Among the world's smallest vertebrates: a new miniaturized Flea-Toad (Brachycephalidae) from the Atlantic rainforest

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The miniaturized toadlets of the genus *Brachycephalus* contains species of two morphotypes: those that are brightly colored with a bufoniform phenotype and others that are smaller, cryptic and with a leptodactyliform phenotype. The diversity of the leptodactyliform species is still underestimated and we generally lack basic information about their biology. However, due to recent sampling efforts, collecting samples for DNA analyses and recordings of the advertisement calls, we can now access the real diversity of this group. Thus, we describe a new species of *Brachycephalus* that is among the smallest vertebrates so far known. The new species is distinguished from its congeners by a combination of morphologic, bioacoustic, and genetic data. Although it is in the lowest bounds of body size of frogs (the second smallest amphibian species in the world), it surprisingly presents skeletal traits typical of larger frogs, including lacking the loss and synostosis of cranial bones typical of other miniature frogs, including other *Brachycephalus*. This description highlights how new discoveries in the megadiverse fauna of the Atlantic Forest, one of the richest biodiversity hotspots of the world, can reveal new information on phenotypes, including important variation in vertebrate body size. By describing this new species it will be possible to explore the previous hypothesis that the type series of *B. hermogenesi* is composed of two species, likely with individuals of the one we are hereby describing.

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Abstract

The miniaturized toadlets of the genus *Brachycephalus* contains species of two morphotypes: those that are brightly colored with a bufoniform phenotype and others that are smaller, cryptic and with a leptodactyliform phenotype. The diversity of the leptodactyliform species is still underestimated and we generally lack basic information about their biology. ~~However,~~ due to recent sampling efforts, collecting samples for DNA analyses and recordings of the advertisement calls, we can now access the ~~real~~ diversity of this group. ~~Thus,~~ we describe a new species of *Brachycephalus* that is among the smallest vertebrates ~~so far~~ known. The new species is distinguished from its congeners by a combination of morphologic, bioacoustic, and genetic data. Although it is in the lowest bounds of body size of frogs (the second smallest amphibian species in the world), it ~~surprisingly~~ presents skeletal traits typical of larger frogs, including lacking the loss and synostosis of cranial bones typical of other miniature frogs, including other *Brachycephalus*. ~~This~~ description highlights how new discoveries in the megadiverse fauna of the Atlantic Forest, one of the richest biodiversity hotspots of the world, can reveal new information on phenotypes, including important variation in vertebrate body size. By describing this new species it will be possible to explore the previous hypothesis that the type series of *B. hermogenesi* is composed of two species, likely with individuals of the one we are hereby describing.

Keywords: *Brachycephalus*, Brachycephalidae, miniaturization, Neotropical biodiversity, pumpkin toads, taxonomy.

Introduction

The complex anatomical structures of vertebrates, including multiple and highly structured organs, puts limits on their body size. The largest vertebrates to have ever lived were extinct archosaur reptiles, such as the colossal dinosaur *Argentinosaurus huinculensis* (likely > 30 m in length: Bonaparte & Coria, 1993), or the prehistoric whale *Perucetus colossus* (weighing > 300 tons: Bianucci et al., 2023). Among extant mammals, elephants and cetaceans are particularly notable for their size, with the Blue Whale (*Balaenoptera musculus*) being the heaviest and one of the largest animals ever (reaching > 32 m in length and ~ 150 tons: Sears & Perrin, 2009). These gigantic creatures face physiological constraints, such as blood pumping, and morphological limitations, such as weight support, that impose a limit on their growth (Vermeij, 2016; Goldbogen, 2018).

Likewise, the smallest known vertebrates, such as the freshwater cyprinid fish *Paedocypris progenetica* (the smallest mature individual reported was 7.9 mm in total length: Kottelat et al., 2006) and direct-developing frogs, as *Paedophryne amauensis* (the smallest adult individual with a snout–vent length [SVL] of 7.0 mm: Rittmeyer et al., 2012) and *Brachycephalus pulex* (the smallest adult individual with a SVL of 6.5 mm: Bolaños et al., 2024), also present unique traits related to their extreme sizes, including the loss, reduction, and/or fusion of bones (Trueb & Alberch, 1985; Hanken & Wake, 1993; Scherz et al., 2019). Among amphibians, the Neotropical genus *Brachycephalus* (pumpkin toadlets and flea toads; Anura: Brachycephalidae) is noteworthy for containing several of the smallest vertebrates, including species that reach maturity at less than 1 cm SVL (Rittmeyer et al., 2012; Bolaños et al., 2024). The skeletons of species of *Brachycephalus* exhibit bone fusions in both the skull and postcrania (Rittmeyer et al., 2012; Izecksohn, 1971; Bornschein et al., 2016), loss of skull bones (Rittmeyer et al., 2012; Yeh, 2002), fewer digits and phalanges (Rittmeyer et al., 2012; Izecksohn, 1971; 1988; Yeh, 2002; Pombal Jr. et al., 1998), loss of vomerine or maxillary teeth (Izecksohn, 1971) but sometimes having maxillary odontoids (Ribeiro et al., 2017), loss of middle ear bones and an external tympanum (Goutte et al., 2017), and having miniaturized semicircular canals (Essner Jr. et al., 2022). In addition, *Brachycephalus* species typically lack metacarpal, metatarsal, and subarticular tubercles (Pombal Jr. & Izecksohn, 2011), enervation connecting the inner ear to the brain (Goutte et al., 2017), a complete atrium septum, and a carotid body (Carrasco-Medina et al., 2023). All species are also thought to have direct-development with reduced fecundity and large eggs (Trueb & Alberch, 1985; Hanken & Wake, 1993). All these traits are associated with the evolutionary reduction in body size, i.e., miniaturization (Hanken & Wake, 1993; Zimkus et al., 2012).

The miniaturized body of these toadlets constrains their morpho-physiology, ecology, and life history, as well as impacts their detectability in the wild and taxonomic identification. These tiny toadlets are hard to detect in the field because, apart from the bright colored species ('bufoniform' species: Lyra et al., 2021), the smallest species are generally cryptic ('leptodactyliform' species: Lyra et al., 2021; or the ‘*B. didactylus* species group’: Ribeiro et al., 2015), matching the background coloration pattern (Rebouças et al., 2019). None of these major phenotypes are monophyletic (Condez et al., 2020; Lyra et al. 2021), and both the lack and convergence of diagnostic traits resulting from miniaturization makes taxonomic identifications challenging (Hedges et al., 2008; Rittmeyer et al., 2012). Despite these challenges, documenting the diversity of miniaturized vertebrates provides important opportunities for research on the anatomical evolution of vertebrates. For example, by understanding the lower limits of organ sizes, we gain insight into their operation and putative functions (Hanken & Wake, 1993; Essner et al., 2022).

Among the 'leptodactyliform' species a recent review made clear that *B. hermogenesi*, *B. sulfuratus* and a still ~~not~~ described species (*Brachycephalus* sp.) are morphologically cryptic and ~~could~~ only be diagnosed through ~~its~~ advertisement call~~s~~ traits (Bornschein et al., 2021). Furthermore, it was suggested that the type series of *B. hermogenesi* might contain individuals of the new species (Bornschein et al., 2021). Therefore, based on additional collections in the region where both species occur (*B. hermogenesi* and the new ~~one~~), we describe a new flea-toad, a leptodactyliform species of the genus *Brachycephalus*, with adults that are among smalle~~r~~ vertebrates ever described.

Materials & Methods

Specimens and field sampling

We conducted fieldwork at the Projeto Dacnis private reserve (Ubatuba, São Paulo, 23°27'38.92"S, 45° 8'24.48"W, WGS84), located in the Brazilian Atlantic Forest Domain. The predominant vegetation cover in the region is formed mainly by secondary Alluvial Ombrophilous Dense Forest and Submontane Ombrophilous Forest. The annual mean temperature is 22.5 °C and the annual mean rainfall is 2,552 mm^3 (Alvares et al., 2013).

The specific site where the individuals were found, within the Projeto Dacnis reserve, was monitored from June 2021 to May 2022. Diurnal visits occurred twice each week and nocturnal visits once every 15 days. The area was sampled using visual and auditory search methods. The visual sampling consisted of careful removal and analysis of the leaf litter at the specific site and at different locations in the Projeto Dacnis reserve.

Collected specimens were deposited at the amphibian collection (ZUEC-AMP) of the Museu de Diversidade Biológica (MDBio), Universidade Estadual de Campinas (Unicamp), Campinas, state of São Paulo, Brazil, and Coleção de Anfíbios do Laboratório de Herpetologia (HCLP-A), Universidade Estadual Paulista, São Vicente, state of São Paulo, Brazil.

External morphology and morphometry

We took 14 body measurements, following Duellman (1970), Heyer et al. (1990), and Napoli (2005): snout–vent length (SVL), head length (HL), head width (HW), eye to nostril distance (END), interorbital distance (IOD), eye diameter (ED), nostril diameter (ND), upper arm length (UAL), forearm length (FAL), hand length (HAL), tibia length (also called shunk length; TBL), thigh length (THL). We also measured foot length, including the tarsus (FL) and without the tarsus (fL). We followed Heyer et al. (1990) for morphological terminology of the snout shape. The measurements were taken with a digital caliper (precision 0.01 mm) and with a micrometric eyepiece attached to a stereo microscope (Leica S9). Examined specimens are listed in Appendix I.

Internal anatomy: organs

One adult female (ZUEC-AMP 25273) was submitted to a dose of 2 % lidocaine hydrochloride anesthetic solution, through administration via skin contact. Dissections were made immediately after the anesthesia procedure (Iuliis & Pulerà, 2006; Sebben, 2007). The individual was then immersed in an isotonic saline solution (NaCl 0.65 %) to allow heart contractions and continuous blood flow as long as possible (enabling blood vessels observation and description). The saline solution guarantees the maintenance of the morphophysiological characteristics of the organs and avoids unwanted reflexes that would otherwise prevent visualization. A Pasteur pipet was used to remove blood, skin, and/or candle wax that could interfere with the observations[48]. Photographs and videos were taken using a stereoscopic microscope (Leica S9), an Asus_A001D cell phone, and a Canon 5D Mark III camera coupled with a Sigma 105 mm lens. Visual files were deposited in MDBio with the voucher numbers ZUEC-PIC 699 (pictures) and ZUEC-VID 998 (videos).

Internal anatomy: skeleton

We generated high-resolution X-ray computed tomography (microCT) scans at the University of Florida’s Nanoscale Research Facility. We scanned the entire body of two specimens of the new species (ZUEC-AMP 24978 and 24979) and one of *Brachycephalus hermogenesi* (ZUEC-AMP 24980) using a GE v|tome|x m with a 240 kV microfocus x-ray tube with a tungsten target with the following settings: 60 kV, 110 mA (250 mA for 24979), a 333 ms detector time (500 ms for 24979) and no filters (see Table S2 for scanning parameters). All scans averaged three images per rotation with a skip of one, and a voxel resolution of 5–7 µm. We processed raw X-ray data using GE’s datos|x software v.2.3 to produce tomogram images that were then viewed, segmented, and analyzed using VGStudioMax 2023.1 (Volume Graphics, Heidelberg, Germany). To produce figures showing the skeleton in VGStudio Max, we used the paintbrush tool with grayscale threshold, and the region growing tool, with enhanced lighting and shadows enabled to create a sense of depth. We use the organization of the osteological description following Ribeiro et al. (2017), which uses the terminology of Trueb et al. (2011) and Gómez & Turazzini (2015); we refer to the manual digits as I–IV rather than II–V to avoid confusion for most taxonomists. We compared the skeletons to existing datasets of species from the *Brachycephalus ephippium*, *B. pernix*, and *B. vertebralis* species groups as well as all flea-toads except for *B. pulex* and *B. puri*. Examined specimens for CT-scan data are in Appendix II.

Bioacoustics

We used a Zoom H2n recorder with 16 bits of resolution and 44.1 kHz to record the calls. We conducted all sound analyses in Raven Pro v.1.6.3. We performed bioacoustic analyses using the following spectrogram settings: window type = Hann, window size = 512 samples, hop size = 2.90 ms, overlap = 75 %, DFT size = 1024 samples, grid spacing = 43.1 Hz. We obtained the sound graphics using Raven software, and used the following spectrogram parameters: Hanning window, FFT = 512, and 70 % overlap. We measured temporal and spectral parameters directly from the oscillogram and spectrogram, respectively, using parameters from Raven Pro, following Köhler et al. (2017): call duration, inter-call interval, note duration, inter-note interval, note rate, number of pulses per note, dominant frequency, minimum frequency (frequency 5%), maximum frequency (frequency 95%), and frequency bandwidth (90%). We adopted the note-centered approach to describe the calls (*sensu* Köhler et al. 2017). All analyzed recordings of the new species are deposited at Fonoteca Neotropical Jacques Vielliard (FNJV), MDBio (FNJV 45418–30; 51341; 51346–8; not all males were collected).

To compare the advertisement call of the new species with that of *B. hermogenesi*, we made new recordings at its type locality, Parque Estadual da Serra do Mar, Núcleo Picinguaba, Ubatuba (FNJV 51343), as well as in the Projeto Dacnis reserve area (FNJV 51344–5), in Natividade da Serra (FNJV 51349), and reexamined the advertisement call from Estação Biológica de Boraceia, Salesópolis (FNJV 32769), all in the state of São Paulo, Brazil. Also, we used data provided by Verdade et al. (2008) for *B. hermogenesi* (FNJV 32769; from Salesópolis, SP), by Condez et al. (2016) for *B. sulfuratus* (FNJV 34498; from São Francisco do Sul, SC), and compared our data with that provided for *B. hermogenesi*, *B. sulfuratus* and the new species in Bornschein et al. (2021). The concept of attenuated notes follows Bornschein et al. (2021). Recordings were also used to confirm species distributions.

Molecular data

We extracted total genomic DNA from ethanol-preserved muscle or liver tissues from individuals collected at Projeto Dacnis reserve, using both DNeasy Tissue Kits (Qiagen Inc., Valencia, CA, USA) or standard salt precipitation methods (Lyra et al., 2021). We amplified two overlapping fragments of the mitochondrially encoded 16S rRNA (16S). The polymerase chain reactions (PCR) conditions and primers used are given in Table S3. All reactions were purified using Wizard® SV PCR Clean-Up System (Promega), following manufacturer's recommendation, or using a mix of thermosensitive alkaline phosphatase (FastAP) and Exonuclease I, as described in Lyra et al. (2017). The amplified products were sequenced in both directions by Macrogen Inc. (Seoul, South Korea), with a Big Dye v.3.0 Sequencing Kit (Applied Biosystems). We trimmed for quality, excluded primers sequences, and assembled consensus sequences using Geneious Prime® 2023.2.1 (Biomatters Ltd.). GenBank accession numbers and voucher information is available in Table S4.

For the phylogenetic inference, we aligned the newly generated sequences with representative 16S sequences of all species of *Brachycephalus* available in GenBank, including putative non-described species, and one individual of *Ischnocnema henselii* as outgroup, totalizing 99 individuals (Table S4). We aligned sequences with MAFFT v.7.427 (Katoh & Standley, 2013) using E-INS-i algorithm in Geneious Prime® 2023.2.1. Phylogenetic analyses were performed under Bayesian Inference (BI) using BEAST v2.7.1 (Bouckaert et al., 2019) under a birth-death tree prior, assuming a strict clock and using substitution rates of the GTR model. The analyses consisted of two independent runs, each with eight chains, for 100 million generations; parameters and trees were sampled every 1000 generations and we discarded the first 25% iterations as burn-in. We assessed stationarity, convergence between runs, and effective sample sizes (> 200) with Tracer (v.1.7; Rambaut et al., 2018). The log files of the independent runs were combined using LogCombiner (v.2.7; Bouckaert et al., 2019), and we extracted the majority-rule consensus tree in TreeAnnotator (V.2.7[53]). The resulting tree was visualized using FigTree 1.4.3 (Rambaut et al., 2018).

We estimated the uncorrected p-distances between the new species and the closest relatives using the 16S rRNA gene fragment limited by the primers 16Sar-L and 16Sbr-H ("Palumbi fragment"; Palumbi et al., 1991), using Mega 11 (Tamura et al., 2021; Stecher et al., 2020) and treating missing data and gaps as pairwise deletions. Estimates were done using the alignment generated in MAFFT v.7.25. For comparative purposes, we also estimate uncorrected p-distances using the Palumbi fragment for all available *Brachycephalus* species.

Species delimitation approach

We found two distinct vocalizations during field activities in an area where only *B. hermogenesi* was known (Projeto Dacnis private reserve, municipality of Ubatuba, state of São Paulo). It also matches the bioacoustic findings reported by Bornschein et al. (2021). Our next step was to address different lines of evidence (vocalization, DNA sequence data, and morphology) to determine whether two different species were present. First, we recorded individuals of the genus *Brachycephalus* in the type locality of *B. hermogenesi* (Picinguaba, Ubatuba; Giaretta & Sawaya, 1998); no other congeners were found at that site. Since *B. hermogenesi* and the new species described here are morphologically similar but can be distinguished by their unique advertisement call and DNA sequences, we used this information to delimitate both species. We assigned the calls recorded from individuals from Picinguaba to *B. hermogenesi* and described the new species. To avoid misidentification, the comparisons were based solely on individuals that had been sequenced or had their advertisement call recorded.

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix http://zoobank.org/. The LSID for this publication is: urn:lsid:zoobank.org:pub:115413F3-9005-4006-863C-EA4BAD3C58AD, and for the new taxon is: urn:lsid:zoobank.org:act:18F93070-2CAE-4817-

8CCD-48C0C5DA5210. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central SCIE and CLOCKSS.

Ethics and permits

Samplings and individual euthanasia were performed in accordance with relevant guidelines and regulations. Sampling permits were provided by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, SISBio #51898-1). Specimens' collection and deposit in scientific collections followed Brazilian animal care guidelines and were previously approved by the University of Campinas (Unicamp) animal care committee (CEUA IB/CLP #03/2020). The access of genetic information was also registered at the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGen #A74AD8B).

Results

Brachycephalus dacnis sp. nov. (Fig. 1)
Brachycephalus sp. (Bornschein et al., 2021)
LSID: urn:lsid:zoobank.org:act:18F93070-2CAE-4817-8CCD-48C0C5DA5210

Etymology – The specific epithet name '*dacnis*' honors the Projeto Dacnis private reserve and NGO that has supported biodiversity research since 2010 in the municipalities of São José dos Campos, Miracatu and Ubatuba (where the new species was discovered), state of São Paulo, Brazil. The name is used as an invariable noun in apposition to the generic name.

Holotype – Adult male (ZUEC-AMP 24982) collected and recorded (FNJV 51341) at Projeto Dacnis reserve, municipality of Ubatuba, state of São Paulo, Brazil, on 18 May 2021 by A. Mariano (Fig. 1; Table 1). This individual had also its DNA sequenced.

Paratypes – Eleven adults, only considered those confirmed by DNA and/or call traits: 03–09 August 2019 by R. Mitsuo and R.C. Menegucci (HCLP-A 267–68; heard, but not recorded); 10 March 2021 by E. Muscat and L.M. Botelho (ZUEC-AMP 24978; sequenced); 05 May 2021 by L.M. Botelho (ZUEC-AMP 24984; sequenced); 18 May 2021 by A. Mariano (ZUEC-AMP 24979; recorded); 24 May 2021 by E. Muscat and L.M. Botelho (ZUEC-AMP 24981; recorded and sequenced); 21, 22 February 2022 by L.M. Botelho (ZUEC-AMP 25270; 25272; 25274–75; sequenced); 14 April 2022 by E. Muscat, L.M. Botelho, L.A.O.S Ferreira (adult male; ZUEC-AMP 25612; Fig. 2; recorded). All paratypes were collected at the same locality as the holotype. Sex determination was not possible in most cases, except for the individual that was dissected (a female; see below) and the calling males.

Diagnosis – The new species is assigned to the genus *Brachycephalus* because of its miniature body size, fewer phalanges and toes than a typical frog, fingers and toe tips not expanded but apically pointed, and toes lacking circumferential grooves. The new species can be diagnosed from its congeners by the following combination of characters: (1) “leptodactyliform” body shape; (2) adult body length (SVL) smaller than 1 cm; (3) distinct and functional toes II and V; (4) presence of vestigial fingers I and IV; (5) distinct iris; (6) absence of dark markings on the skin over the pectoral region; (7) dark black or pale brown marbled venter with small white blotches in preserved specimens; (8) and advertisement call composed of one or two multi-pulsed (3–7 pulses) note with dominant frequency between 8.01 and 8.44 kHz, note duration between 0.03–0.08 s (when isolated), up to 0.41 s (when in pairs), and absence of attenuated notes.

Holotype description – Body slender and leptodactyliform (Fig. 1; SVL = 7.55 mm); head as wide as long (1.78 mm; Table 1), and one-fourth of total body length (HW/SVL = HL/SVL = 23.6%); snout short and slightly mucronate in dorsal view and between rounded and vertical in lateral view; nostrils oval, not protuberant and directed anterolaterally; *canthus rostralis* distinct; loreal region slightly concave; lips nearly sigmoid; eyes slightly protruding in dorsal and lateral view, and directed anterolaterally, 27.5% ED/HW and ED/HL (as HW = HL); tympanum indistinct; vocal sac not externally expanded; vocal slits present; tongue longer than wide, with posterior half free; choanae small and rounded; vomerine teeth absent. Forearm more than twice (2.3 times) length of upper arm length; hand length shorter than forearm or upper arm lengths; hands with all fingers distinct; fingers I and IV externally vestigial; fingers II and III robust; tips of fingers I and IV (when visible) rounded, fingers II and III pointed; relative finger length I ≤ IV < II < III; subarticular tubercles absent; inner and outer metacarpal tubercles absent. Leg relatively long, total leg length (TBL + THL + FL = 12.1 mm) 160 % of SVL (7.6 mm); shank slightly shorter than thigh (SHL/SVL = 40.6 % and THL/SVL = 47.5 %); foot longer than tarsus and the shank; toe I externally absent; toes II, III, IV and V externally distinct and functional. Skin smooth without dermal ossifications. Measurements of holotype presented in Table 1.

Color in life of the holotype – The general coloration of the dorsal body surface is yellowish-brown. A dark brown stripe extends laterally from the tip of the snout to the flanks. Dorsal surfaces of arms and legs interrupted by dark brown stripes. An x-shaped or dorsal mark is present. The lateral view of the body is dark brown; the region overlying the upper jaw is dark brown with distinct white spots. The ventral body surface is brown with small white blotches. The ventral surface of the hands and feet are dark brown with white blotches over each phalanx of the fingers and toes. The pupil is black, and the iris is bronze.

Color variation – Color and ornamentation patterns in this species vary considerably among individuals. Some frogs have a leaf-like dorsal coloration, while others have a rock-like dorsal coloration (Fig. 3). We did not notice significant variation in the ventral pattern of the individuals.

Color in preservative of the holotype (less than one year in preservative) – The general coloration of the dorsal surface is reddish-brown (Fig. 1). The white spots overlying the upper jaw and ventral region can become pale cream; no *linea masculina* is visible. The stripes on the arms and legs become more evident. The extremities of the fingers and toes become pale cream.

Internal anatomy: organs (ZUEC-AMP 25273; female) – After cutting the skin, the transparent abdominal muscles were observed with a slight dark-brown coloration (melanocytes). Beneath the musculature, the liver and small intestine were also observed with similar dark-brown pigmentation. However, it was more prominent in the intestine than in the ventral musculature and liver (Fig. 4A). After removing the abdominal muscles and opening the pericardium, we observed the heart to have melanocytes scattered throughout the atria, ventricle, *conus arteriosus*, and *truncus arteriosus*. After cutting the pectoral girdle in the medial region of the weakly mineralized procoracoid cartilages, we observed that the dark pigmentation extends from the cranial region of the heart to the carotid and systemic arteries. We did not see this pigmentation in the pulmocutaneous artery (Fig. 4b, c). A slight extension of this dark pigmentation occurs in the musculature lateral to the systemic and carotid arteries (Fig. 4b, c). Near the bifurcation of the internal and external jugular arteries and between the systemic and carotid arteries, there were two black, ovoid parathyroid glands on each side (Fig. 4c). Less black pigmentation was observed in the thoracic region; however, a few small spots were observed close to the white eggs and in the oviduct. The oviduct was transparent and between the left and right oviduct two mature eggs were observed, and each oviduct joined with its counterparts near the pubis (Fig. 4d, e).

Internal anatomy: skeleton (ZUEC-AMP 24978; undetermined sex). The skeleton of *B. dacnis* is broadly similar to that of other small species in the genus, including by lacking osteoderms and having unornamented dermal skull bones that are not co-ossified to the skin. This individual (Fig. 5) is osteologically mature with ossified mesopodials and complete, ossified distal long bones; the specimen is missing the right leg, extracted for DNA analysis.

The skull is compact, slightly longer than wide, and lacking ornamented dermal roofing bones. The frontoparietals, sphenethmoids, prootics, exoccipitals, and parasphenoid are incompletely synostosed with each other and often have clear separations between the bones. There are well-defined and bony margins to both the optic fenestrae and prootic foramina. The premaxillae are broad, clearly separated from one another, and have teeth; each has a robust *pars dentalis*, and a robust alary process that is approximately as tall as wide and widely separated from the nasal. In ventral view, the maxillae are nearly straight and bear odontoids on the anterior third. The quadratojugals are thin and angled dorsally, and do not articulate with the maxillae. The pterygoids are slender, each with a long, straight, and forked anterior ramus that approaches but does not articulate with the adjacent maxilla, a short, subtriangular posterior ramus adjacent to but not articulating with the ventral ramus of the squamosal, and a short, broad medial ramus that is well separated from the prootic. Distinct neopalatines are not observable and likely not synostosed to the sphenethmoid. Distinct, small c-shaped vomers are present bordering the anterior margins of the choanae. Tall, thin, and curved septomaxillae are present at the anterior margin of the nasal capsule. The parasphenoid is triradiate with a long, rectangular anterior ramus. The squamosals are robust, and each has a prominent zygomatic ramus that is expanded ventrally as a sheet-like flange along its anterior margin and a long, slender posterior ramus that is adjacent but not fused to the prootic. The portions of the prootic are not completely synostosed and the broad fenestra ovalis contains a small, irregularly shaped operculum; a stapes (or columella) is not present. The posteromedial processes of the hyoid are ossified and slender. The arytenoid cartilages are not obviously mineralized.

There are eight distinct, procoelous, non-imbricating presacral vertebrae that are not fused to one another. The atlas lacks transverse processes and cotyles that are widely separated, a distance greater than the posterior diameter of the atlas centrum. Transverse processes of presacral II are distinctly shorter than those of III–VIII. The sacrum is procoelous with stout transverse processes. A sesamoid at the distal transverse process of the sacrum is present at its articulation with the ilium. The urostyle bears a tall dorsal ridge that decreases in height posteriorly along the anterior two-thirds of the urostyle.

The pectoral girdle is not heavily ossified. The coracoids that are expanded medially but do not meet at the midline. The coracoids are thin and articulate at the midline with a small, irregularly shaped element that may represent the omosternum. The scapula is short with a well-developed anterior process and a prominent supraglenoid foramen. The epicoracoid and procoracoid cartilages are weakly mineralized and do not form solid elements synostosed to the coracoid and clavicle.

The pelvic girdle is a robust element composed of synostosed ilium, pubis, and ischium; the publis and ischium appear to be incompletely ossified. The circular acetabulum is incompletely ossified but has well-defined margins. The shaft of the ilium is stout and mostly straight in both lateral and dorsal views, and has both a well-developed dorsal protuberance and a weakly developed dorsal crest. There is a broad ventral acetabular expansion comprising both the ilium and ossified pubis, but it is incompletely ossified.

The forearm is somewhat shorter than the humerus. The distal carpals (Element Y and II–V) are fused. The radiale and ulnare are large and subequal in size. The phalangeal formula for the manus is 1–2–3–1. There appears to be a single, minute ossified prepollex. There is one small palmar sesamoid. The tips of the terminal manual phalanges are arrow-shaped in digits II and III but blunt and globular in I and IV. The tibiofibula and femur are similar in length, and a small ossified fabella sesamoid near their articulation. There are two large distal tarsals, and there is an ossified tarsal sesamoid near their articulation with the tibiofibula. The phalangeal formula for the pes is 1–2–3–4–2 and there is a single small ossified prehallux. Medially, there are two small plantar sesamoids, whereas laterally there are two large plantar sesamoids. The tips of the terminal pedal phalanges are arrow-shaped in digits I–IV but blunt in V.

Calls and calling activity – The new species is vocally active throughout the year, with peaks of activity when the humidity was higher than 90 % (> 50 males calling). The calling activity was mostly detected during daytime, with a higher number of individuals calling between 6:00 and 11:00 AM, from 3:00 to 6:00 PM, or anytime of the day after heavy rains. From August to April, we also heard calls during the night. Males frequently ceased calling for a few minutes after detecting the researcher's approach.

The advertisement call of *B. dacnis* (Fig. 6b) can be simple or complex, respectively containing one or two multipulsed (3 to 7 pulses) notes, emitted in mean intervals of 2.1 ± 0.32 s (1.67 – 2.69). Out of the 17 recordings made, only 2 of them presented calls with two notes. The mean note duration was 0.05 ± 0.01 s (0.03–0.08), and the call with 2 notes could reach up to 0.41 s. The call frequency varied from 6.89 (minimum measured frequency) to 8.96 (maximum measured frequency) kHz, while the mean dominant frequency was 8.22 ± 0.1 kHz (8.01–8.44) (Table 2).

Phylogenetic inference and genetic distances – Our phylogenetic analysis supported the distinction of *B. dacnis* from all the other members of the genus. All the specimens are clustered within a strongly supported clade (posterior probability, PP = 1.0). The new species is recovered as the sister species of *B. hermogenesi*, and this clade sister of *B. sulfuratu*s + *B. pernix* species group (Fig. 6c; Fig. S1, Table S4). The phylogenetic inference recovered *B. pulex* sister to two diverse clades, one containing ((*B. puri* + two putative distinct species) + (*B. didactylus* + (*B. clarissae* + (*B. vertebralis* species group + *B. ephippium* species group)) and the other containing ((*B. hermogenesi* + *B. dacnis*) + (*B. sulfuratus* + *B. pernix* species group) (Fig. 6c, Fig. S1, Table S4). The 16S uncorrected genetic distance between the new species and *B. hermogenesi* ranged from 6.8 to 7.8 % (mean 7.1 %; Table S1). These distances are higher than distances found between species of *B. ephippium* and *B. vertebralis* species groups (3.2 to 5.9 %, Table S1). The relationships between species within each of the species' groups containing the bright colored species (*B. ephippium*, *B. vertebralis* and *B. pernix* species groups) are in general low supported (Fig. S1). Furthermore, some species were not recovered as monophyletic (i.e., *B. pitanga* and *B. guarani*; *B. mariaeterezae* and *B. olivaceus*; Fig. S1) and the genetic distances estimated were extremely low, less than 1% (Table S1).

Comparison with other species – *Brachycephalus dacnis* is distinguished from all species of the *B. ephippium* (*B. atelopoide*, *B. darkside*, *B. ephippium*, *B. garbeanus*, *B. ibitinga*, *B. margaritatus*, and *B. rotenbergae*), *B. vertebralis* (*B. alipioi*, *B. bufonoides*, *B. crispus*, *B. guarani*, *B. herculeus, B. nodoterga*, *B. pitanga*, *B. toby*, and *B. vertebralis*), and *B. pernix* (*B. actaeus*, *B. albolineatus*, *B. auroguttatus*, *B. boticario*, *B. brunneus*, *B. coloratus*, *B. curupira*, *B. ferruginus*, *B. fuscolineatus*, *B. izecksohni*, *B. leopardos*, *B. mariaeterezae*, *B. mirissimus*, *B. olivaceus*, *B. pernix*, *B. pombali*, *B. quiririensis*, *B. tabuleiro*, *B. tridactylus*, and *B. verrucosus*) species groups by the combination of its small adult body size, a leptodactyliform body form (*vs.* bufoniform), and a dark-brown coloration (*vs.* vibrant coloration such as greenish, yellowish, orange or reddish) (Ribeiro et al., 2015; Lyra et al., 2021). *Brachycephalus dacnis* is also distinguished from species of the *B. ephippium* and *B. vertebralis* species groups by its the smooth skin, distinct frontoparietals, nasals, vomers, and parasphenoid that are not synostosed via a bony sphenethmoid (Figs. 5, 7), presence of a quadratojugal, robust anterior process of the pterygoid, lack of hyperossification in the skull, distinct coracoid and clavicle and lack of mineralized epicoracoid cartilages, lack of vertebral fusions, lack of bony plates in the axial skeleton, and presence of phalanges on all digits (Lyra et al., 2021; Clemente-Carvalho et al., 2009; 2012; Condez et al., 2014; 2016; Haddad et al., 2010; Pombal Jr., 2010). From the other six leptodactyliform species, a.k.a. flea-toads (*B. didactylus*, *B. clarissae*, *B. hermogenesi*, *B. pulex*, *B. puri*, and *B. sulfuratus*), *B. dacnis* is distinguished by skin texture, toe morphology, coloration, and some skeletal traits. From *B. clarissae*, *B. dacnis* is distinguished by having a smooth dorsal texture (granular in *B. clarissae*), a distinct and functional toe V (vestigial in *B. clarissae*), lacking synostosis among cranial bones (sphenthmoids, parasphenoid, and frontoparietals synostosed in *B. clarissae*; Figs. 5, 7), phalanges on pedal digits I and V (lacking in *B. clarissae*), lacking mineralized epicoracoid cartilages (heavily mineralized and fused with pectoral bones in *B. clarissae*), and by having a distinct iris (indistinct in *B. clarissae*) (Folly et al., 2022). *Brachycephalus dacnis* has a distinct, and functional toe V (like *B. sulfuratus* and *B. hermogenesi*), whereas it is vestigial in *B. didactylus* (Haddad et al., 2010; Giaretta & Sawaya, 1998). Neither *B. dacnis* nor *B. hermogenesi* lack phalanges on the toes, whereas both *B. didactylus* (pedal digit count: 0, 2, 3, 4, 0) and *B. sulfuratus* (0, 2, 3, 4, 2) lack phalanges on one or more digit. In *B. didactylus*, there is a trapezoidal chest mark, which is absent in *B. dacnis* (Almeida-Silva et al., 2021), as well as a fully synostosed braincase (Fig. 7), a wire-like anterior process of the pterygoid, no quadratojugal, heavily mineralized epicoracoid cartilages fused with the pectoral bones, fused presacral vertebrae VI, VII, and VIII, and no phalanges on manual digit I or IV. From *B. sulfuratus*, *B. dacnis* is distinguished by the absence of an inverted V-shaped chest mark (Condez et al., 2016). Although this trait varies in *B. sulfuratus* (see Bornschein et al. 2021), it still can distinguish some of its individuals. From *B. sulfuratus*, *B. dacnis* is distinguished by its thin quadratojugal (Fig. 7), and medially expanded coracoid. From *B. puri*, *B. dacnis* is distinguished by having a distinct toe II (reduced in *B. puri*), by its dark black or pale brown marbled venter with small white blotches (uniformly brown in *B. puri*), and by sometimes having an X-shaped dorsal mark (inverted V-shaped in *B. puri*) (Almeida-Silva et al., 2021). From *B. pulex*, *B. dacnis* is distinguished by the presence of vestigial fingers I and IV (absent in *B. pulex*), the presence of distinct and functional toe II and V (absent or vestigial, respectively, in *B. pulex*), and lacking a chest mark (presence of an inverted V-shaped chest mark in *B. pulex*) (Almeida-Silva et al., 2021). *Brachycephalus dacnis* and *B. hermogenesi* are morphologically similar, including in their skeletons (Figs. 5, 7), but can be recognized as distinct through acoustic and genetic traits.

The advertisement call of *B. dacnis* is distinguished from *B. hermogenesi* and *B. sulfuratus* by generally having fewer notes per call (1 or 2 notes per call in *B. dacnis*, 2–6 in *B. hermogenesi*, 4–6 in *B. sulfuratus*), shorter note duration (≤ 0.08 s in *B. dacnis*; ≥ 0.17 s in *B.*

hermogenesi, and ≥ 1.5 s in *B. sulfuratus*), and shorter call duration (≤ 0.08 s in *B. dacnis* in calls with one note and ≤ 0.41 s in calls with two notes; ≥ 0.41 s in *B. hermogenesi*, and ≥ 0.13 s in *B. sulfuratus*). *Brachycephalus dacnis* generally presents fewer pulses per note (3–7) than *B. sulfuratus* (7–11; Condez et al., 2016), and higher than *B. hermogenesi* (1–3) (see also Bornschein et al. 2021). The absencez of attenuated notes (*sensu* Bornschein et al. 2021) in the calls of *B. dacnis*, distinguishes it from *B. hermogenesi* (which can present attenuated advertisement call notes) (see Bornschein et al. 2021). The dominant frequency of the advertisement call of *B. dacnis* overlaps with that of *B. hermogenesi*, but it is higher than that of *B. sulfuratus*. However, the minimum and maximum frequencies of the call of *B. dacnis* are lower and do not overlap with those of *B. hermogenesi*. Call parameters of these three species are presented in Table 2.

Natural history – We observed *Brachycephalus dacnis* occupying areas with dense vegetation, tall canopy, reduced sunlight incidence, a thick layer of litter, and moist soil. These areas lie between small hills, forming a valley. The leaf litter along the slopes lies in small clusters at the base of trees and creeping plants. These areas have a small stream nearby with a substrate composed of rock and sand. Water flow intensity varies with precipitation, and can soak the adjacent soil. The new species also occurs in an area characterized by thinner vegetation, mainly shrubs, with more solar incidence, and a thick litter layer, and consequently, less moisture. In these sites, finding frogs was difficult because of their small size, cryptic coloration, cryptozoic habits, and the behavior of ceasing calling with any disturbance in their surroundings. After thorough searches, we found the individuals either between juçara palm (*Euterpes edulis*) roots or under rotten trunks. In these areas, *B. dacnis* was syntopic with *B. hermogenesi*. When approached, some individuals occasionally exhibited mouth-gaping (Fig. 3c). We also observed a 0.7 mm *Brachycephalus* cf. *dacnis* individual (not collected – but likely a *B. dacnis,* as no *B. hermogenesi* was found in the same area) jumping 21.8 cm (31 times its SVL) (ZUEC-VID 796).

We identified the stomach contents of two individuals. During dissection, we found one individual (ZUEC-AMP 25273) with four items in its stomach, identified to order: one Collembola, one Coleoptera, one Hymenoptera (Hexapoda), and one Acari (Sarcoptiformes) (Fig. S2). In the microCT scan of another individual (ZUEC-AMP 24978), we identified one item, an Anobiinae beetle (Coleoptera: Ptinidae) (Fig. S2).

Distribution – *Brachycephalus dacnis* is known from its type locality, at Projeto Dacnis private reserve in Ubatuba, state of São Paulo (SP), Brazil, and two additional localities, based on acoustic records previously published in Bornschein et al. (2021): Corcovado, municipality of Ubatuba, SP, and Trilha do Corisco, municipality of Paraty, Rio de Janeiro (Fig. 6).

Discussion

We describe a new species of *Brachycephalus*, that was firstly recognized by Bornschein et al. (2021) as a new entity, which ranks among the smallest vertebrates ever recorded (Bolaños et al., 2024). One of the individuals measured 6.95 mm, which is currently the second smallest adult vertebrate ever described, only larger than another individual of other congeneric species (Bolaños et al., 2024). Although *B. dacnis* is at the lower bounds of anuran body size, it does not exhibit all the traits typically associated with extreme miniaturization in frogs. For example, among the flea-toads, *Brachycephalus pulex* has fewer functional toes, and *B. puri* and *B. didactylus* have more reduced (or vestigial) toes. The skeleton of *B. dacnis* is remarkable for not having the fusions and loss of elements typical of many miniaturized anurans (Yeh, 2002). The skeletons of *B. dacnis* and *B. hermogenesi* are nearly indistinguishable, but both differ substantially from skeletons of most other (even larger) species in the genus *Brachycephalus*. The genus *Brachycephalus*, especially *B. ephippium*, was previously considered a remarkable case of hyperossification associated with phylogenetic decreases in body size (Trueb & Alberch, 1985). The polyphyletic assemblage of flea-toads—the tiniest *Brachycephalus* species—reveals that miniature species in the genus vary widely in the extent of fusions and loss. The phylogeny suggests, that leptodactyliform species are among the earliest diverging species of the genus *Brachycephalus*, then the “normalcy” of their skeletons suggests that many of the unique and remarkable traits of *Brachycephalus* evolved in association with the evolution of slightly larger body sizes, new body shape and conspicuous coloration within this genus. Thus, though extremely small, the skeleton of *B. dacnis* is not otherwise remarkable. However, small body sizes in cryptically colored species could be under strong positive selection as it may provide advantages among leaf litter frogs by reducing predator detectability and increasing agility (Blanckenhorn, 2000).

This is the seventh flea-toad species described. It is likely that the diversity of flea-toads is ~~extremely~~ underestimated due to their cryptic behavior (inhabiting the forest floor and having a cryptozoic lifestyle), small size (the smallest vertebrates on Earth), and overall color-matching of their habits (camouflage coloration). In addition, their lack of basic natural history data, such as recordings of advertisement calls, makes it difficult to identify new species (e.g., Bornschein et al. 2021). Recent taxonomic studies have focused largely on the brightly colored and larger *Brachycephalus* species (those in the species groups of *B. ephippium*, *B. pernix*, and *B. vertebralis*) (*e.g.*, Nunes et al., 2021; Mângia et al. 2023; Folly et al. 2024). A renewed focus on the tiny and cryptic species of *Brachycephalus* will likely reveal many more undescribed species at the lower bounds of body size in vertebrates.

Our phylogenetic analysis (Fig. S1), besides corroborating the fact that *B. dacnis* is indeed a distinct evolutionary lineage, also reveals great genetic differences between flea-toad species, even within close related species. On the other hand, the analysis also suggests that some bright colored species need to be revised, since it reveals some cases of non-monophyletic species or very low divergent species. Those are the cases of (i) *B. olivaceus* and *B. mariaeterezae*, (ii) *B. coloratus* and *B. pernix*, and (iii) *B. pitanga* and *B. guarani*. If each of these pairs includes synonyms, only half of the species should be valid. This result agrees with recently published phylogenies (Folly et al., 2022; Condez et al., 2020; Mângia et al., 2023). Together these data support the urgency of a taxonomic revision in these species groups to provide additional support for the putative synonyms. The analyses should include more individuals per species and localities, and the assessment of nuclear genetic markers. Besides those cases, it is possible that the type series of *B. hermogenesi* is composed with individuals of *B. dacnis* (see Bornschein et al. 2021). As those individuals had their advertisement call not recorded, and both species are morphologically cryptic, it would be interesting to investigate their DNA sequences. Recent molecular techniques for preserved specimens (i.e., ancient DNA; aDNA) are available (Straube et al. 2021) and the description of *B. dacnis* will facilitate such resolution.

We observed individuals producing advertisement calls throughout the year. This may indicate a continuous reproduction strategy, as suggested by Monteiro et al. (2018) for *B. actaeus*. Calling activity may also be related to climate stability in Ubatuba, which provides a moist microhabitat in the leaf litter the whole year. We observed an increase in vocal activity in the wetter periods, with intensified activity after rains, including several males calling simultaneously throughout the day and some individuals calling at night, as observed by Verdade et al. (2008) and Oliveira et al. (2011) for *B. hermogenesi*. Most *Brachycephalus* species have diurnal activity, with calling emission strongly influenced by climatic conditions such as temperature, humidity, and rainfall (Oliveira & Haddad, 2017).

The substantial efforts for sampling and describing biodiversity reflect the continuously growing number of newly discovered species. By describing this new species in an integrative way, e.g., by providing details of internal organs and the skeleton and descriptions of natural history (e.g., diet, advertisement calls), we hope to facilitate the future description of more species in this surprisingly diverse and cryptic genus of tiny vertebrates.

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Figure Legends

Figure 1. *Brachycephalus dacnis* holotype (ZUEC-AMP 24982) adult male, SVL = 7.55 mm). (a) Dorsal and (b) ventral views (photo credit: Mariana Pedrozo).

Figure 2. *Brachycephalus dacnis* paratype (ZUEC-AMP 25612) adult male, SVL = 7.89 mm). (a) Dorsal view, (b) ventral view, (c) ventral view of hand, (d) ventral view of feet (photo credit: Mariana Pedrozo).

Figure 3. *Brachycephalus dacnis* paratype individuals found at the Projeto Dacnis private reserve, municipality of Ubatuba, state of São Paulo, Brazil. (a) ZUEC-AMP 25272; (b) ZUEC-AMP 25274; (c-d) ZUEC-AMP 25275 displaying mouth-gaping behavior and the same individual on top of one herpetologist fingertip (photo credit: L. F. Toledo).

Figure 4. Ventral dissection of *Brachycephalus dacnis* (ZUEC-AMP 25273) with the highlighted structures. a) Abdominal muscles, b) Liver, c) Small intestine; d) Atrium, e) Ventricle, f) *Conus arteriosus*, g) *Truncus arteriosus*, h) Carotid artery, i) Systemic artery, j) Parathyroid gland, k) Eggs, l) Oviduct (photo credit: A. S. Carrasco-Medina).

Figure 5. Skeletal anatomy of *Brachycephalus dacnis* (ZUEC-AMP 24978; undetermined sex) based on CT-scans. (a) the entire skeleton, with insets of (b) the skull (in dorsal, ventral, and right lateral views), (c) right hand and (d) left foot (both in dorsal view), (e) vertebral column (in dorsal view), (f) pectoral girdle (in ventral view), and (g) pelvic girdle (in right lateral view). Abbreviations: c, carpus; cl, clavicle; cor, coracoid; dt, distal tarsals; ex, exoccipital; fp, frontoparietal; il, ilium; isc, ischium; m, maxilla; mdI, manual digit I; mdV, manual digit V; min, mineralized cartilage; n, nasal; op, operculum; pd I, pedal digit I; pd V, pedal digit V; pm, premaxilla; pr, prootic; ps, parasphenoid; pt, pterygoid; pub, pubis; qj, quadratojugal; sac, sacrum; sc, scapula; ses, sesamoid; sm, septomaxilla; sp, sphenethmoid; sq, squamosal; ur, urostyle; v, vomer (photo credit: J. A. Gray and D. C. Blackburn).

Figure 6. Geographic distribution, advertisement calls and phylogeny. (a) Type localities of *Brachycephalus dacnis* (red star) and *B. hermogenesi* (black star), and additional distribution records of *B. dacnis* (red triangles, based on call recordings published in Bornschein et al. 2021). Map was produced in QGis using shape files from public databases. (b) *Brachycephalus dacnis* holotype's advertisement call (FNJV 51341), recorded at Projeto Dacnis private reserve (type locality), Ubatuba, São Paulo (left) and *B. hermogenesi* topotype's advertisement call (FNJV 51343), recorded at Picinguaba (type locality), Ubatuba, São Paulo (right). (c) Schematic representation of the Bayesian phylogenetic inference of *Brachycephalus* based on the mitochondrially encoded 16S rDNA fragment. Black dots indicate fully supported clades (Posterior probability, PP => 99). White dots indicate PP >0.95. Node supports below pp=0.95 are not shown. Complete tree in the supplement (Fig. S1).

Figure 7. Selected skulls of other *Brachycephalus* species. Cranial elements labeled are discussed in comparisons of *B. dacnis* to other species. Top row is the leptodactyliform “flea toads”, including *B. hermogenesi* (sister species of *B. dacnis*), *B. clarissae*, *B. sulfuratus*, and *B. didactylus*. Bottom row contains representatives of the three named species groups: *pernix* group (*B. albolineatus*, *B. pernix*), *vertebralis* group (*B. vertebralis*), and *ephippium* group (*B. ephippium*). Museum catalog numbers are listed below each species name. Abbreviations: fp, frontoparietal; n, nasal; ps, parasphenoid; pt, pterygoid; qj, quadratojugal; sp, sphenethmoid; sq, squamosal; v, vomer (photo credit: J. A. Gray and D. C. Blackburn).

Table 1(on next page)

Table 1. Measurements of the holotype and adult paratypes (one adult that was dissected for internal anatomy was not measured) of *Brachycephalus dacnis*.

Values presented as mean ± standard deviation (minimum − maximum) in millimeters.

Table 1. Measurements of the holotype and adult paratypes (one adult that was dissected for internal anatomy was not measured) of *Brachycephalus dacnis*. Values presented as mean ± standard deviation (minimum − maximum) in millimeters. Raw data is presented in Table S5.

Trait	Holotype	Adult paratypes (n = 10)
SVL	7.55	8.29 ± 1.03 (6.95 – 9.90)
HL	1.78	2.59 ± 0.45 (1.87 – 3.18)
HW	1.78	2.97 ± 0.55 (1.82 – 3.63)
END	0.37	0.48 ± 0.23 (0.18 – 1.02)
IOD	1.42	1.48 ± 0.32 (0.84 – 2.10)
ED	0.49	0.94 ± 0.24 (0.50 – 1.30)
ND	0.31	0.27 ± 0.08 (0.16 – 0.40)
UAL	1.30	1.57 ± 0.22 (1.30 – 2.02)
FAL	2.95	2.13 ± 0.30 (1.80 – 2.70)
HAL	1.00	1.28 ± 0.23 (0.92 – 1.63)
TBL	3.59	3.82 ± 0.48 (2.98 – 4.50)
THL	4.00	3.77 ± 0.51 (3.25 – 5.00)
FL	4.50	6.10 ± 0.41 (5.60 – 6.75)
fL	2.75	3.16 ± 0.47 (2.35 – 4.00)

Table 2(on next page)

Table 2. Advertisement call traits described for three flea-toads species from *Brachycephalus* genus.

Call traits present as mean ± standard deviation (range).

Table 2. Advertisement call traits described for three flea-toad species from *Brachycephalus* genus. Most call traits present as mean ± standard deviation (range). *Attenuated notes follow the definition of Bornschein et al. (2021).

	Brachycephalus dacnis 6 individuals (including the holotype) Dacnis, Ubatuba	*Brachycephalus dacnis* Corcovado, SP and Trilha do Corisco, RJ	*Brachycephalus hermogenesi* 5 individuals Ubatuba	*Brachycephalus hermogenesi* Several locations	*Brachycephalus sulfuratus* 4 topotypes São Francisco do Sul	*Brachycephalus sulfuratus* Several locations
Call duration (s)	0.05 ± 0.01 (0.03 – 0.41)	-	0.56 ± 0.14 (0.41 – 0.78)	-	1.70 ± 0.10 (1.50 – 2.30)	-
Inter-call interval (s)	2.1 ± 0.06 (1.67 – 2.69)	-	2.70 ± 0.15 (2.52 – 2.91)	-	4.5 ± 1.7 (3.1 – 7.4)	-
Number of notes per call	1 – 2	-	2 – 6	-	4 – 6	-
Presence of attenuated notes*	No	No	Yes	Yes	No	No
Note duration (s)	The same as call duration	-	0.25 ± 0.04 (0.17 – 0.27)	-	0.17 ± 0.01 (0.13 – 0.21)	-
Inter-note interval (s)	n/a	-	2.70 ± 0.15 (2.52 – 2.91)	-	n/a	-
Note repetition rate (notes/s)	19.58 ± 1.84 (12.55 – 29.41)	-	4.12 ± 0.48 (3.58 – 4.88)	-	0.2 ± 0.1 (0.2 – 0.3)	-
Number of pulses per note	3 – 7	1 – 16	1 – 2	2 – 3	7 – 9	2 – 14

inimum frequency (Frequency 5%) (kHz)	7.27 ± 0.12 (6.89 – 7.50)	-	7.94 ± 0.08 (7.84 – 8.01)	-	4.9 ± 0.3 (4.5 – 5.2)	-
ominant frequency (kHz)	8.22 ± 0.10 (8.01 – 8.44)	-	8.38 ± 0.18 (8.18 – 8.61)	-	6.6 ± 0.1 (6.5 – 6.7)	-
aximum frequency (Frequency 95%) (kHz)	8.62 ± 0.03 (8.44 – 8.96)	-	8.91 ± 0.19 (8.70 – 9.13)	-	9.1 ± 0.2 (9.0 – 10.7)	-
Frequency bandwidth (90%) (kHz)	1.35 ± 0.12 (0.94 – 1.89)	-	0.97 ± 0.18 (0.78 – 1.29)	-	n/a	-
References	Present study	Bornschein et al. 2021	Present study	Bornschein et al. 2021	Condez et al. 2016	Bornschein et al. 2021

Figure 1

Brachycephalus dacnis holotype (ZUEC-AMP 24982) adult male, SVL = 7.55 mm).

(a) Dorsal and (b) ventral views.

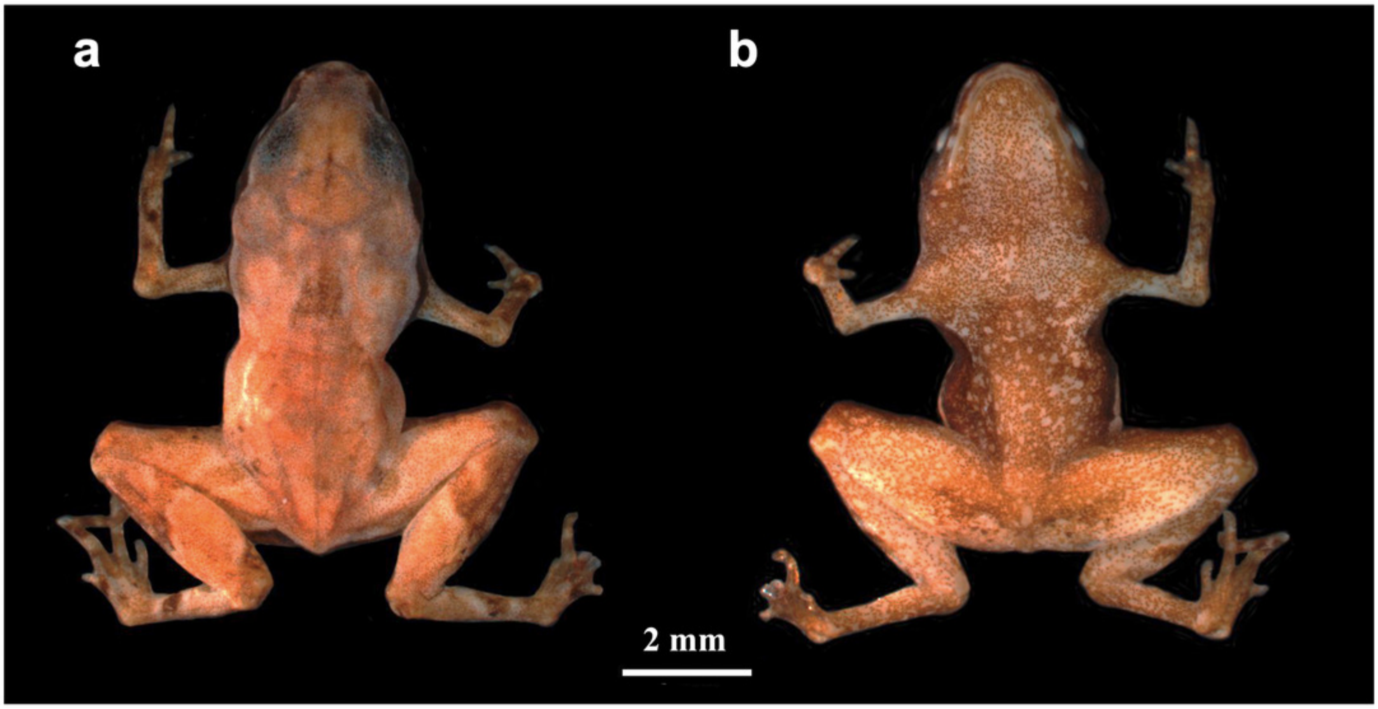
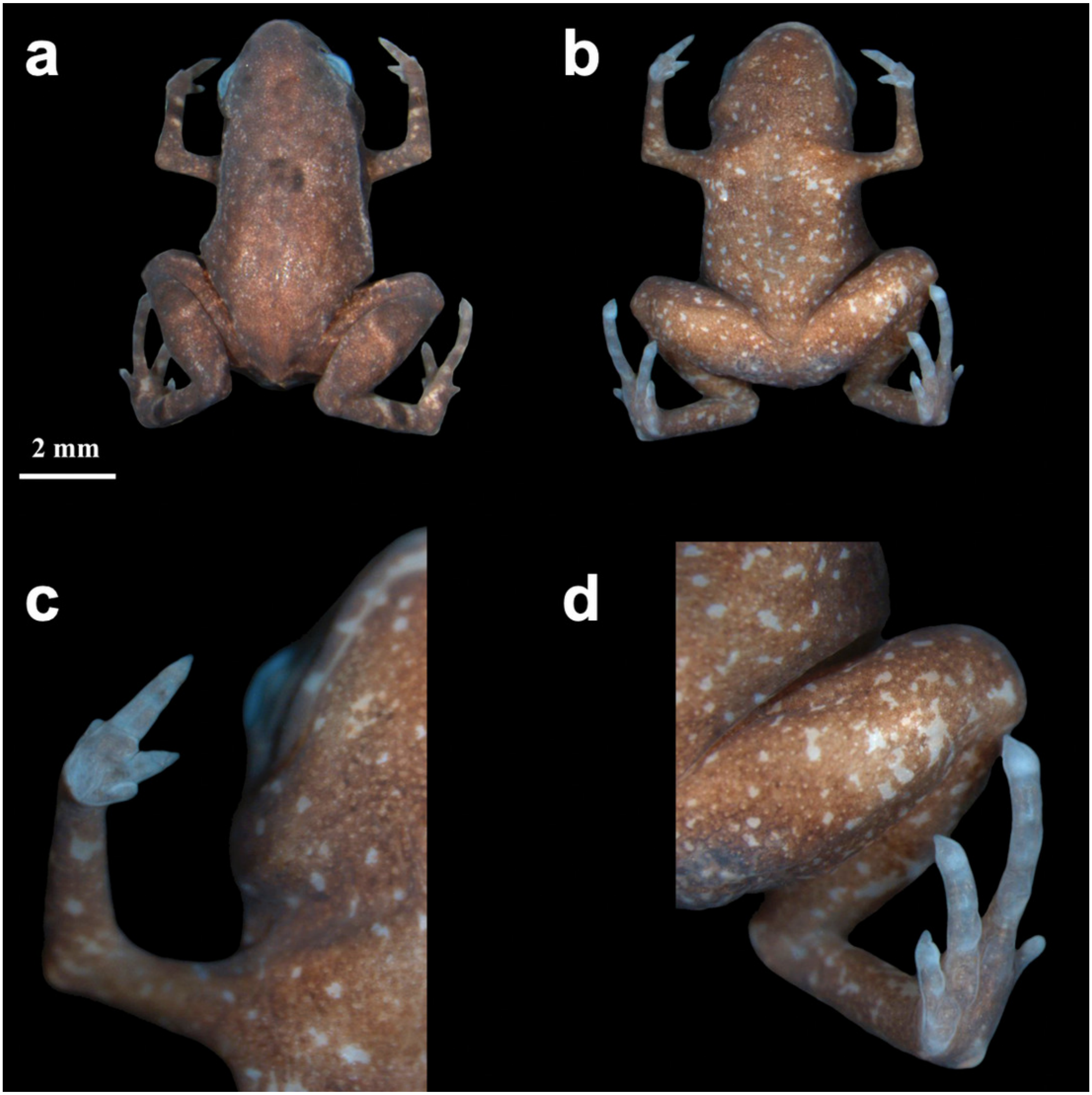


Figure 2

Brachycephalus dacnis paratype (ZUEC-AMP 25612) adult male, SVL = 7.89 mm).

(a) Dorsal view, (b) ventral view, (c) ventral view of hand, (d) ventral view of feet.



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(a) ZUEC-AMP 25272; (b) ZUEC-AMP 25274; (c-d) ZUEC-AMP 25275 displaying mouth-gaping behavior and the same individual on top of one herpetologist fingertip.

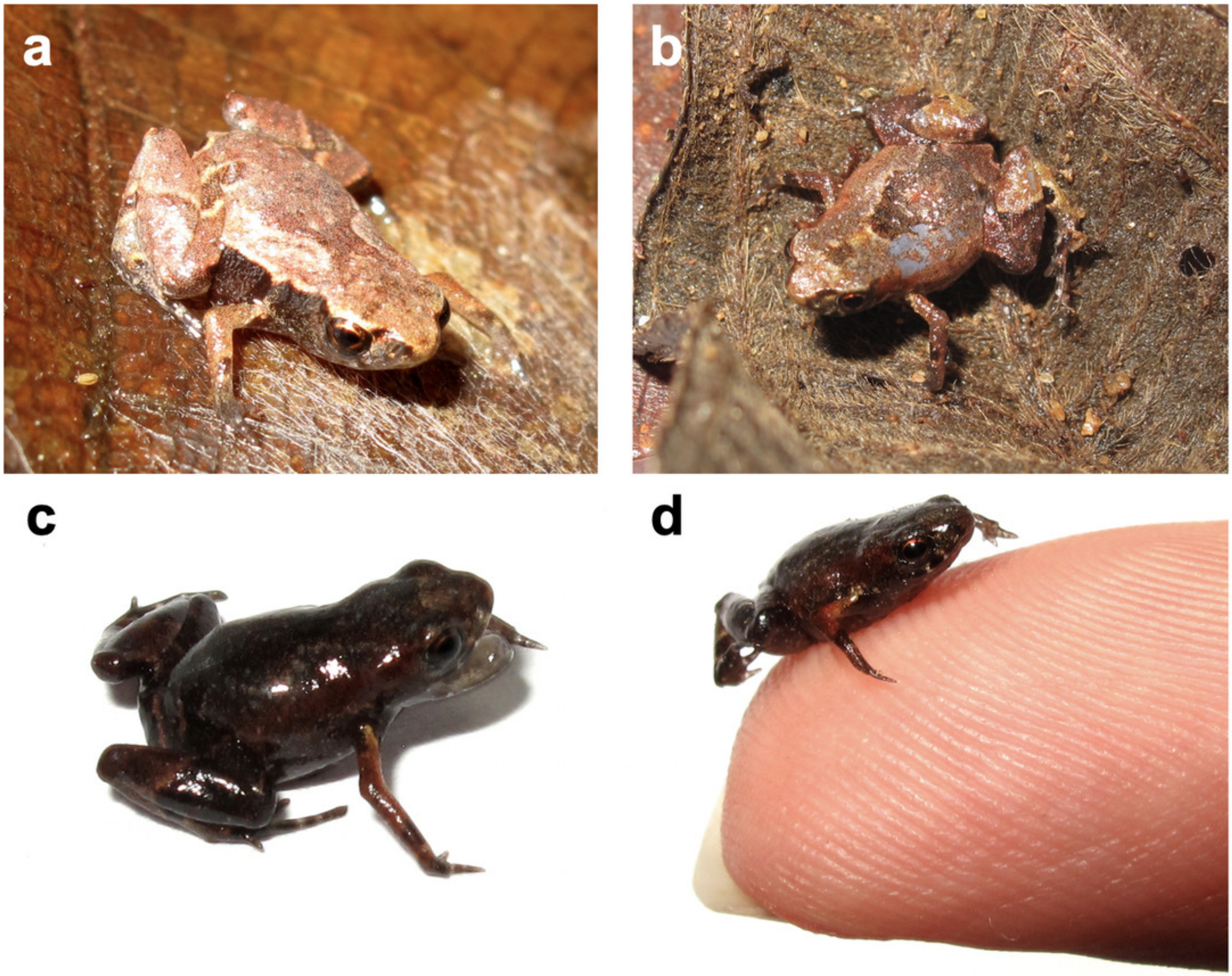

Figure 4

Ventral dissection of *Brachycephalus dacnis* (ZUEC-AMP 25273) with the highlighted structures.

a) Abdominal muscles, b) Liver, c) Small intestine; d) Atrium, e) Ventricle, f) *Conus arteriosus*, g) *Truncus arteriosus*, h) Carotid artery, i) Systemic artery, j) Parathyroid gland, k) Eggs, l) Oviduct.

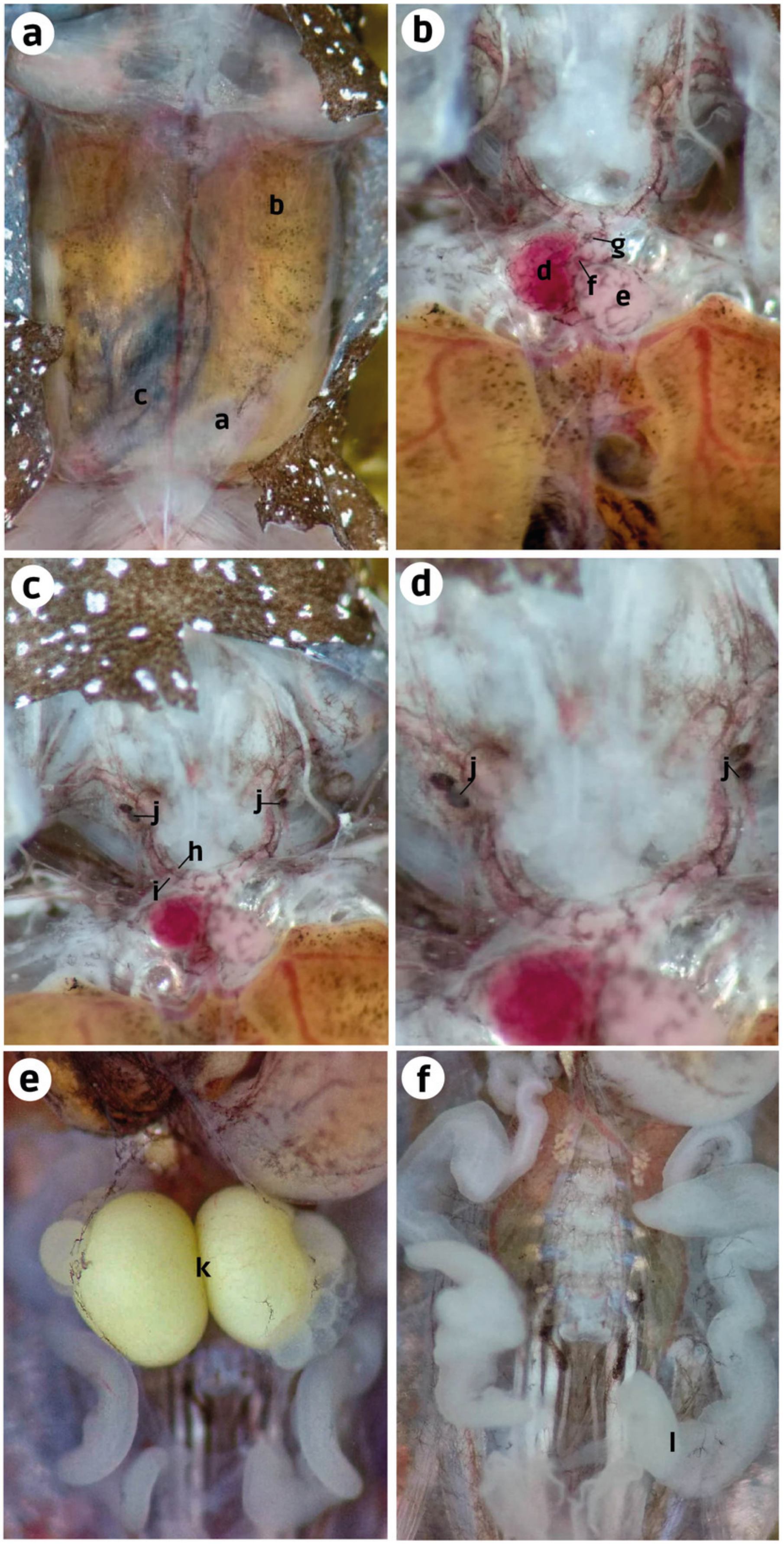
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Figure 5

Skeletal anatomy of *Brachycephalus dacnis* (ZUEC-AMP 24978; undetermined sex) based on CT-scans.

(a) the entire skeleton, with insets of (b) the skull (in dorsal, ventral, and right lateral views), (c) right hand and (d) left foot (both in dorsal view), (e) vertebral column (in dorsal view), (f) pectoral girdle (in ventral view), and (g) pelvic girdle (in right lateral view). Abbreviations: c, carpus; cl, clavicle; cor, coracoid; dt, distal tarsals; ex, exoccipital; fp, frontoparietal; il, ilium; isc, ischium; m, maxilla; mdI, manual digit I; mdV, manual digit V; min, mineralized cartilage; n, nasal; op, operculum; pd I, pedal digit I; pd V, pedal digit V; pm, premaxilla; pr, prootic; ps, parasphenoid; pt, pterygoid; pub, pubis; qj, quadratojugal; sac, sacrum; sc, scapula; ses, sesamoid; sm, septomaxilla; sp, sphenethmoid; sq, squamosal; ur, urostyle; v, vomer.

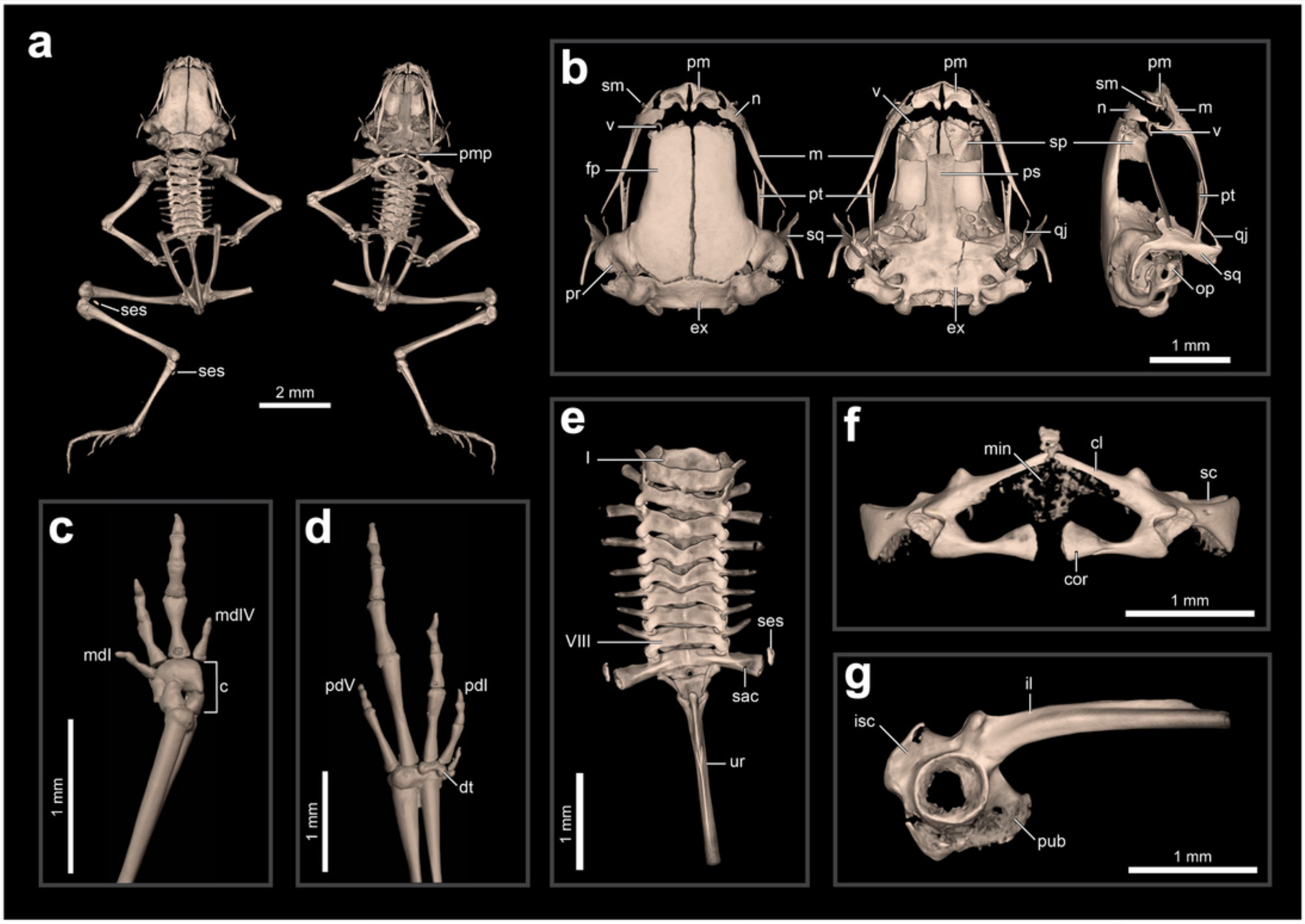
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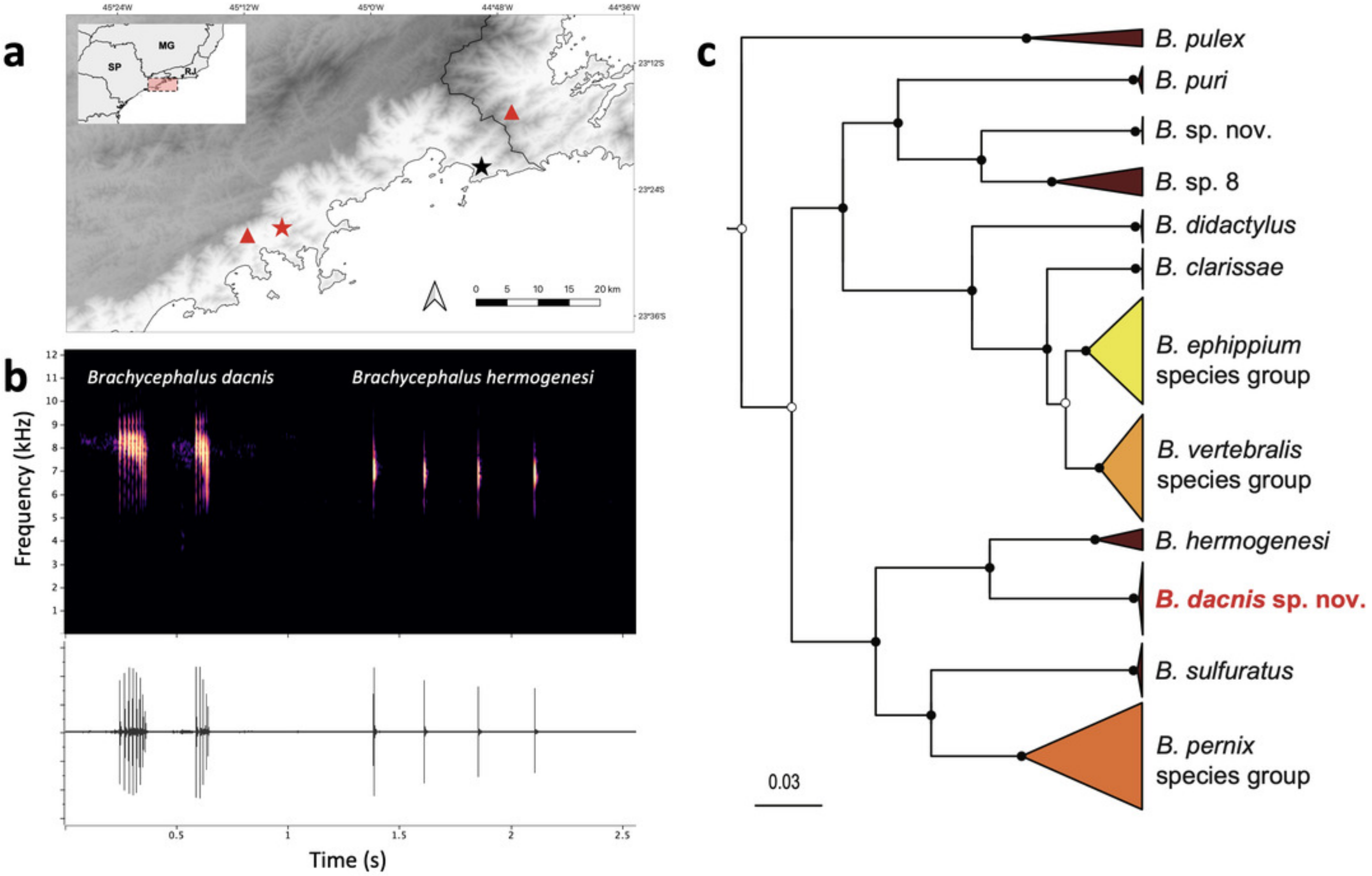
Figure 6

Geographic distribution, advertisement calls and phylogeny.

(a) Type localities of *Brachycephalus dacnis* (red star) and *B. hermogenesi* (black star), and additional distribution records of *B. dacnis* (red triangles, based on call recordings published in Bornschein et al. 2021). (b) *Brachycephalus dacnis* holotype's advertisement call (FNJV 51341), recorded at Projeto Dacnis private reserve (type locality), Ubatuba, São Paulo (left) and *B. hermogenesi* topotype's advertisement call (FNJV 51343), recorded at Picinguaba (type locality), Ubatuba, São Paulo (right). (c) Schematic representation of the Bayesian phylogenetic inference of *Brachycephalus* based on the mitochondrially encoded 16S rDNA fragment. Black dots indicate fully supported clades (Posterior probability, PP => 99). White dots indicate PP >0.95. Node supports below pp=0.95 are not shown. Complete tree in the supplement (Figure S1).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Brachycephalus hermogenesi
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1.5
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Time (s)
c
B. pulex
B. puri
B. sp. nov.
B. sp. 8
B. didactylus
B. clarissae
B. ephippium species group
B. vertebralis species group
B. hermogenesi
B. dacnis sp. nov.
B. sulfuratus
B. pernix species group
0.03

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