dermal folds on lower lip absent; oral disc 26% of body width. Labial tooth row formula 2(2)/3(1); $A\text{-}1 = A\text{-}2$, $P\text{-}1 = P\text{-}2 > P\text{-}3$; A2 gap large. Jaw sheaths present, serrate, melanized; upper jaw sheath arch-shaped; lower jaw sheath V-shaped. Labia tooth diameter uniform, base flattened, wide; body narrow; head convex, cuspidate; tooth body-head continuous (Fig. 2C, D).

Spiracle sinistral, tubular, lateral, located ventral to midline of body, directed posterolaterad in dorsal view, dorsad at angle of 30–45° in lateral view; inner wall present, fused to body proximally, free distally, longer than external wall; opening elliptical, smaller than spiracle width, margin irregular (Fig. 1E). Intestinal tube coiled, switchback position medial (Fig. 1C). Vent tube dextral, conical, positioned above level of ventral fin, fused to ventral fin, right margin shorter than left, opening elliptical (Fig. 1E, F). Tail long (TAL/TL 0.6), high (BH/TH 0.85); caudal muscles not reaching rounded tip; dorsal fin arched, originating on body/tail junction, ventral fin arched; dorsal fin slightly higher than ventral fin. Myotomes V-shaped, arranged in serial blocks; maximum tail height 23.4% of total length. Lateral line stitches inconspicuous, including angular, dorsal, infraorbital, jugal, medial, oral, supraorbital, temporal, and ventral lines.

Measurements.—TL 35.9±4.0 (30.2–43.1); BL 14.2±2.0 (11.3–16.2); TAL 21.6±3.0 (18.0–26.8); BW 9.1±2.1 (6.1–11.9); BH 7.2±1.4 (5.3–8.7); TH 8.3±1.9 (6.0–10.1); NSD 2.0±0.4 (1.3–2.3); ESD 3.8.0±0.6 (2.8–4.4); IOD 4.0±0.7 (3.0–4.6); END 2.0±0.3 (1.4–2.2); IND 1.8±0.2 (1.4–2.0); ODW 2.6±0.4 (1.9–3.0); ED 1.2±0.2 (0.9–1.57).

Color of the tadpole in preservative.—Skin translucent; in dorsal view, body light brown anteriorly. Snout pale yellow, with brown dots. In lateral view, body dark brown. Tail fins translucent, with light brown dots and five light brown stripes. Tail muscles pinkish (Fig. 1).

Buccopharyngeal morphology.—Buccal floor triangular (Fig. 3A). Two pairs of infralabial papillae; medial pair branched, branches of different lengths; lateral pair flap-like. Lingual bud elliptical; eight lingual papillae, long, finger-like; three lingual papillae bifurcated. Buccal floor arena bell-shaped; ca. 16 and 10 papillae laterally and posteriorly, respectively. Buccal floor arena partially covered with pustulations (about 150); conical papillae present on central buccal floor arena. Prepocket papillae present, few, short, conical. Buccal pockets shallow, discrete, oblique slit-shaped. Ventral velum preset; spicular support inconspicuous; secretory pits poorly developed. Branchial basket triangular, wider than long; ca ¼ of buccal length and ½ of buccal width. Three filter cavities, well defined, partially covered by ventral velum. Filter plates parallel to branchial slits; third filter plate covering fourth.

Buccal roof elliptical (Fig. 3B); prenarial arena bell-shaped, without papillae, few pustulations present posteriorly. Internal nares elliptical, transversally oriented; posterior valve free, with projection rounded and low. Postnarial arena diamond-shaped, four to six conical postnarial papillae. Lateral ridge papillae hand-like, with four branches. Median ridge low, wide, trapezoidal, with irregular border. Buccal roof arena circular, delimited laterally by six to eight conical papillae. Central area of buccal roof arena covered with scattered, rounded pustulations. Glandular zone well defined. Dorsal velum medially discontinued, conical papillae present, v-shaped.

Comparisons with other tadpoles.—Data for comparisons were extracted from personal examination of specimens or from the literature (see Material Examined). For a summary of phenotypic traits, see Dias et al. (2014: tables 2, 3, 4). The

Table 2. Species, voucher numbers, locality, and GenBank accession number of the DNA sequences employed in this study.

Species	Voucher number	Locality	GenBank accession number
P. avelinoi 1	JF1948	Missiones, AR	DQ283039
P. avelinoi 2	DB1246	Missiones, AR	FJ685691
P. bigibbosa	CFBH 212995	Mato Castelhano, RS	MG798659
P. bigibbosa	CFBH 212996	Mato Castelhano, RS	MG798660
P. bigibbosa	DB2313	Missiones, AR	FJ685692
P. brauni	MZUSP 135823	Praia Grande, SC	—

tadpoles of *P. bigibbosa* can be differentiated from its congeners (character states in parenthesis), except *P. avelinoi*, by the conical vent tube (tubular). It is different from all species of *Proceratophrys*, except *P. avelinoi*, *P. mantiqueira*, *P. minuta,* and *P. palustris*, by the dorsal fin originating on the body/tail junction (posterior third of the body). Furthermore, it is diagnosed from *P. cururu*, *P. dibernardoi*, *P. laticeps*, *P. palustris*, *P. renalis*, *P. schirchi,* and *P. tupinamba* by presenting a rounded tail tip (acute). From *P. appendiculata*, *P. avelinoi* (polymorphic), *P. cururu*, *P. dibernardoi*, *P. izecksohni*, *P. laticeps*, *P. mantiqueira*, *P. moehringi*, *P. palustris*, *P. renalis*, *P. schirci*, and *P. tupinamba* by the absence of two folds in the lower lip (two folds). From *P. appendiculata*, *P. cristiceps*, *P. izecksohni*, *P. palustris*, *P. schirchi*, and *P. tupinamba* by the free inner wall of the spiracle (fused to the body). From *P. appendiculata*, *P. izecksohni,* and *P. tupinamba* by presenting an interruption on the second superior tooth row (absent).

Regarding the buccopharyngeal morphology, the elliptical buccal roof differentiates it from all its congeners (triangular, circular, trapezoid, rectangular, hexagonal), and the elliptical buccal floor from all other species (ovoid, triangular, circular) but *P. cristiceps*. The presence of four infralabial papillae differentiates it from *P. boiei*, *P. cururu,* and *P. renalis* (two). From *P. cristiceps* (three), *P. appendiculata*, *P. avelinoi*, *P. boiei*, *P. cururu*, *P. izecksohni* (four), and *P. moratoi* (four to six) by the presence of six lingual papillae. From *P. cururu* and *P. mantiqueira* by the absence of a medial notch on the ventral vellum (present). From *P. boiei* and *P. cristiceps* by the presence of prepocket papillae (absent). From *P. cururu*, *P. cristiceps,* and *P. moratoi* by the presence of projections/ornamentation on the anterior margin of internal nares (absent). From *P. appendiculata*, *P. cururu*, *P. izecksohni*, *P. moratoi* (triangular), and *P. cristiceps* (semi-circular) by the trapezoidal median ridge. From *P. avelinoi* and *P. cristiceps* by the presence of six postnarial papillae (four). From *P. cururu* by the branched lateral ridge papillae (simple). Finally, from *P. cristiceps* by the presence of papillae/projections bordering the dorsal vellum (absent).

DISCUSSION

Knowledge on tadpoles of *Proceratophrys* has increased recently as almost 50% of the descriptions were published in the last ten years (Vieira et al., 2007; Fatorelli et al., 2010; Nascimento et al., 2010; Napoli et al., 2011; Provete et al., 2013, 2017; Dias et al., 2014; Peixoto et al., 2014; Santos et al., 2017). Notwithstanding, few authors (e.g., Dias et al., 2014) have attempted to explore all these data in an evolutionary context, despite the increase in phylogenetic knowledge on the genus (Amaro et al., 2009; Pyron and Wiens, 2011; Teixeira Jr. et al., 2012; Dias et al., 2013a; Pyron, 2014). For instance, many authors (e.g., Peixoto et al., 2014; Santos et al., 2017) employed restricted morphological comparisons within phenetic groups even when a large amount of data (e.g., Amaro et al., 2009; Teixeira Jr. et al., 2012; Dias et al., 2013a) refutes the monophyly of such assemblages. Even with the small taxon sampling of the available phylogenetic hypothesis and the relatively small number of described tadpoles, some evolutionary interpretations can be made.

The *Proceratophrys biggibosa* group is the only phenetic group whose monophyly is supported by molecular data (Amaro et al., 2009; Pyron and Wiens, 2011; Teixeira Jr. et al., 2012; Dias et al., 2013a; Pyron, 2014). Larval morphology provides four putative synapomorphies (conical vent tube, dorsal fin at the body tail junction, large A2 gap, and trapezoidal median ridge) for the group. One is the conical vent tube; this character state was observed only in *P. bigibbosa* and *P. avelinoi* (see Material Examined). This character is poorly described in the literature (e.g., Dias et al., 2014) and any further discussion is compromised until new data are acquired.

Another putative synapomorphy for the *P. bigibbosa* group is the dorsal fin originating at the body/tail junction, contrasting with the origin on the posterior third of the body in other *Proceratophrys*. Besides *P. bigibbosa* and *P. avelinoi*, Giaretta and Sazima (1993) also reported the former condition for *P. palustris*, another member of the *P. bigibbosa* group (see also de Sá and Langone, 2002). Other species of *Proceratophrys* that have this character state are *P. minuta* (Napoli et al., 2011) and *P. tupinamba* (Fatorelli et al., 2010), which were not recovered as being closely related to the *P. bigibbosa* group in all the available phylogenetic hypothesis (Amaro et al., 2009; Pyron and Wiens, 2011; Teixeira Jr. et al., 2012; Dias et al., 2013a; Pyron, 2014), thus not interfering in the optimization of this character state as a putative

Table 3. Pairwise genetic similarity between the species of the *Proceratophrys bigibbosa* group.

	CFBH 9659	CFBH 9664	*P. bigibbosa*	*P. brauni*	*P. avelinoi* 1	*P. avelinoi* 2
CFBH 9659	—					
CFBH 9664	99.8	—				
P. bigibbosa	99.1	99.1	—			
P. brauni	95.5	95.5	96.1	—		
***P. avelinoi* 1**	95.1	95.1	95.6	96.6	—	
***P. avelinoi* 2**	95.2	95.2	96	97.3	99.6	—

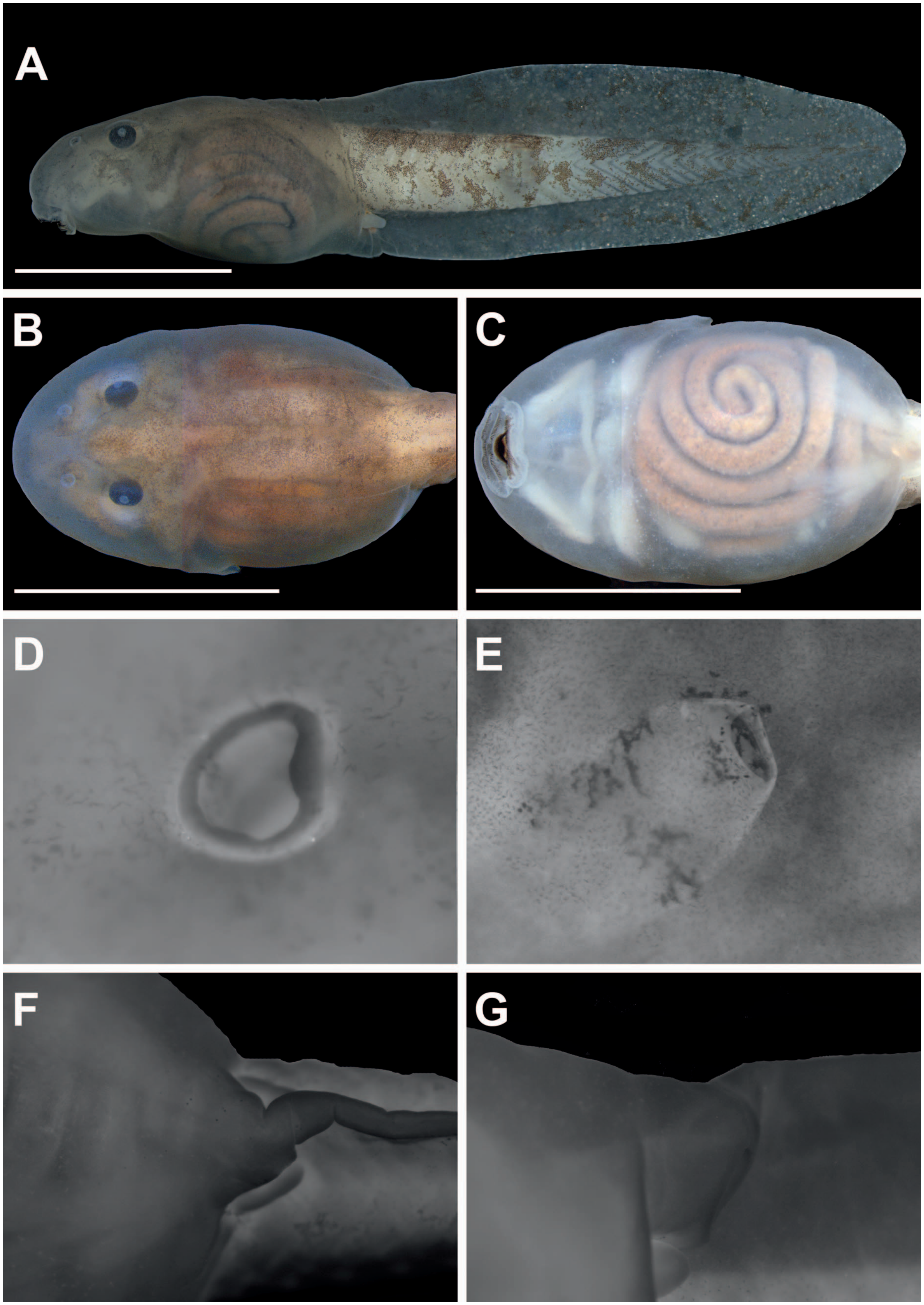

Fig. 1. The tadpole of *Proceratophrys bigibbosa* (CFBH 21293) at stage 31. Lateral (A); dorsal (B); and ventral (C) views. Details of the nostril (D), spiracle (E), and vent tube in ventral (F) and lateral (G) views. Scale bar = 10.0 mm.

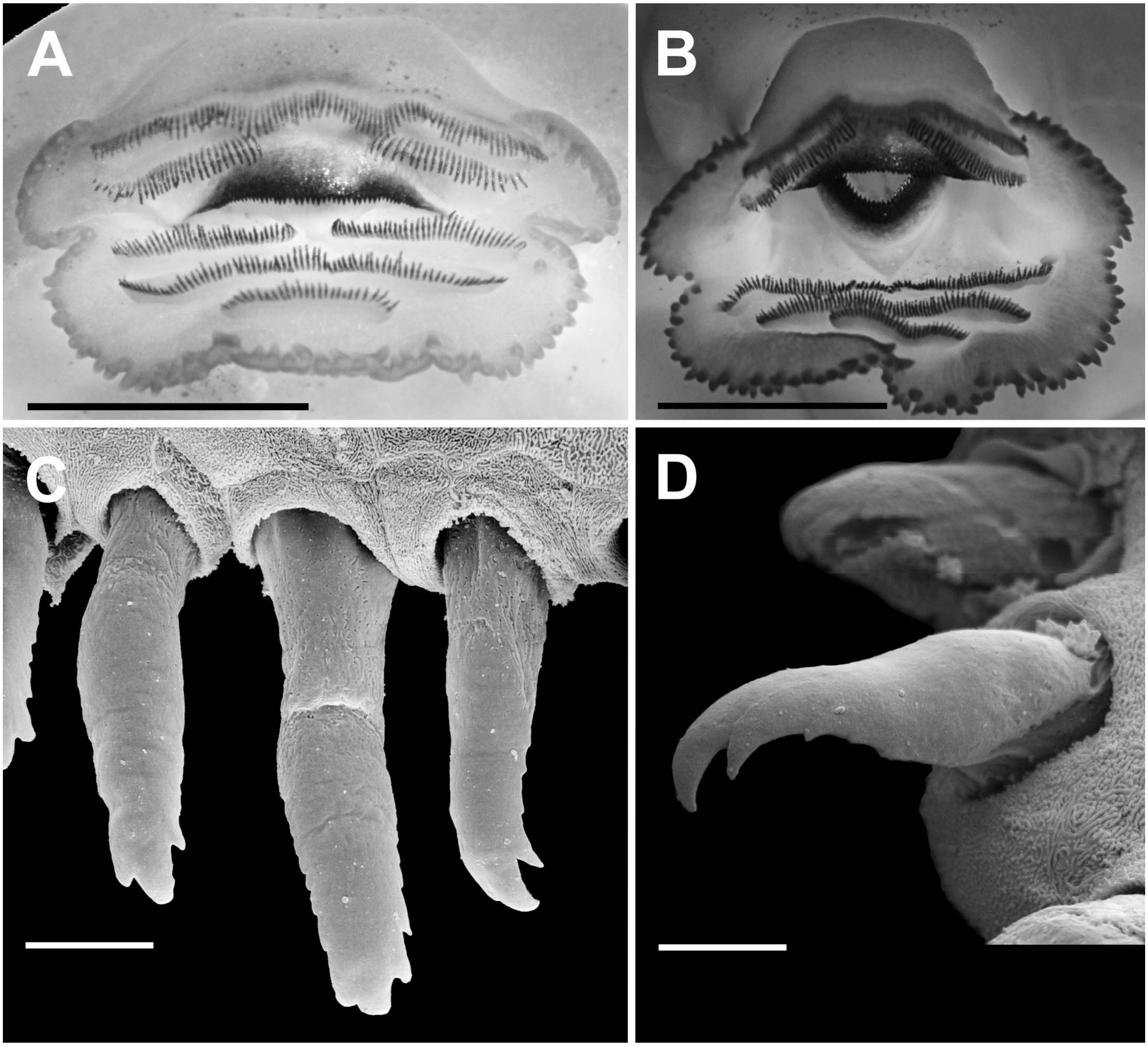

Fig. 2. Oral disc of the tadpole of *Proceratophrys bigibbosa* (CFBH 21293) at stage 31 closed (A) and open (B); scale bar = 1.0 cm. Labial tooth of *Proceratophrys bigibbosa* (CFBH 21292) at stage 27 in frontal (C) and lateral (D) views; scale bar = 5 μm.

synapomorphy—*P. minuta* is nested within a clade including *P. redacta*, *P. cristiceps*, and *P. schirchi*, whereas *P. tupinamba* is nested within the *P. appendiculata* clade.

The interruption on the second superior tooth row is an important character in the identification of larvae of *Proceratophrys*. Dias et al. (2014), for instance, suggested the absence of the gap was a synapomorphy for what they called the *P. appendiculata* clade, which included *P. appendiculata*, *P. belzebul*, *P. izecksohni*, and *P. tupinamba*. However, the configuration of the A2 gap was never further explored. In most species of *Proceratophrys*, this gap is short, present only in the most apical area of the upper jaw sheaths, whereas in *P. bigibbosa* and *P. avelinoi* it is large, extending itself until the descending border of the upper jaw sheath. I observed the same state in *P. minuta,* and the illustrated tadpoles of *P. cristiceps* and *P. dibernardoi* (Vieira et al., 2007; Santos et al., 2017) seem to also have the same condition. Assuming *P. dibernardoi* is closely related to *P. goyana* (see Brandão et al., 2013), the most parsimonious optimization for this character suggests it may represent a synapomorphy for the *P. bigibbosa* group.

Regarding the buccopharyngeal anatomy, some characters deserve further investigation. For instance, the trapezoidal median ridge may represent another putative synapomorphy for *P. bigibbosa* group (present in *P. biggibosa* and *P. avelinoi*; de Sá and Langone, 2002), although it also appears in other non-related taxa—*P. boiei*, *P. mantiqueira*, *P. melanopogon*, and *P. renalis*. Data on the buccopharyngeal anatomy of *P. brauni* and *P. palustris* are still lacking.

Tadpoles represent a key element in the evolution and diversification of anurans (Wassersug, 1975; Altig, 2006; Roelants et al., 2011; Dias et al., 2013b; Fabrezi et al., 2016), and the usage of larval morphology as a powerful source of evidence it is not new (e.g., Lataste, 1879; Boulenger, 1892;

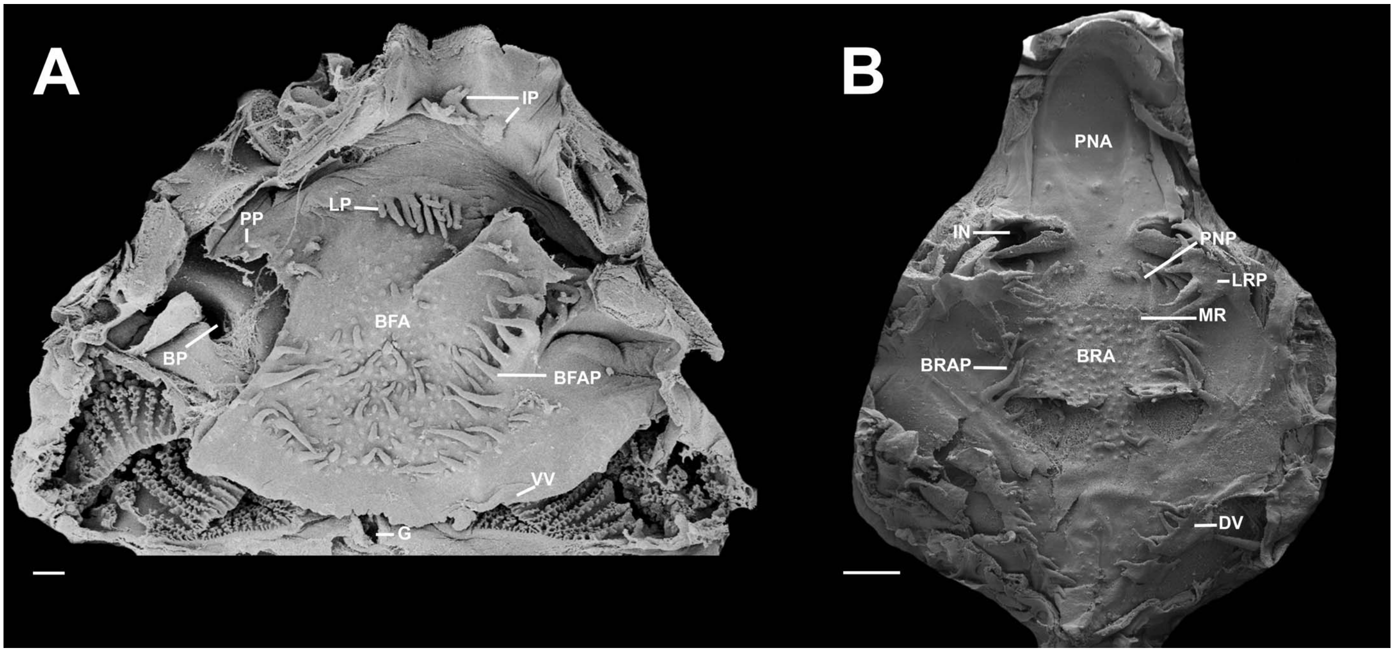

Fig. 3. Buccopharyngeal cavity of the tadpole of *Proceratophrys bigibbosa* (CFBH 21292) at stage 27. Buccal floor (A) and buccal roof (B). BFA, buccal floor arena; BFAP, buccal floor arena papillae; BP, buccal pocket; BRA, buccal roof arena; BRAP, buccal roof arena papillae; DV, dorsal velum; G, glottis; IN, internal nares; IP, infralabial papillae; LP, lingual papillae; LRP, lateral ridge papilla; MR, median ridge; PNA, prenarial arena; PNP, postnarial papilla; PP, prepocket papillae; VV, ventral velum. Scale bar = 200 μm.

Noble, 1925, 1927, 1929; Orton, 1953). Unfortunately, with some exceptions (e.g., Maglia et al., 2001; Haas, 2003; Grant et al., 2006; Miranda et al., 2014), few studies have attempted to provide explanations for larval evolution in a phylogenetic context. This challenge is particularly accentuated in the genus *Proceratophrys*, given our poor understanding of its phylogenetic relationships—to date only 18 species were included in a phylogenetic analysis—and larval morphology—only 19 tadpoles have been described so far. Nevertheless, my data on the larva of *P. bigibbosa* show that tadpoles can provide phenotypic support for many phylogenetic relationships, as demonstrated by the four new putative synapomorphies hypothesized for the *P. bigibbosa* group. However, more data are still necessary to properly test these hypotheses in a cladistic framework.

MATERIAL EXAMINED

(given in lots)

Proceratophrys appendiculata: Brazil, Rio de Janeiro, Teresópolis, Parque Nacional da Serra dos Órgãos: UNIRIO 4036.

Proceratophrys avelinoi: Argentina, Missiones: JF 781.

Proceratophrys biggibosa: Brazil, Rio Grande do Sul, Mato Castelhano: CFBH 21291, 21292, 21293, 21296; São Francisco de Paula: MCP 3888, 3912, 3914, 8354.

Proceratophrys concavitympanum: Brazil, Rondônia, Espigão D'Oeste: ZUEC 11545.

Proceratophrys cururu: Brazil, Minas Gerais, Serra do Cipó: ZUEC 9575.

Proceratophrys minuta: Brazil, Bahia, Chapada Diamantina: UFBA 10756.

Proceratophrys moratoi: Brazil, São Paulo: JJ 6943, 6944, housed at DZSJRP.

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