Article

On the stream-dwelling *Crossodactylus timbuhy* (Anura, Hylodidae): taxonomy, natural history, and geographic distribution

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Abstract

The genus *Crossodactylus* has taxonomic problems due to the difficulty in properly identifying some populations to the species level. The deficient species diagnosis rises mostly due to high intraspecific variation and lack of information on topotypes. *Crossodactylus timbuhy* was described based only on external morphology of preserved adult specimens. Based on topotypic specimens, we provide further information on *C. timbuhy* regarding color in life (both adult and larvae), tadpole external morphology, vocal repertoire, natural history, and geographic distribution. The species identification was confirmed through comparison to the type series, 16S rRNA analysis, and bioacoustics. *Crossodactylus timbuhy* vocalizes during daylight along shallow slow-flowing streams. The advertisement call has ascendant amplitude modulation, duration of 2.1–8.4 s, 23–75 pulsed notes, and peak frequency of 3.6–4.8 kHz. Our report on male unilateral sac inflation is the first within *Crossodactylus*. Although overall tadpole external morphology and coloration resemble other species of hylodids, we report ventral depression and golden iridophores also for the first time within *Crossodactylus*. Adult males display five antipredator mechanisms (interrupt calling, escape, aggression, posture, and aposematism). Finally, we restrict its geographic distribution to the Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil.

Key words: Amphibia, Atlantic Forest, Santa Teresa, tadpole, vocalization

Introduction

As presently defined, Hylodidae Günther comprises 13 species of *Crossodactylus* Duméril & Bibron, 26 species of *Hylodes* Fitzinger, eight species of *Phantasmarana* Vittorazzi, Augusto-Alves, Neves-da-Silva, Carvalho-e-Silva, Recco-Pimentel, Toledo, Lourenço, and Bruschi, and one species of *Megaelosia* Miranda-Ribeiro. These 48 stream-dwelling species are distributed throughout the Atlantic Forest from northeastern to southern Brazil and northeastern Argentina. Hylodids are mostly diurnal species and are commonly found at narrow streams inside the forest (e.g., Weygoldt & Carvalho-e-Silva 1992; Haddad & Giaretta 1999; Narvaes & Rodrigues 2005; Caldart *et al*. 2014). The genus *Crossodactylus* has been taxonomically problematic due to the difficulty to adequately identifying some populations to species level. The deficient species diagnosis rises due mainly to the high intraspecific variation and lack of information on topotypes (Pimenta *et al*. 2008, 2014; Vidigal *et al*. 2018).

   Although anuran vocalization is an inheritable characteristic and thus widely studied in evolutionary (e.g.,

Gerhardt 1994; Cocroft & Ryan 1995; Wilczynksi *et al.* 2001; Robillard *et al.* 2006; Goicochea *et al.* 2010; Toledo *et al.* 2015) and taxonomic approaches (Köhler *et al.* 2017), many species of hylodids still lack description of vocal repertoire. To date, seven *Crossodactylus* species have the vocalization described: *C. caramaschii* Bastos & Pombal, 1995; *C. cyclospinus* Nascimento, Cruz & Feio, 2005; *C. franciscanus* Pimenta, Caramaschi & Cruz, 2015; *C. gaudichaudii* Duméril & Bibron; *C. schmidti* Gallardo, 1961; *C. trachystomus* (Reinhardt & Lütken); and *C. werneri* Pimenta, Cruz & Caramaschi, 2014 (see Weygoldt & Carvalho-e-Silva 1992; Bastos & Pombal 1995; Nascimento *et al.* 2005; Pimenta *et al.* 2008; Caldart *et al.* 2011; Pimenta *et al.* 2015; Vidigal *et al.* 2018).

Larval studies have also great relevance in different fields of biology as ecology (e.g., Thurnheer & Reyer 2000; Eterovick & Barros 2003), biological control (e.g., Salinas *et al.* 2018; Naz *et al.* 2019), evolution (e.g., Grosjean *et al.* 2004; Sherratt *et al.* 2017), and taxonomy (e.g., Rossa-Feres & Nomura 2006; Grosjean *et al.* 2015). Nevertheless, at the moment, only were described the tadpoles of *C. caramaschii*, *C. cyclospinus*, *C. gaudichaudii*, *C. schmidti*, *C. trachystomus*, and *C. werneri* (see Caramaschi & Sazima 1985; Caramaschi & Kisteumacher 1989; Francioni & Carcerelli 1993; Faivovich 1998; Nascimento *et al.* 2005; Pimenta *et al.* 2015; Silva-Soares *et al.* 2015; Vidigal *et al.* 2018; Toscano *et al.* 2019).

Crossodactylus timbuhy Pimenta, Cruz & Caramaschi, 2014 was described based on preserved specimens from the Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil. Formerly referred to as *C.* cf. *dispar*, its advertisement call was briefly described by Weygoldt (1986), but it is neglected in bioacoustic comparations of the genus (see Pimenta *et al.* 2014, 2015; Vidigal *et al.* 2018). The species description also lacks data on larval morphology, color in life, and natural history. Based on topotypic specimens, the present study aims to provide data on *Crossodactylus timbuhy* regarding: 1) color in life (both adult and larvae); 2) tadpole external morphology; 3) vocal repertoire; and 4) notes on natural history. We further provide information on the species geographic distribution.

Material and methods

Color in life of adult specimens, tadpole samples, vocal repertoire recordings, and natural history observation took place at a permanent shallow, slow-flowing stream "Córrego Roda D'água" (19°54'30" S; 40°32'29" W; 908m asl; Fig. 1A) inside a forested area in the Atlantic Forest, located in the Reserva Biológica Augusto Ruschi (type locality), Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil (see Results). We also used 16S rRNA samples from other four localities from the Municipality of Santa Teresa: Sítio da Penha Briel (19°52'05.30"S; 40°32'05.70"W); Sítio da Irmã Vulpi (19°50'45.50"S; 40°33'25.30"W); and two localities in the Reserva Biológica Augusto Ruschi (19°55'15.78"S; 40°33'8.90"W and 19°54'52.57"S; 40°33'7.92"W) (Fig. 1B). We further included samples from surrounding areas (i.e., outside the type locality) in the molecular comparisons (Fig. 1B): Reserva Biológica Duas Bocas, Municipality of Cariacica (MNRJ 39465); Municipality of Viana (RBF 806); Rio Muqui, Fazenda Bandarra, Municipality of Mimoso do Sul (CFBHT 12726); Municipality of Bom Jesus do Norte (RU 8367); Sítio Recanto da Mata, Municipality of Muniz Freire (CFBHT 10799); and a second locality also in the Municipality of Muniz Freire (CFBHT 11961).

We euthanized voucher specimens (adults) in 5% lidocaine, fixed them in 10% formalin, and then transferred to 70% ethanol and deposited them at Museu de Biologia Mello Leitão (MBML 11856–7) from Instituto Nacional da Mata Atlântica (INMA). Tadpoles were fixed in 10% formalin (MNRJ 93668–70). Before fixation, specimens had tissue (liver in adults and tail muscle in tadpoles) samples extracted and stored in 96% ethanol for whole genomic DNA extraction.

For molecular analysis, we sequenced 14 individuals (Table 1; GenBank OK144256–7 and OK664948–59) using a fragment of 16S ribosomal gene (16Sar-L [CGCCTGTTTATCAAAAACAT] and 16Sbr-H [CCGGTCT-GAACTC AGATCACGT] [Palumbi *et al.* 1991]). The genomic DNA was extracted with DNeasy (QIAGEN, Valencia, CA) isolation kit, following the manufacturer's guidelines or with the phenol-chloroform protocol of Sambrook and Russell (2001). We used two protocols for amplification, purification, and sequencing. 1) The 16S rRNA amplification was carried out using a handle mix (2.5 μL of buffer, 2 μL of Mg, 1 μL of DNTP, 0.15 μL of Taq, and 1 μL of each primer). PCR cycles consisted of an initial denaturing step of 3 min at 96° C, followed by 35 cycles of amplification (denaturation for 30 s at 96° C, annealing for 30 s at 48° C, and extension for 60 s at 60° C [following Lyra *et al.* 2017]), with a final extension step set to 60° C for 7 min. PCR products were cleaned using Agencourt AMpure XP, and sequenced in Macrogen Inc. (Seoul, Korea). 2) The 16S rRNA amplification was carried out using 1x buffer, 4 μL of mix SOLIS, 14 μL of miliq water, 0.5 μL of each primer at 10 μM, and 2 μL of template DNA (total reaction volume of 20 μL). PCR cycles consisted of an initial denaturing step of 12 min at 95° C, followed by

35 cycles of amplification (denaturation for 20 s at 95° C, annealing for 30 s at 48° C, and extension for 1 min at 72° C. PCR products were purified with Ethanol/Sodium Acetate, and sequenced in Plataforma de Sequenciamento da Universidade Federal de Pernambuco (Brazil).
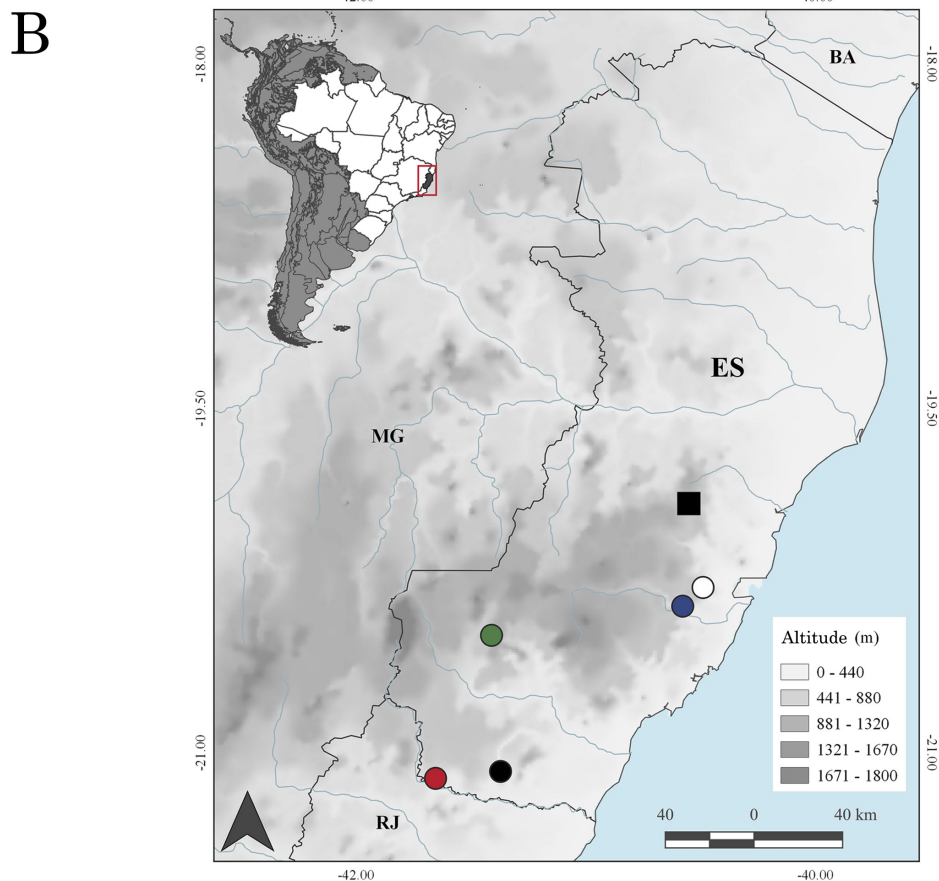

FIGURE 1. A) Córrego Roda D´água, the shallow slow-flowing stream where *Crossodactylus timbuhy* was collected (both adults and tadpoles) and recorded, at the species type locality Reserva Biológica Augusto Ruschi, Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil. B) Sampled Municipalities from the State of Espírito Santo, Southeastern Brazil: *Crossodactylus timbuhy* from the Municipality of Santa Teresa (black square); *Crossodactylus* sp. (circles) from the Municipalities of Cariacica (white), Viana (blue), Muniz Freire (green), Mimoso do Sul (black), and Bom Jesus do Norte (red).

Chromatograms were read, contigs assembled, and complete sequences were edited and aligned using Geneious v. 9.1.2 (Kearse *et al.* 2012) with MUSCLE algorithm (and default parameters; Edgar 2004). We aligned our 16S sequences with 16S sequences of other species of *Crossodactylus* available in GenBank. We estimated uncorrected *p* distances among species/individuals using Mega v. 10.1.1 (Kumar *et al.* 2018). Moreover, we estimated haplotype networks among species using Median-Joining algorithm and the default epsilon = 0 in PoPArt (Leigh & Bryant 2015) to explore the relationship among haplotypes.

Research protocol was approved by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, Permit Number: 63575–5). Vocalization recordings were deposited at Fonoteca Neotropical Jacques Vielliard (FNJV 50641–50) and video at the Audiovisual Collection (Video collection - ZUEC-VID) of the Museum of Zoology (ZUEC-VID 981).

Species confirmation. We confirmed species identification from morphological comparison of topotypes to the *Crossodactylus timbuhy* type series: MZUSP 69117–8, MZUSP 69120–1, MZUSP 69127, USNM 318101, USNM 318103, USNM 318106, USNM 318109–14, and USNM 318118–20. We also ran barcoding using 16S rRNA for species identification, including one tadpole used in the description. Because the sympatric and morphologically indistinguishable *C.* cf. *gaudichaudii* lacks molecular samples, we confirmed identification through bioacoustics comparison (see *Discussion* section).

Tadpole description. Tadpoles stages follow Gosner (1960) and measurements Lavilla & Scrocchi (1986), Altig & McDiamarid (1999), and Grosjean (2005): total length (TL); body length (BL); body width (BW); body height (BH); tail length (TaL); tail height (TH); tail muscle height (TMH); tail muscle width (TMW); dorsal fin height (DFH); ventral fin height (VFH); internostril distance (IND); interorbital distance (IOD); eye diameter (ED); nostril diameter (ND); eye-nostril distance (END); nostril-snout distance (NSD); eye-snout distance (ESD); snout-spiracle distance (SSD); and oral disc width (ODW). Tadpole descriptions and measurements were based on 12 tadpoles in stages 30–40. Measurements were taken with a caliper to nearest 0.01 mm under a stereomicroscope. Lateral line system terminology follows Lannoo (1987). Our samples were compared to the other *Crossodactylus* species based on the description of external larval morphology (Caramaschi & Sazima 1985; Caramaschi & Kisteumacher 1989; Francioni & Carcerelli 1993; Faivovich 1998; Nascimento *et al.* 2005; Silva-Soares *et al.* 2015; Vidigal *et al.* 2018; Toscano *et al.* 2019). We did not consider the external morphology of *C. dispar* from the region of Paranapiacaba (Bokermann 1963) because of the lack of certainty of the tadpole identification (see Pimenta *et al.* 2014).

Vocal repertoire. Recordings were carried out with temperatures varying from 17–21° C and were performed using a Tascam DR-40 recorder (with internal microphone) at 44.1 kHz with a 16-bit sampling size. The sound files were then analyzed using Raven pro 1.5 (Bioacoustics Research Program 2017). Spectrograms were generated using a window size of 256 samples, 81% overlap, hop size of 128 samples, Discrete Fourier Transform (DFT) of 256 samples, and *Hann* window type. We filtered out low frequencies up to ca. 500 Hz (below the minimum frequency reached by *Crossodactylus timbuhy*). Bioacoustical terminology follows Köhler *et al.* (2017): call duration (s), number of notes per call, note duration (ms), internote interval (ms), note repetition rate (notes/sec), number of pulses per note, pulses repetition rate (pulses/sec), minimum frequency at 5% of energy (kHz), maximum frequency at 95% of energy (kHz), peak frequency (kHz), and amplitude modulation. We then compared our recordings to the literature available for other *Crossodactylus* species (Weygoldt 1986; Weygoldt & Carvalho-e-Silva 1992; Bastos & Pombal 1995; Nascimento *et al.* 2005; Pimenta *et al.* 2008; Caldart *et al.* 2011; Pimenta *et al.* 2015; Vidigal *et al.* 2018).

Natural history observation. Observations on natural history did not follow a standard sampling design. Vocal sac inflation and visual display were recorded using a Samsung J7 Prime2 cellphone. Antipredator mechanisms were induced during fieldwork, and the classification follows Ferreira *et al.* (2019).

Results

Species confirmation. Our specimens from the Municipality of Santa Teresa were identified as *Crossodactylus timbuhy* based on morphology and bioacoustics (see below for further details on the acoustic differences from the sympatric *C.* cf. *gaudichaudii*) and genetic data. Mitochondrial partial sequence of 16S rRNA comparison revealed a very short genetic distance (0.1%; Table 1) among topotypes and other sampled populations from the Municipality of Santa Teresa. In contrast, these samples of *C. timbuhy* have high genetic distance (9.6%; Table 1) compared

to populations of *Crossodactylus* from other Municipalities of Espírito Santo (herein referred to as *Crossodactylus* sp.; Fig. 1B). In the haplotype network (Fig. 2), our samples of *C. timbuhy* from Santa Teresa generated only two haplotypes, distinguished from the other species from many mutational steps.
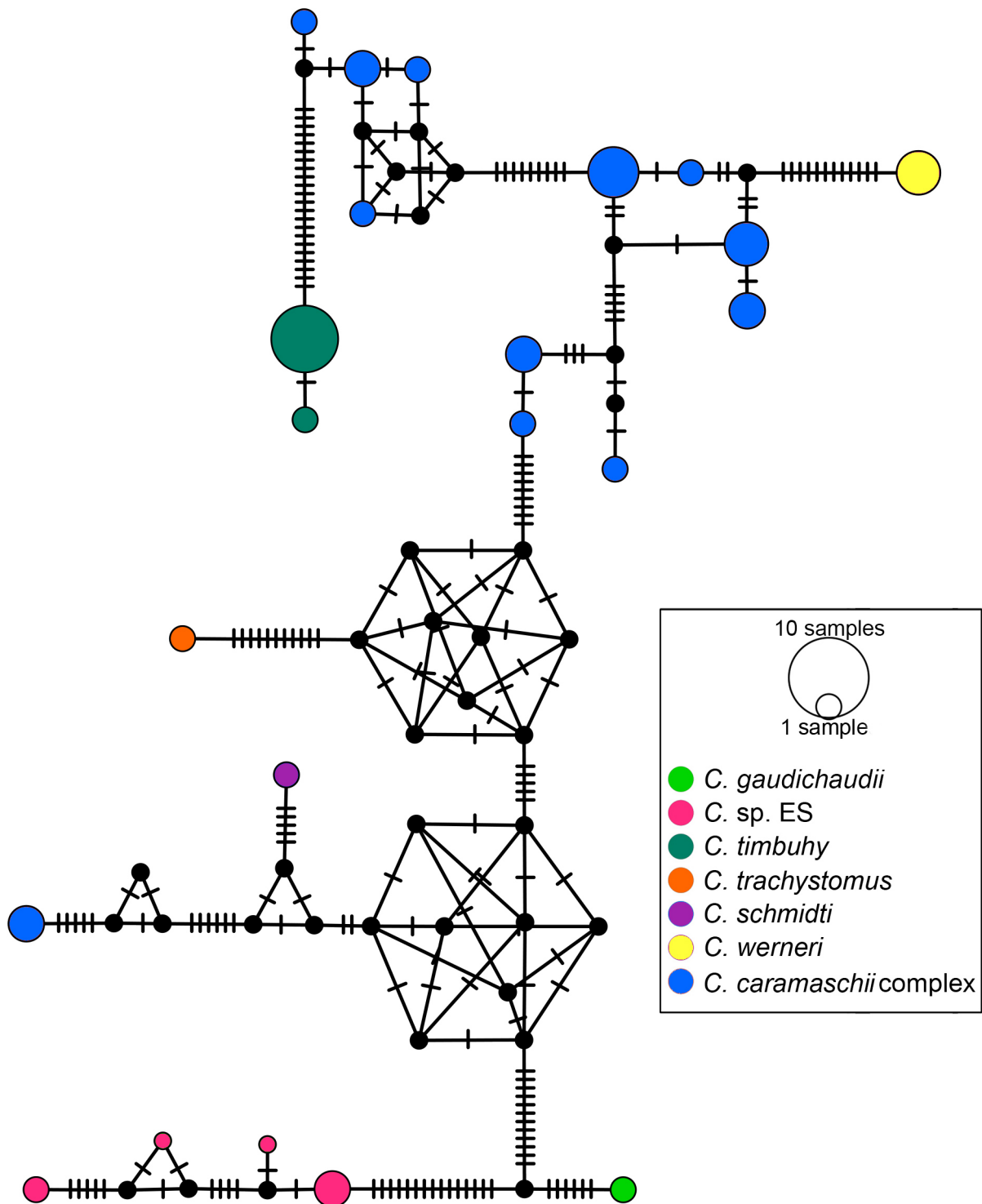

FIGURE 2. Haplotype network based on 16S rRNA of *Crossodactylus* species using POPART. Haplotype circle is proportional to its frequency (indicated in legend). Each color represents distinct species and/or species complex, and black dots represent inferred unsampled or extinct haplotypes. Mutational steps between alleles are represented by lines.

TABLE 1. Uncorrected *p* distances for partial 16S rRNA among species of *Crossodactylus*. Species (number of samples). Intraspecific variation in bold. Colors corresponds the haplotypes. *Crossodactylus gaudichaudii* was identified as *C. aeneus* in genbank (see Vittorazzi *et al*. 2021). Samples of *C. timbuhy* includes seven adults and one tadpole for species confirmation. *Crossodactylus caramaschii* is likely a species complex including specimens identified in genbank as *C.* aff. *caramaschii*.

		1	2	3	4	5	6	7
1	*C. gaudichaudii* (1)	n/c						
2	*C.* sp. (6)	0.065	**0.015**					
3	*C. timbuhy* (8)	0.108	0.096	**0.001**				
4	*C. trachystomus* (1)	0.096	0.094	0.099	**n/c**			
5	*C. schmidti* (1)	0.084	0.103	0.108	0.081	**n/c**		
6	*C. werneri* (3)	0.122	0.113	0.099	0.081	0.078	**< 0.001**	
7	*C. caramaschii** (22)	0.102	0.104	0.084	0.076	0.079	0.059	**0.034**

Color in life (Fig. 3). General pattern varying among different tones of brown and gray usually with darker tones on the flanks (almost black in some individuals); irregular darker blotches along the dorsum; some individuals have a lighter mild vertebral line; glandular ridges are darker but isolated tubercles may also be lighter or even white; a black line along the canthus rostralis appearing to be continuous with pupil, extending dorsally to the tympanum and reaching different heights of the dorsolateral region of the body (it may end at the shoulders height or may extend to near the sacral region); white and usually poorly delimited area from snout to shoulder with darker spots/blotches on it; postrictal tubercles are white; transversal dark bars along the forearms (two bars), thigh (five), tibia (four), and tarsus-foot (four) (some of these bars may not be visible on some individuals); gular, chest and belly white with darker reticulation (less intense on the gular region); ventral surfaces of the legs unpigmented; palmar and plantar surface dark brown or gray.

Tadpole description. Morphometric traits are summarized in Table 2. Tadpoles (Fig. 3G, 4) have an elliptical to ovoid body shape in dorsal view; in lateral view, it varies from oval to almost triangular; a slight depression ventrally in the medial region, right anterior to the gut; rounded snout in dorsal and lateral views; eyes dorsolaterally positioned and laterally directed; nostrils dorsofrontally positioned and directed; nostrils rounded with a tiny projection in the inner margin giving a reniform aspect to some individuals; nostrils about the same distance between the snout and the eyes; spiracle sinistral, posterodorsally directed, short, tubular, attached to the body, opening slightly posterior to mid-body and slightly below the body midline; vent tube dextral, short, conical (base wider than its opening); tail maximum height equal or close to the body maximum height; dorsal fin slightly higher than body; maximum height of the caudal musculatures less than the maximum body height; dorsal fin extending onto the end of the body (ca. the posterior fifth of the body); dorsal fin ascending slightly in the anterior third (or anterior half) then descending more abruptly in the posterior third (or posterior half); ventral fin relatively parallel to tail musculature, and curved at its distal third; dorsal fin higher than the ventral one; maximum tail musculature height higher than maximum dorsal fin height; tail musculature reaches the tip of the tail; tail ending pointed; lateral line system having supraorbital, posterior supraorbital, infraorbital, posterior infraorbital, longitudinal oral, angular, ventral, middle (tail portion of middle line not visible in some individuals), and dorsal lines; oral disc ventral with two lateral emarginations and a third one in the middle of lower labium; marginal papillae uniseriate, anteriorly interrupted; scattered submarginal papillae distributed laterally; tooth row formula 2(2)/3(1) the third being posterior tooth row shorter than others; jaw sheaths developed and serrated; upper jaw sheath as an inverted U-shape, thickened medially and thinner lateral margins, with a subtle medial projection in some specimens; lower jaw sheath more developed than the upper one.

Tadpoles in life (Fig. 3G) are overall light brown colored with darker pigmentation forming irregular (sometimes circular) darker brown blotches through the body and tail (some individuals have black blotches in the tail). The flanks are usually lighter and translucent; the ventral surface of the body is lighter, translucent, and may present a metallic reflex. The tail is translucent in some areas where pigmentation is absent, visualizing the whitish or beige musculature; golden iridophores spread through the entire body and tail. Our samples were collected in June, July and September 2019 and then analyzed in October of the same year, when individuals became overall more opaque, especially the ventral metallic reflex and golden blotches of iridophores.

TABLE 2. Morphometric traits (mm) for tadpoles of *Crossodactylus timbuhy* from its type locality, Reserva Biológica Augusto Ruschi, Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil. Abbreviations: total length (TL); body length (BL); body width (BW); body height (BH); tail length (TaL); tail height (TH); tail muscle height (TMH); tail muscle width (TMW); dorsal fin height (DFH); ventral fin height (VFH); internostril distance (IND); interorbital distance (IOD); eye diameter (ED); nostril diameter (ND); eye-nostril distance (END); nostril-snout distance (NSD); eye-snout distance (ESD); spiracle-snout distance (SSD); oral disc width (ODW). Values are presented as minimum–maximum (mean±standard deviation).

| Morphometric Traits	Tadpole stages (number of measured individuals)						
	30 (2)	34 (1)	35 (3)	36 (1)	38 (4)	40 (1)	Total (12)
TL	48.5–55.5	52.2	51.3–54.9 (53.7±2.0)	53.3	53.4–56.5 (54.8±1.6)	54.2	48.4–56.5 (53.6±2.2)
BL	17.7–17.8	18.1	18.3–19.7 (19.0±0.6)	18.6	18.2–19.8 (19.0±0.6)	20.7	17.7–20.7 (18.8±0.9)
BW	12.4–13.2	12.3	13.6–15.2 (14.4±0.8)	12.9	13.4–15.0 (14.1±0.7)	15.2	12.3–15.2 (13.8±1.0)
BH	11.0	9.6	11.3–12.1 (11.8±0.4)	10.0	10.9–12.3 (11.6±0.6)	12.0	9.6–12.3 (11.3±0.8)
TaL	33.0–30.1	34.0	32.9–36.4 (34.9±1.8)	35.6	34.5–38.0 (35.8±1.5)	34.8	30.1–38.0 (34.6±2.0)
TH	11.0–11.5	10.8	11.3–12.4 (11.9±0.7)	11.7	11.2–13.1 (12.3±0.9)	12.2	10.8–13.1 (11.8±0.8)
TMH	6.85–7.2	7.0	6.7–7.3 (7.0±0.3)	6.6	6.4–7.8 (7.4±0.7)	8.6	6.4–8.6 (7.2±0.6)
TMW	5.3–5.5	5.1	5.3–5.4 (5.4±0.1)	5.2	5.3–6.6 (5.9±0.6)	6.7	5.1–6.7 (5.6±0.5)
DFH	4.0–4.5	4.3	3.8–4.9 (4.3±0.5)	3.9	4.3–5.0 (4.6±0.3)	5.0	3.8–5.0 (4.4±0.4)
VFH	2.9–3.4	3.0	2.1–3.4 (2.9±0.7)	2.8	2.8–3.9 (3.2±0.5)	4.0	2.1–4.0 (3.2±0.5)
IND	3.5–3.8	3.5	3.5–4.0 (3.7±0.3)	3.5	3.5–3.9 (3.7±0.1)	3.8	3.5–4.0 (3.7±0.2)
IOD	6.1	5.8	6.3–6.6 (6.5±0.1)	6.2	6.1–6.9 (6.6±0.3)	7.0	5.8–7.0 (6.4±0.4)
ED	2.0–2.2	2.3	2.3–2.6 (2.4±0.1)	2.2	2.5–3.0 (2.7±0.2)	2.3	2.0–3.0 (2.4±0.2)
ND	0.5	0.4	0.4–0.6 (0.6±0.1)	0.6	0.4–0.5 (0.5±0.1)	0.6	0.4–0.6 (0.5±0.1)
END	1.7–1.8	1.8	1.7–2.0 (1.9±0.1)	2.0	1.6–2.0 (1.8±0.2)	2.2	1.6–2.2 (1.9±0.2)
NSD	2.7–3.2	3.3	2.7–3.8 (3.2±0.6)	2.8	3.0–3.7 (3.3±0.3)	3.7	2.7–3.8 (3.2±0.4)
ESD	5.4–5.6	5.3	5.1–6.4 (5.8±0.6)	5.3	5.3–5.6 (5.5±0.1)	6.4	5.1–6.4 (5.6±0.4)
SSD	10.1–10.4	10.7	10.9–11.5 (11.2±0.3)	10.75	11.3–12.0 (11.8±0.3)	11.7	10.1–12.0 (11.2±0.6)
ODW	3.8–4.5	4.35	4.2–4.6 (4.4±0.2)	4.0	3.9–4.4 (4.2±0.2)	4.5	3.8–4.6 (4.3±0.2)

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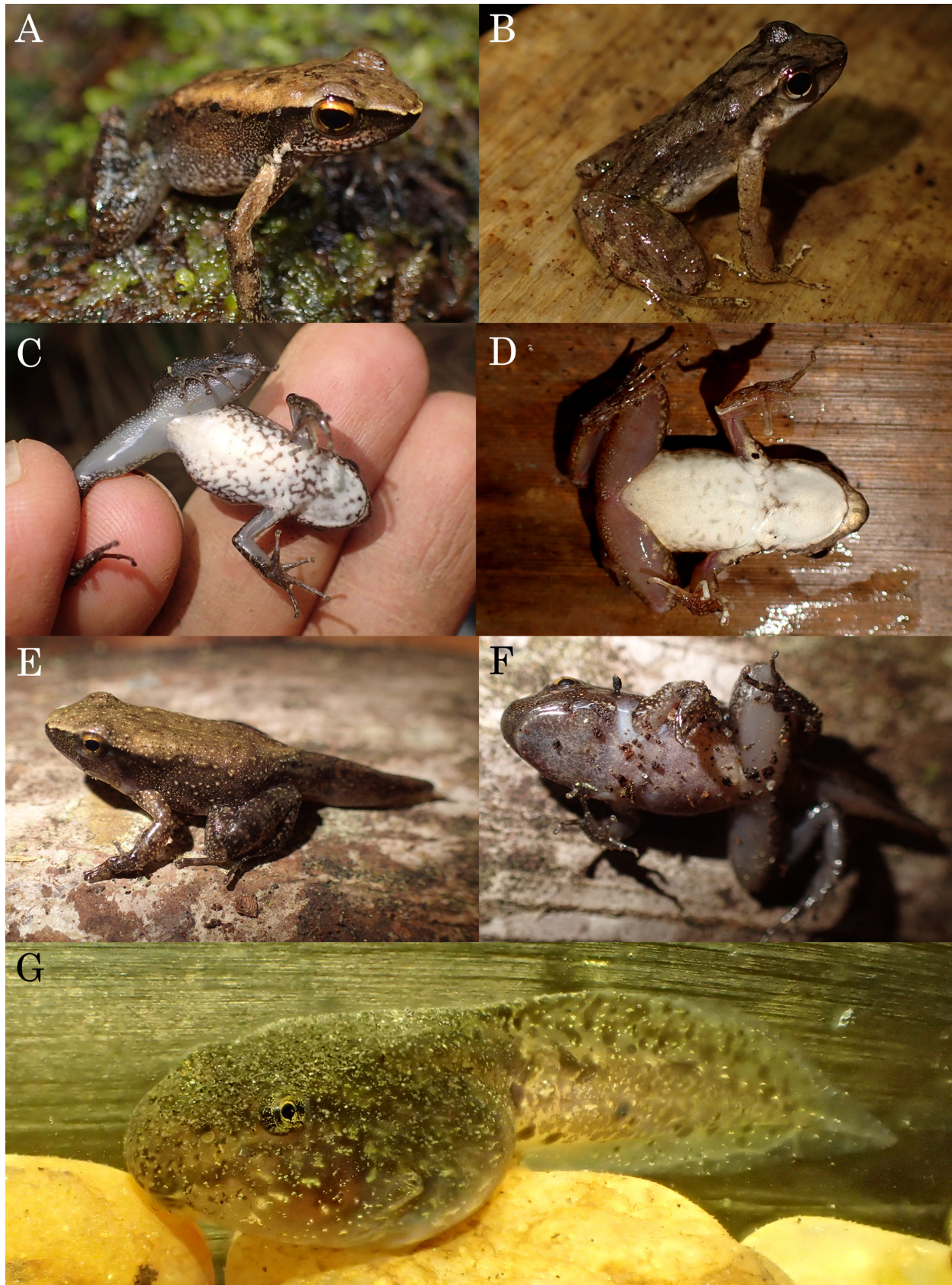
FIGURE 3. *Crossodactylus timbuhy* at Córrego Roda D´Água, Reserva Biológica Augusto Ruschi, Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil: (A–B) different tones of overall color pattern in two unvouchered specimens; (C–D) different intensities of reticulation on the belly, chest and gular surfaces in two unvouchered specimens; (E) dorsal, lateral and (F) ventral color pattern in a froglet; and (G) tadpole (MNRJ 93668). Photos by JVAL.
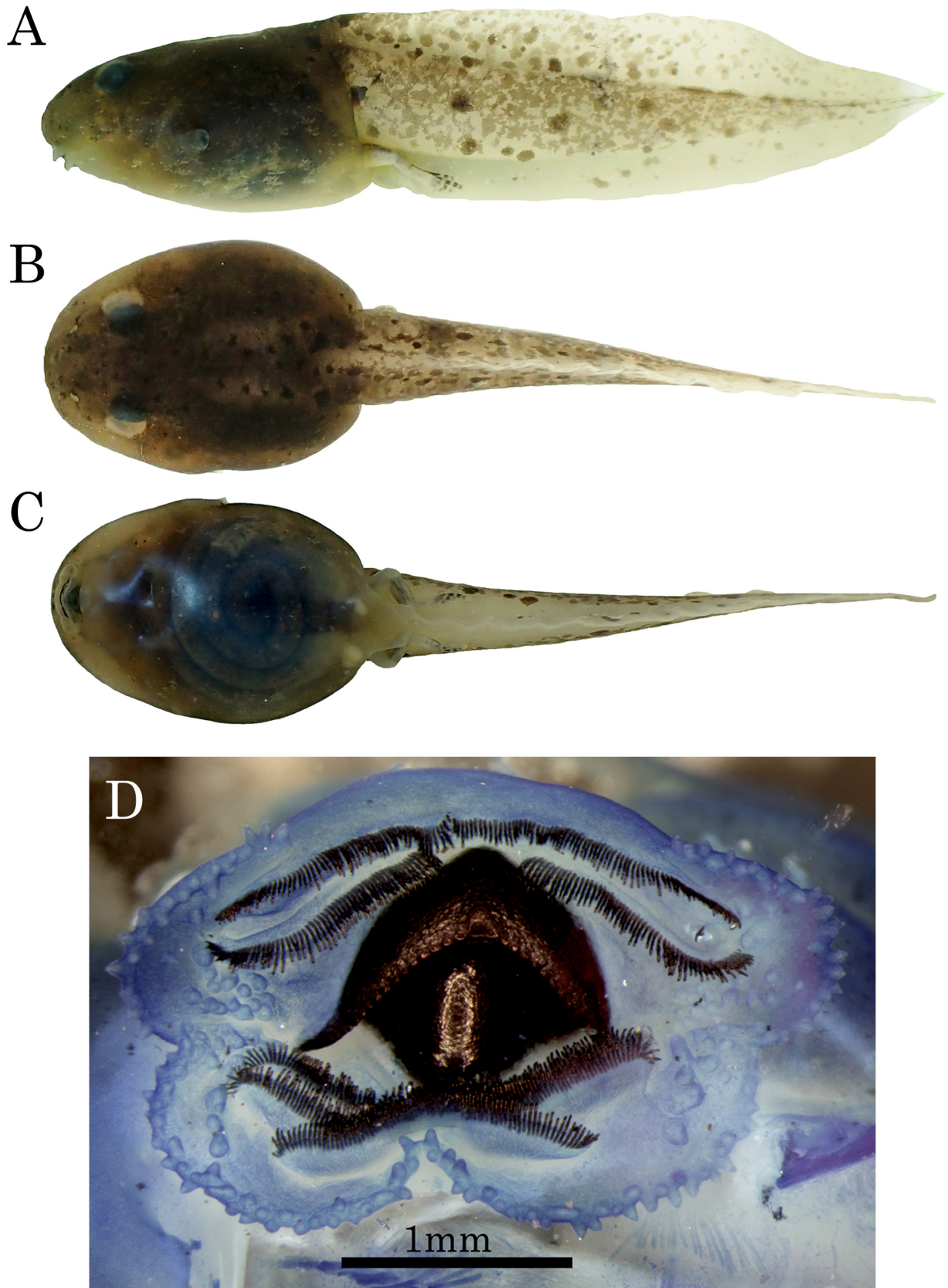
FIGURE 4. *Crossodactylus timbuhy* tadpole at stage 38 (TL=53.4 mm; MNRJ 93668): A) lateral view, B) dorsal view, and C) ventral view. D) Oral disc from a tadpole stage 25 (MNRJ 93670). Both collected at the species type locality (Reserva Biológica Augusto Ruschi, Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil. Photos by JVAL (A–C) and Daniela Fonseca (D).

Vocal repertoire. The vocal repertoire of *Crossodactylus timbuhy* has three types of calls (type A, type B and type C; Fig. 5; Table 3). Call type A was mostly emitted isolated (only twice during a call series) and could be heard from a long distance. Calls type B and C were emitted in series (both types in the same call series) at lower relative amplitudes. Call series have ascendant amplitude modulation being difficult to hear at those from the beginning. We recorded 14 complete call series emitted by seven males with duration of 7.3–79.8 sec (33.2±23.8) and 7–80 calls/call series (35.5±25.8) emitted at a rate of 0.6–1.6 calls/sec (1.1±0.3). Call series duration and number of calls per call series may depend on the social context, increasing significantly when two males interact.

TABLE 3. Acoustic traits of the vocal repertoire of *Crossodactylus timbuhy* (calls type A, B and C). *Notes with pulses merged to the point they could not be individualized were not considered (see Discussion). Values are presented as minimum–maximum (mean±standard deviation).

Type of call	Call Duration (s)	Notes/call	Note Duration (ms)	Internote Interval (ms)	Notes/sec
A	2.1–8.4 (6.1±1.3) n=24	23–75 (59.8±11.8) n=24	3.0–46.0 (27.2±6.0) n=1250	44.2–109.0 (74.8±13.9) n=1226	8.8–11.0 (9.9±8.8) n=24
B	0.1–0.8 (0.4±0.1) n=198	1	0.1–0.8 (0.4±0.1) n=198)	x	x
C	0.01–0.07 (0.04±0.01) n=227	1	0.01–0.07 (0.04±0.01) n=227	x	x

continued.

Type of call	Pulses/note*	Pulses/sec*	Minimum frequency (kHz)	Peak Frequency (kHz)	Maximum Frequency (kHz)
A	1–18 (9.0±3.0) n=527	142.8–681.8 (401.6±113.4) n=523	2.9–3.6 (3.3±0.2) n=20	3.6–4.8 (4.4±0.4) n=24	4.6–5.3 (5.0±0.2) n=24
B	11–95 (40.6±18.7) n=197	37.2–162.8 (93.5±28.1; n=197	0.5–3.2 (2.6±0.4) n=197	2.0–4.8 (3.4±0.4) n=197	4.1–6.2 (4.9±0.4) n=197
C	7–23 (15.2±3.9) n=34	219.5–793.1 (473.3±121.4) n=34	0.6–3.6 (2.7±0.4) n=227	2.5–4.8 (3.7±0.6) n=227	3.4–6.3 (4.5±0.6) n=227

Call type A has a series of pulsed notes with the following features: call with an ascendant amplitude modulation; duration 2.1–8.4 sec (6.1±1.3; n=24 calls); 23–75 notes (59.8±11.8; n=24 calls); notes emitted at a rate 8.8–11.0 notes/sec (9.9±0.9; n=24 calls); peak frequency 3.6–4.8 kHz (4.4±0.2; n=24 calls); maximum frequency at 95% of energy 4.5–5.3 kHz (4.9±0.2; n=24 calls); minimum frequency at 5% of energy 2.9–3.6 kHz (3.3±2.4; n=20 calls); note duration 3.0–46.0 ms (27.2±6.0; n=1250 notes); interval between notes 44.2–109 ms (74.8±13.9; n=1226 notes); 723 notes had poorly defined pulses (thus not quantified), those with well-defined pulses have 1–18 pulses/note (9.0±3.0; n=527 notes) emitted at a rate of 142–681.8 pulses/sec (401.6±113.4; n=523 notes); some notes presented sidebands artifacts.

Call type B has a single multipulsed note with the following features: usually an ascendant amplitude modulation on the first third/half of the call and descendant modulation on the ending of the call (see Fig. 5B); duration 0.12–0.8 sec (0.43±0.14; n=198 calls); 11–95 pulses (40.6±18.7; n=197 calls); pulses emitted at a rate 37.2–162.8 pulses/sec (93.5±28.1; n=197 calls); pulses usually emitted at lower rates at the first half of the call (see Fig. 5B); peak frequency 2.0–4.8 kHz (3.4±0.4; n=197 calls); maximum frequency at 95% of energy 4.1–6.2 kHz (4.9±0.4; n=197 calls); minimum frequency at 5% of energy 0.5–3.2 kHz (2.6±0.4; n=197 calls).
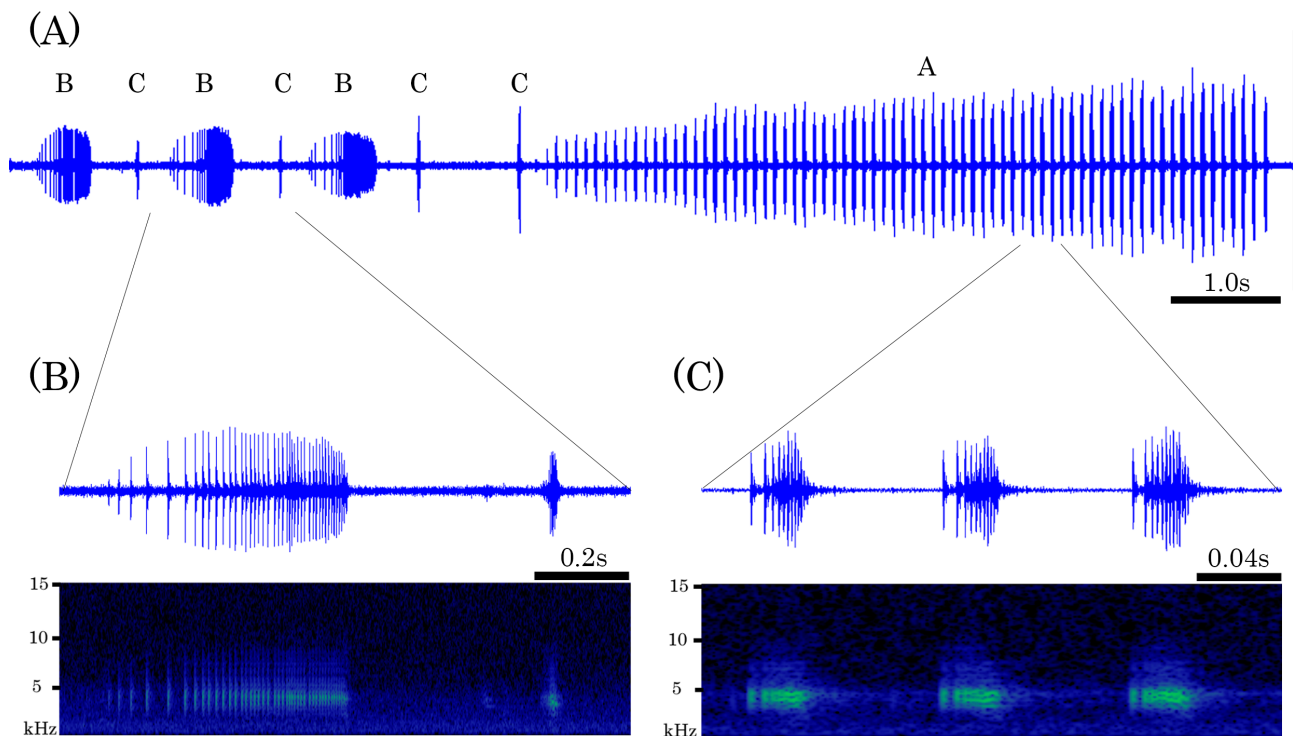
FIGURE 5. Vocal repertoire of *Crossodactylus timbuhy* recorded at the type locality Reserva Biológica Augusto Ruschi, Municipality of Santa Teresa, State of Espírito Santo, Southeastern Brazil (21° C): (A) oscillogram of a call series having one call type A, three calls type B, and four calls type C; (B) oscillogram and spectrogram of calls type B and C; (C) oscillogram and spectrogram of three notes in detail from call type A.

Call type C has a single multipulsed short note with: duration 0.014–0.073 sec (0.038±0.009; n=227 calls); peak frequency 2.5–4.8 kHz (3.7±0.6; n=227 calls); maximum frequency at 95% of energy 3.4–6.3 kHz (4.6±0.6; n=227 calls); minimum frequency at 5% of energy 0.6–3.6 kHz (2.8±0.5; n=227 calls); most of the notes had poorly defined pulses (thus not quantified), only 34 notes had clearly defined pulses (7–23; 15.2±3.8).

Natural history. Adults of *Crossodactylus timbuhy* called during the day from stream banks (on the ground, rocks, crevices, or perched on roots) and from the stream (on rocks, fallen branches and trunks or half-submerged). Calling activity was less frequent during colder days (ca. 15° C). Some individuals were found on the leaf litter about 200 m away from a stream. *Aplastodiscus weygoldti* (Cruz & Peixoto, 1987) was the only species breeding at the same stream portion. Tadpoles of *C. timbuhy* were in shallow (ca. 3 cm) and deep (ca. 40 cm) stream portions. Tadpoles were mostly hidden underneath the fallen leaves on the bottom.

Three males were observed regarding vocal sac inflation. Two replied to our playback stimuli, and another one was calling isolated. These three males emitted calls type B and C in very low amplitude, raising it progressively along with the call series. Males also displayed arms and legs lifting when calls were getting louder (see ZUEC-VID 981). Males usually end up with a call type A with no arms or legs lifting. During call type B, vocal sacs inflate partially. On the other hand, vocal sac inflation occurred during calls type A and C (males responding to playback stimuli called with only one vocal sac inflated; see ZUEC-VID 981).

Adult males (N=9) displayed five antipredator mechanisms (interrupt calling, escape, aggression, posture and aposematism) and five variations (jump away, swim, kick, death feigning and hidden aposematism). Interrupt calling was displayed by all calling males. During our approach, seven (N=7; 78%) males jumped away and two (N=2; 22%) swam for escaping. During handling, five (N=5; 56%) males kicked off the observer with the hind legs in an attempt to break free. Two (N=2; 22%) males displayed death feigning posture with the dorsum on the substrate and partially loose limbs, remaining in this position for about 1 min showing the bright coloration of the belly. Then, they returned to the regular position (dorsum up) and jumped away. Skin secretions and distress calls were not displayed during manipulation.

Discussion

Species identification. The only morphological difference between our samples and Pimenta *et al.* (2014) was the presence of paired lateral vocal sacs instead of a median subgular one. It is worth mentioning that Pimenta *et al.* (2014) described *Crossodactylus timbuhy* based only on external morphology of preserved specimens, which may had hampered the proper evaluation of this character. In fact, this lateral vocal sac observed in *C. timbuhy* was later reported as a synapomorphy for Hylodidae (Elias-Costa *et al.* 2017). Mitochondrial partial sequence of 16S rRNA comparison revealed very short genetic distances among topotypic and other sampled populations from the Municipality of Santa Teresa, and strong haplotypic relationship between them (0.1%; Table 1; Fig. 2). These genetic distances are safely below the threshold suggested by similar studies concerning species boundaries for hylodid species (Montesinos *et al. in prep*) and also for other anurans (3%; Fouquet *et al.* 2007). On the other hand, populations of *Crossodactylus* sp. from other Municipalities of Espírito Santo (Fig. 1; Table 1) revealed considerably high genetic distances compared to those from Santa Teresa (9.6%; Table 1), formed haplotypes related to each other and distinct from the other species (Fig. 2), and represent a new species (Montesinos *et al. in prep*).

Weygoldt (1986) reported two species of *Crossodactylus* for the Municipality of Santa Teresa: the abundant *C.* cf. *gaudichaudii* and the rare *C.* cf. *dispar*. Pimenta *et al.* (2014) described *C. timbuhy*, including specimens of *C.* cf. *dispar* from Weygoldt (1986). Because both species are indistinguishable regarding adult's external morphology (see Weygoldt 1986) and tadpoles or molecular data of *C.* cf. *gaudichaudii* are not known so far, it is required bioacoustic data to confirm identification. The advertisement call of *C.* cf. *gaudichaudii* has note emission rate (20 notes/sec; referred to as pulses/sec by Weygoldt 1986) twice higher than *C. timbuhy* (9 notes/sec; referred to as pulses/sec and *C.* cf. *dispar* by Weygoldt 1986).

Tadpole comparisons. Tadpole external morphology, oral features and coloration seem conserved in *Crossodactylus* species. Most *Crossodactylus* species, including *C. timbuhy*, have: ovoid or elliptical body shape; rounded snout; visible lateral line system (lateral line system absent only in *C. gaudichaudii*; see Francioni & Carcerelli 1993); dextral ventral tube; oral disc ventral with two lateral emarginations and a third one in the middle of lower labium; marginal papillae uniseriate and anteriorly interrupted; tooth row formula 2(2)/3(1) being the third posterior tooth row shorter than others; jaw sheaths developed and serrated.

Crossodactylus gaudichaudii (referred to as *C. aeneus* by Silva-Soares *et al.* 2015) and *C. werneri* distinguishes from other hylodid sympatric species by lacking a ventral depression anterior of the coiled intestine (ventral depression present in *Hylodes* spp.; see Haddad & Pombal 1995; Nascimento *et. al.* 2001; Pombal *et al.* 2002; Haddad *et al.* 2003; Costa *et al.* 2009, 2010; Laia *et al.* 2010) (see Silva-Soares *et al.* 2015; Vidigal *et al.* 2018). The present study is the first report on ventral depression for the genus *Crossodactylus* (Fig. 4C). Thus, we suggest this character should no longer be used to distinguish species of *Crossodactylus* from *Hylodes*. Furthermore, the present study is the first to report the presence of golden iridophores spread throughout the body and tail of living specimens (Fig. 3G).

Vocal comparisons. We assumed that call type A is the advertisement call, call type B is the territorial call (see Vidigal *et al.* 2018), and call type C has an unclear social context. Comparative data on advertisement vocalization of *Crossodactylus* species are provided in Table 4. Despite the little overlap, *C. timbuhy* calls have considerably more notes than *C. werneri* (23–74 notes in *C. timbuhy* and 12–25 in *C. werneri*). Interval between notes is shorter in *C. timbuhy* than in *C. werneri* and *C. franciscanus* (44–76 ms in *C. timbuhy* and 90–278 combining values from *C. werneri* and *C. franciscanus*). The advertisement call of *C. timbuhy* further differs from *C. werneri* by having notes emitted at a higher rate (8.8–11.0 notes/sec in *C. timbuhy* and 3.2–7.5 in *C. werneri*) and higher dominant frequency (3.6–4.8 kHz in *C. timbuhy* and 3.0–3.6 in *C. werneri*). Calls from *C. timbuhy* differ from *C. caramaschii*, *C. cyclospinus*, *C. franciscanus*, *C. schmidti* and *C. trachystomus* by lacking harmonic structures (present in these species; it is worth mentioning this feature depends on the quality of the records). Although frequency bands may be visible from some notes of *C. timbuhy*, we considered it as a sidebands artifact. These bands were noticed only in the second half of few notes where the pulse rate was higher to the point they could not be individualized. An increase in the amplitude modulation rate probably increases the frequency interval between sidebands (see Gerhardt 1998).

Natural history. Like other hylodid species, *Crossodactylus timbuhy* reproduces during daylight (e.g., Weygoldt & Carvalho-e-Silva 1992; Haddad & Giaretta 1999; Narvaes & Rodrigues 2005; Caldart *et al.* 2014). However, contrary to most hylodid, *C. timbuhy* was found only in shallow, slow-flowing streams (as observed for *C. werneri*; see Vidigal *et al.* 2018).

TABLE 4. Comparative acoustic traits of the advertisement call of *Crossodactylus* species with acoustic data available: *C. caramaschii* (Bastos & Pombal 1995); *C. cyclospinus* (Nascimento *et al.* 2005); *C. franciscanus* (Pimenta *et al.* 2015); *C. gaudichaudii* (Weygoldt & Carvalho-e-Silva 1992); *C. schmidti* (Caldart *et al.* 2014); *C. timbuhy* (present study); *C. trachystomus* (Pimenta *et al.* 2008); and *C. werneri* (Vidigal *et al.* 2018). Presence of harmonics (Harm), number of notes per call, call duration (sec), note duration (ms), internote interval (ms), notes per second, and dominant frequency (kHz).

Species	Harm	Notes/ call	Call dura- tion	Note dura- tion	Internote interval	Notes/s	Dominant frequency
C. caramaschii	y	49–69	4.7–6.1	x	x	10	~5
C. cyclospinus	y	35–98	3.6–6.2	3–40	29–65	x	3.4–5.4
C. franciscanus	y	36–84	4.8–11.6	6–20	90–184	x	2.3–4
C. gaudichaudii	x	25–130	2.0–13.0	40–50	40–50	x	2–5.5
C. schmidti	y	13–45	2.2–5.7	9–61	17–249	x	2–4.2
C. timbuhy	n	23–74	2.1–6.9	11–46	44.2–76.3	8.9–11.0	3.6–4.8
C. trachystomus	y	1–33	56–265	x	x	x	1.8–4.8
C. werneri	n	12–25	1.9–6.3	11–27	128–278	3.2–7.5	3–3.6

Hylodid species typically perform complex visual displays such as toe flagging, arms and legs lifting, arm waving, leg kicking, foot flagging, and throat display (e.g., Wogel *et al.* 2004; Hartmann *et al.* 2005; Silva & Benmaman 2008; Forti & Castanho 2012; Caldart *et al.* 2014; de Sá *et al.* 2016; Augusto-Alves *et al.* 2018; Furtado *et al.* 2019). *Crossodactylus timbuhy* displayed arms and legs lifting, but it is required further observation using mirror self-image presentation, picture or video presentation, or artificial models presentation to understand how these behaviour relate to different social contexts (see Pombal *et al.* 1994; Reicher & Höbel 2015).

The capacity of performing the unilateral sac inflation is known to occur only in *Hylodes* (see de Sá *et al.* 2016) with homoplasy in *Staurois guttatus* (Günther) (Ranidae; Elias-Costa & Faivovich 2019). Unilateral sac inflation was reported only from four species of *Hylodes*: *H. asper* (Müller) (Hödl *et al.* 1997); *H. heyeri* Haddad, Pombal, & Bastos, 1996 (Struett *et al.* 2021); *H. japi* de Sá, Canedo, Lyra & Haddad, 2015 (de Sá *et al.* 2016); and *H. meridionalis* (Mertens) (de Sá *et al.* 2018; Furtado *et al.* 2019). Based on morphological characters, Elias-Costa *et al.* (2017) proposed that unilateral sac inflation likely occurs in the other hylodids genera, although it has not been reported. Thus, our study provides the first record of unilateral sac inflation within *Crossodactylus*. Sac inflation in *C. timbuhy* was associated with calling and not only with visual display.

Distribution. Pimenta *et al.* (2014) stated that *C. timbuhy* occurs in Santa Teresa and Cachoeiro de Itapemirim, but did not cite any specimen voucher from the latter Municipality (see Appendix from Pimenta *et al.* 2014). The map provided by Pimenta *et al.* (2014) shows a second locality, but it clearly is not Cachoeiro de Itapemirim. Thus, we recognize *C. timbuhy* as endemic to the Municipality of Santa Teresa, State of Espírito Santo. Although Weygoldt (1989) reported the decline and possible disappearance of *C. timbuhy* (referred to as *C.* cf. *dispar*) in the region, Pimenta *et al.* (2014) mentioned that it could be found in different areas of Santa Teresa. We agree with Pimenta *et al.* (2014) as since 2005, we have often found *C. timbuhy* in preserved streams of Santa Teresa.

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