Zootaxa 5691 (2): 231–256
https://www.mapress.com/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.5691.2.3
http://zoobank.org/urn:lsid:zoobank.org:pub:7F5EF807-28B2-424E-8B02-CAE094C9C6EB

A new species of flea-toad of the genus *Brachycephalus* Fitzinger, 1826 (Anura: Brachycephalidae) from the cloud forests of Serra do Mar, southeastern Brazil

IVAN NUNES[1,8]*, MARIANA L. LYRA[2], THAYNARA M. MACHADO[3,4,10], ANDRES SANTIAGO CARRASCO-MEDINA[3,5,11], FELIPE SILVA DE ANDRADE[6,12], ISABELLE AQUEMI HAGA[6,13], LUCAS M. BOTELHO[7,14], MARIANA PEDROZO[7,15], ISABEL G. VELASCO[1,8,16], FERNANDO DE CASTRO JACINAVICIUS[9], JAIMI A. GRAY[17,18,19], DAVID C. BLACKBURN[17,20], TIANA KOHLSDORF[6,21], EDÉLCIO MUSCAT[7,22] & L. FELIPE TOLEDO[3,4,5,23]

[1]*Laboratório de Herpetologia (LHERP), Instituto de Biociências, Campus do Litoral Paulista, Unesp, São Vicente, São Paulo, Brazil.*
[2]*New York University Abu Dhabi, Saadiyat Island, Abu Dhabi, P.O. Box 129188, United Arab Emirates; and CBioClima – Center for Research on Biodiversity Dynamics and Climate Change, Institute of Biosciences, Universidade Estadual Paulista-UNESP, Rio Claro, São Paulo, Brazil.*
marillyra@gmail.com; https://orcid.org/0000-0002-7863-4965
[3]*Laboratório de História Natural de Anfíbios Brasileiros (LaHNAB), Departamento de Biologia Animal, Instituto de Biologia, Unicamp, Campinas, São Paulo, Brazil.*
[4]*Programa de Pós-Graduação em Ecologia, Unicamp, Instituto de Biologia, Campinas, São Paulo, Brazil.*
[5]*Programa de Pós-Graduação em Biologia Animal, Unicamp, Instituto de Biologia, Campinas, São Paulo, Brazil.*
[6]*Laboratório de Evolução e Biologia Integrativa (LEBI), Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP), Universidade de São Paulo (USP), Ribeirão Preto, São Paulo, Brazil.*
[7]*Projeto Dacnis. Estrada do Rio Escuro, 4754, Sertão das Cotias, Ubatuba, São Paulo, Brazil.*
[8]*Programa de Pós-Graduação em Ciências Biológicas (Zoologia), Unesp, Instituto de Biociências, Botucatu, São Paulo, Brazil.*
[9]*Laboratório de Coleções Zoológicas, Instituto Butantan, São Paulo, Brazil.*
fcjacinavicius@gmail.com; https://orcid.org/0000-0002-5503-3120
[10] *thaynara.herpeto@gmail.com; https://orcid.org/0000-0001-6626-7953*
[11] *andress.carrascomedina@gmail.com; https://orcid.org/0000-0002-1869-9986*
[12] *felipe_andrade@ymail.com; https://orcid.org/0000-0003-0514-142X*
[13] *hagaisabelle@gmail.com; https://orcid.org/0000-0002-3788-0238*
[14] *lucasbotelho77@gmail.com, https://orcid.org/0000-0002-8308-2026*
[15] *mariana.pedrozo.24@gmail.com; https://orcid.org/0000-0002-7571-9787*
[16] *ig.velasco@unesp.br; https://orcid.org/0000-0001-6929-6928*
[17]*Florida Museum of Natural History, University of Florida, Gainesville, Florida US.*
[18] *High-Resolution X-Ray CT Facility, University of Texas, Austin, USA.*
[19] *jaimigray@utexas.edu; https://orcid.org/0000-0002-4758-8572*
[20] *dblackburn@flmnh.ufl.edu; https://orcid.org/0000-0002-1810-9886*
[21] *tiana@usp.br; https://orcid.org/0000-0002-3873-2042*
[22] *edelciomuscat@terra.com.br; https://orcid.org/0000-0003-3162-5209*
[23] *toledosapo@gmail.com; https://orcid.org/0000-0002-4929-9598*
**Corresponding author: ivan.nunes@unesp.br; https://orcid.org/0000-0001-7985-2836*

Abstract

Species of the genus *Brachycephalus* are endemic to the Brazilian Atlantic Forest, where they inhabit amidst the leaf litter. Depending on their phenotypic traits, these small-sized amphibians are commonly referred to as either flea-toads or pumpkin toadlets. Among them, the pumpkin toadlets (with a bufoniform phenotype) have received more extensive research attention. These individuals are often conspicuous, and prevalent in their natural habitat. In contrast, the flea-toads (leptodactyliform species) are brownish, cryptic with the background. This scarcity has led to their reduced scientific attention, including in the realm of taxonomy. Thus, we herein describe a new species of the genus *Brachycephalus* from montane forests of the Estação Ecológica de Bananal, in the municipality of Bananal, state of São Paulo, Brazil. The new species is characterized by having a leptodactyliform body; adult SVL 6.9–9.0 mm for males and 9.2–9.6 mm for females; smooth skin lacking hyperossification; snout rounded in dorsal view; finger II with pointed tips; toe I absent (lacking phalanges), and toes II, III, IV and V distinct; toe II with rounded tips; brown background with few golden spots scattered

Accepted by M. Rivera-Correa: 26 Jul. 2025; published: 9 Sept. 2025

aleatory in the general coloration of dorsal surfaces and cloacal region; presence of a dark brown stripe along the flanks and the thigh; yellow blotches on the ventral surfaces; presence of dark brown strips on the legs. Based on molecular phylogeny, we recognize the *Brachycephalus puri* species group, based on a clade composed by *B. puri* sister to the new species plus a candidate new species. The new species probably has a restricted geographic distribution, and its discovery reinforces the importance of deeper taxonomic effort with the brownish leptodactyliform species of the genus.

Key words: Atlantic Forest, taxonomy, natural history, phylogeny, Brachycephaloidea, direct-developing anurans

Introduction

The genus *Brachycephalus* Fitzinger 1826 (Brachycephalidae) is a group of small-sized anurans endemic to the Brazilian Atlantic Forest (Frost 2024). The specimens of this genus have either cryptic or conspicuous (Rebouças *et al*. 2019) coloration and can be found from sea level up over 2,000 m elevation forests in the leaf litter. Its geographical distribution ranges from the state of Santa Catarina to southern Bahia, along almost 1700 Km in straight-line distance (Bornschein *et al*. 2019; Frost 2024). Despite such wide distribution for the genus, most of *Brachycephalus* species have microendemic distributions (Pie *et al*. 2018, Frost 2024).

The taxonomy of the genus has advanced notably over the past decade, with most species being described in the 21st Century (Frost 2024), and recent studies have shown several candidate species yet to be described (e.g., Condez *et al*. 2020). Also, our knowledge of phylogenetic relationships among these species is unstable as both new species and additional genomic markers reshape phylogenetic inferences (see Clemente-Carvalho 2011; Ribeiro *et al.* 2015; Condez *et al.* 2020; Almeida-Silva *et al.* 2021; Lyra *et al.* 2021; Folly *et al.* 2022). Currently, the genus *Brachycephalus* includes 42 species (Frost 2024), historically placed in two recognizable phenotypic and non-monophyletic groups based on body plan: the flea-toads (with leptodactyliform phenotype, n = 6), and the pumpkin toadlets (with bufoniform phenotype, n = 35) (Lyra *et al.* 2021). *Brachycephalus clarissae* Folly, Vrcibradic, Siqueira, Rocha, Machado, Lopes, and Pombal, 2022, is so far the only species with intermediate body plan (Folly *et al.* 2022). The flea-toads *B. didactylus* (Izecksohn, 1971), *B. hermogenesi* (Giaretta & Sawaya, 1998), *B. pulex* Napoli, Caramaschi, Cruz, and Dias, 2011, *B. sulfuratus* Condez, Monteiro, Comitti, Garcia, Amaral, and Haddad, 2016, *B. puri* Almeida-Silva, Silva-Soares, Rodrigues, and Verdade, 2021, and *B. dacnis* Toledo, Botelho, Carrasco-Medina, Gray, Ernetti, Gama, Lyra, Blackburn, Nunes, and Muscat, 2024, are also characterized by having a cryptic brown coloration. In turn, the pumpkin toadlets are known to present orange, red or green components in the body parts. Previous works with the systematics of this genus suggested that both leptodactyliforms and bufoniforms are paraphyletic (Condez *et al.* 2020, Lyra *et al.* 2021).

During zoological surveys at Estação Ecológica de Bananal in the Serra da Bocaina Mountain range (state of São Paulo, southeastern Brazil), some authors collected specimens of a *Brachycephalus* species with cryptic brown coloration, leptodactyliform body plan, and that have sufficient phenotypic differences to be described as a new species, also corroborated by phylogenetic analyses. Herein, we describe this species based on an integrative approach (see Padial *et al.* 2010), using molecular and morphological data. In addition, we provide some natural history data, and information on visceral anatomy and diet of the new species.

Material and Methods

Site and field expeditions. We carried out field expeditions at Estação Ecológica de Bananal, municipality of Bananal, state of São Paulo. The individuals were found at 'Trilha do Ouro' (22S48'14"; 44W22'17", 1220 m above sea level [a.s.l.], datum = WGS84). We carried out field expeditions in December 2021 and in March 2022. We actively searched the area with careful removal and analysis of the leaf litter at the site, dedicating seven hours for day search and two hours for night search, totalizing 40 hours of effort/person. The first expedition involved seven participants, while the second expedition included six participants. In all expeditions, we used a digital Thermo-hygrometer (Hikari HTH-913) to obtain temperature and relative air humidity data.

The municipality of Bananal is located in the Serra da Bocaina Mountain range. The region is inserted in dense montane rainforest and grasslands, with areas in recovery stages and small patches of primary forest up to about 2,100 m a.s.l. The area has a dry and cold winter and hot and rainy summer (Garey *et al*. 2014).

Collection specimens examined. We compared adult specimens with animals deposited in museums (see Appendix I) and with data available in the literature. Specimens used for comparisons were deposited in the following Brazilian zoological collections: Coleção Científica do Laboratório de Zoologia da Universidade de Taubaté, SP (CCLZU); Coleção de Anfíbios do Laboratório de Herpetologia, Instituto de Biociências, Universidade Estadual Paulista, Campus do Litoral Paulista, SP (HCLP-A); Museu Nacional, Universidade Federal do Rio do Janeiro, RJ (MNRJ); Museu de Zoologia Joao Moojen de Oliveira, Universidade Federal de Viçosa, MG (MZUFV), and Museu de Zoologia, Universidade de São Paulo, SP (MZUSP); Museu de Diversidade Biológica (MDBio), Universidade Estadual de Campinas, SP (ZUEC-AMP); Coleção Zoológica do Instituto de Biociências da Universidade Federal do Mato Grosso do Sul, MS (ZUFMS).

Molecular data and Phylogenetic Analysis. The individuals from Estação Ecológica de Bananal collected in the field were euthanized by applying 5% lidocaine to the skin, according to recommendations of the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists (available at https://asih.org/sites/default/files/2018-05/guidelines_herps_research_2004.pdf) and approved by SISBio/Institute Chico Mendes de Conservação da Biodiversidade (ICMBio). Muscle tissue samples were obtained and stored in 99% ethanol for molecular analyses. Specimens were fixed in 10% formalin, stored in 70% ethanol, and deposited in the Museu de Diversidade Biológica (MDBio), Universidade Estadual de Campinas, São Paulo (ZUEC-AMP). Specimens were collected under collection permits issued by ICMBio (SISBio #77947-3).

We generated new sequence data for four specimens (holotype ZUEC-AMP 25581 and three paratopotypes ZUEC-AMP 25577–8, 25580). We extracted total DNA from ethanol-preserved muscle using DNeasy® Blood & Tissue Kit (Qiagen Inc.) and amplified two overlapping fragments of the mitochondrially encoded *16S rRNA* (*16S*). Primers used for amplification and PCR conditions are given in Appendix II. PCR products were purified using a GFX PCR DNA kit (GE Healthcare) and sent to CREBIO (Centro de Recursos Biológicos e Biologia Genômica FCAV-UNESP, Jaboticabal, São Paulo, Brazil), or Macrogen (Seoul, South Korea) to be sequenced. The new DNA sequences were quality trimmed and assembled using Geneious Prime® 2022.1.1 (www.geneious.com) and submitted to GenBank (accession numbers: OR250404, OR250405, OR399156, OR399157). We also registered the access to genetic information on the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGen # A74AD8B).

For the phylogenetic inferences, we included representatives of all *Brachycephalus* species available in GenBank (including few candidate species, see Appendix III), and we used *Ischnocnema henselii* (Peters, 1870) to root the trees (representative of sister genus based on Padial *et al*. 2014). The total matrix contained 96 individuals. The new species hypothesis is in accordance with the General Lineage Concept and the Phylogenetic Species Concept (de Queiroz 1998, 2007).

Sequence alignment was performed with the software MAFFT version 7.402 (Katoh & Standley 2013) using E-INS-i, and the final length of the alignment was 1385 bp, including gaps. Alignments indicated that some samples obtained from Firkowski *et al.* (2016) presented an artificial gap in one of the hypervariable regions of the 16S. This region was probably accidentally deleted before the authors submitted them to GenBank. To include these sequences in our analyses, we filled the region with question marks, denoting missing data based on alignments.

We performed the phylogenetic inferences using two distinct methods: Bayesian Inference (BI) and Maximum Likelihood (ML). The ML analysis was conducted online using the software PhyML v. 3.0 (Guindon *et al*. 2010; available at http://www.atgc-montpellier.fr/phyml-sms/). For that, we applied the Smart Model Selection option (SMS; Lefort *et al*. 2017) under Akaike Information Criterion, and branch supports were estimated using standard bootstrap (100 replicates). The SMS selected GTR as the best-fitting evolution model. The BI analysis was performed using BEAST v2.7.7 (Bouckaert *et al*. 2019) under a Birth Death tree prior, assuming a strict clock and using substitution rates of the GTR model. (Heled & Bouckaert 2013) The Markov chain Monte Carlo (MCMC) parameters were set to 2 independent runs of 10 million iterations each, sampling every 10000 iterations and discarding 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 Maximum Clade Credibility (MCC) tree in TreeAnnotator (V.2.7, Bouckaert *et al*. 2019).

We calculated the uncorrected pairwise genetic distances (p-distances) among the new species and, the other flea-toads and *B. clarissae*, which has intermediate body plan, using Mega 11 (Tamura *et al*. 2021; Stecher *et al*. 2020) using pairwise deletion (Appendix IV). Distances were first calculated based on the *16S* fragment flanked by primers 16Sar-L and 16Sbr-H (Palumbi *et al*. 1991), as extensively used for species comparisons and delimitations (Palumbi fragment; see Fouquet *et al*. 2007; Lyra *et al*. 2017; Vieites *et al*. 2009). Unfortunately, we could only use one sample from *Brachycephalus* sp. 8 (from Ilha Grande, Angra dos Reis, RJ, accession number HQ435692) to estimate genetic distances. The other sample (from Bananal, SP, accession number MK697410; CTMZ1522/MZUSP133731) does not have the fragment between primers 16Sar-L and 16Sbr-H, and, consequently, does not have a homologous sequence to be compared with *B. puri*. To be able to compare genetic divergence between the new species and *Brachycephalus* sp. 8 presents in Bananal, SP, we used 727 bp of *16S* (16S-UP), which is common between all samples of the new species, *B. puri* and *Brachycephalus* sp. 8.

External morphometry. Under a stereomicroscope coupled to an ocular micrometer (Stemi DV4 Zeiss), and a digital caliper (Mitutoyo Absolute Model CD-6 CX-B; of 0.01 mm of precision), we took 16 body measurements. SVL was measured with the caliper and all other measurements with the stereomicroscope. We followed Duellman (1970) for the measurements of snout-vent length (SVL), head length (HL), head width (HW), eye diameter (ED), interorbital distance (IOD), internarial distance (IND), and tibia length (TL). We followed Heyer *et al*. (1990) for upper arm length (UAL), forearm length (FAL), hand length (HAL), thigh length (THL), and shank length (SHL), foot length (FL, including tarsus). Nostril diameter (ND) and eye-nostril distance (END) follows Napoli (2005). Morphological terminology of the snout shape follows Heyer *et al*. (1990). We determined the sex of individuals by the presence of vocal slits, internal organs observation and the sexual size dimorphism.

Internal anatomy. We anesthetized two specimens (ZUEC-AMP 25259 and 25578; female and male, respectively) with a dose of 2% lidocaine hydrochloride. The route of administration was through the skin contact with the anesthetic solution. We made dissections immediately after the preparatory anesthesia procedure (Iuliis & Pulerà 2006; Sebben 2007). We then immersed individuals in an isotonic saline solution (Sodium Chloride; NaCl; 0.65% solution), to allow heart contractions and continuous blood flow for as long as possible. The saline solution guarantees the maintenance of the morphophysiological characteristics of the organs and avoids unwanted reflexes that prevent the correct visualization or clotting of the blood. We made photographs (ZUEC-PIC 647–649) and videos (ZUEC-VID 992–993) 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. They were deposited in Museu de Diversidade Biológica (MDBio). The procedure was done in consent with the Animal Ethics Committee of Unicamp (CEUA #5903-1/2021).

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 25580 and 25610) 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, 95–250 mA, a 500 ms detector time and no filters. All scans averaged three images per rotation with a skip of one, and a voxel resolution of 5–6 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 (from Appendix II of Toledo *et al*., 2024) 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*.

Additional information (measurements raw data, and genetic distances) can be found in Appendix I-IV.

Results

Phylogenetic and molecular analyses. Results from both phylogenetic analyses (BI and ML) were consistent, presenting concordant topologies for most species relationships and clades (Fig. 1). The phylogenetic analyses supported the distinction of the new species proposed here from all other members of the genus, being that the four individuals sequenced cluster in a highly supported clade (Posterior probability, PP=1, Bootstrap, BS=1). In both analyses the new species was recovered in a well-supported clade (PP=99, Bootstrap, BS=74). The clade is harbored by *B. puri* as sister taxon of a less inclusive clade comprised of a candidate species (*Brachycephalus* sp. 8; *sensu*

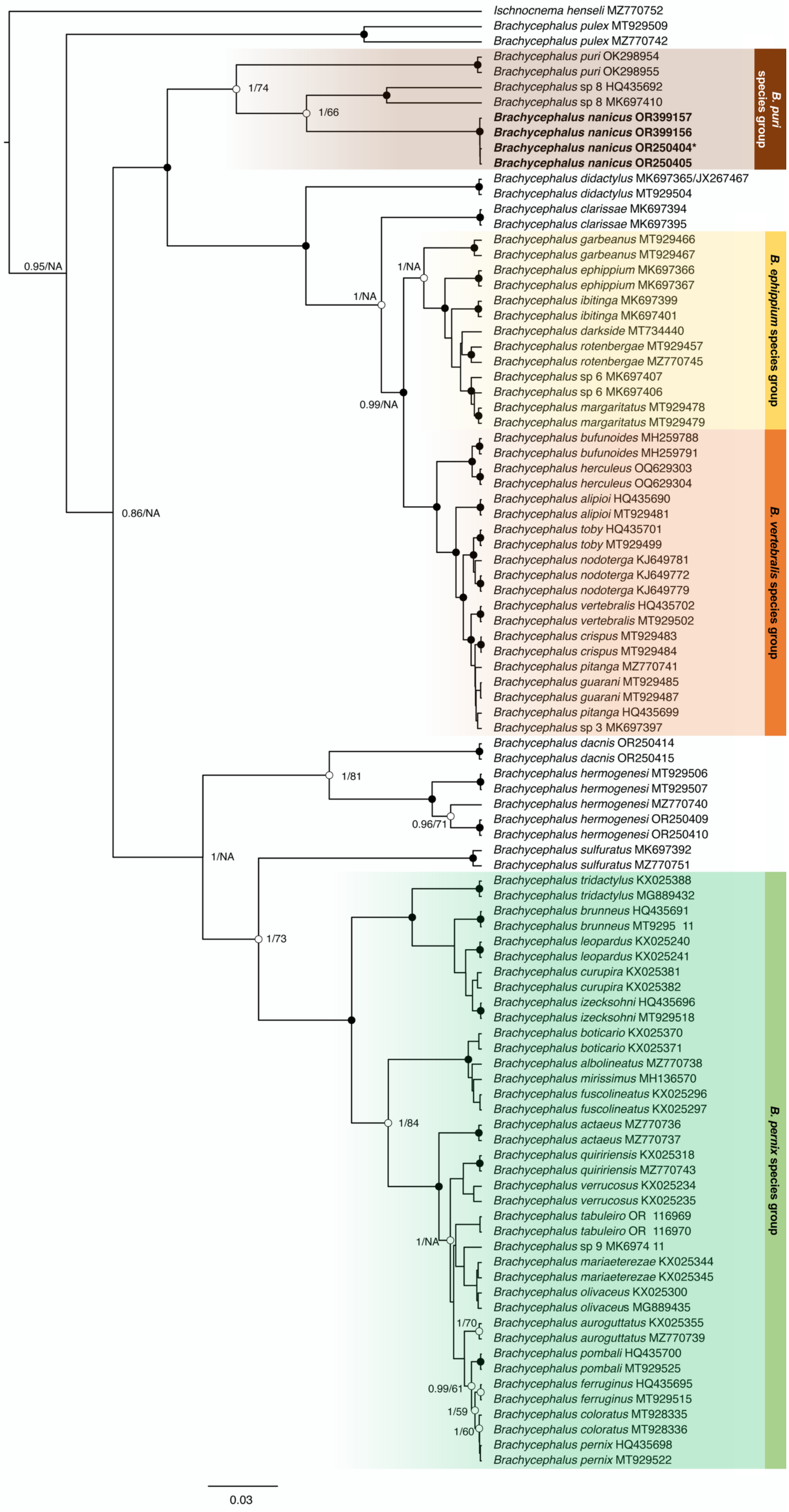

FIGURE 1. Phylogenetic relationships of *Brachycephalus* based on the mitochondrially encoded 16S rRNA fragment (1375 pb). Topology inferred from the Bayesian inference. Posterior probabilities (PP) and Maximum Likelihood bootstrap (BS) are shown at each node (PP/BS). Black dots indicate high support for both phylogenetic inferences (PP ≥ 99; BS ≥ 85). White dots indicate high support for only Bayesian inferences (PP ≥ 99). NA= node is not present in ML tree. Scale bar represents the number of substitutions per site.

Condez *et al*. 2020) + new species (Fig. 1). The sequences obtained for the four individuals of the new species were identical. The *16S* genetic distance for the "Palumbi" fragment between new species and *Brachycephalus* sp. 8 was estimated to be 12.7% and between new species and *B. puri* was around 15.8–16% (Appendix IV). For the 16S-UP fragment, the genetic distance between new species and *B*. sp. 8 from Bananal, SP (MK697410) was 14.7% and between new species and *B*. sp. 8 from Ilha Grande (HQ435692) was 16%. Most species presented very low intraspecific diversity (from 0–2.5% in the Palumbi region; 0–5.6% in the 16S-UP fragment), with two exceptions, *B. pulex* and *B*. sp. 8 (Appendix IV). The intraspecific distance of *Brachycephalus* sp. 8 was 9.2% 16S-UP fragment, and between 6.9% and 10% for *B. pulex*.

The monophyly of the *B. pernix*, and *B. vertebralis* species groups were also recovered, with strong support in both analyses, however the *B. ephippium* species group was not recovered monophyletic in the ML analysis, due to the alternative position of *B. clarissae* and *B. garbeanus* (Supplementary data S2). Relationships between species within clades are not well supported in general. All the remaining flea-toads' species were placed at distant locations throughout the tree (Fig. 1). In the BI tree, the deepest split within the genus occurs with the separation of *B. pulex* from all other species. From this position, we have two major clades. One comprised by (*B. puri* + (*Brachycephalus* sp. 8 + new species)) as sister clade of (*B. didactylus* + (*B. clarissae* (*B. ephippium* species group + *B. vertebralis* species group))). The other clade is comprised by ((*B. hermogenesi* + *B. dacnis*) + (*B. sulfuratus* + *Brachycephalus pernix* species group)). The position of *B. hermogenesi*+ *B. dacnis* and *B. pulex* are not supported by the ML tree, in which *B. hermogenesi*+ *B. dacnis* is sister taxa of *B. pulex*+all other species.

Description of the new species

Brachycephalus nanicus sp. nov.

https://zoobank.org/B7A9AA10-0F6A-41FE-AFE3-AAF78FB87B4D
Figure 2

Chresonymy

Brachycephalus sp. Toledo *et al*. 2024

Holotype. Adult male (ZUEC-AMP 25581; field number LaHNAB 251) collected in Trilha do Ouro, Estação Ecológica de Bananal (22S 48' 14", 44W 22' 17", ~1.220m a.s.l.; WGS84 Datum; Figure 3), municipality of Bananal, state of São Paulo, Brazil by L.M. Botelho, L.A.O.S. Ferreira, E. Muscat, M. Moroti, T.M. Machado, and J.P. Bovolon on 28 March 2022.

Paratopotypes. ZUEC-AMP 25577–25579, 25610 and 25617 (Figure 4) adult males, and 25580 indetermined sex, all of them collected with the holotype by the same collectors; and ZUEC-AMP 25259, an adult female, also collected in the type-locality by J. Prado on 10 December 2021.

Etymology. The specific epithet *nanicus* an adjective in Latin (meaning those with reduced size) in allusion to the extremely small body size of the adult individuals of this *Brachycephalus* species (less than 1 cm).

Diagnosis. *Brachycephalus nanicus* can be assigned to the genus *Brachycephalus* by observed phalangeal reduction, and phylogenetic affinities. Member of the *Brachycephalus puri* species group (new group proposal), with the content of *B. puri*, *B. nanicus* and *Brachycephalus* sp. 8 (*sensu* Condez *et al*. 2020). The new species can be diagnosed from its congeners by the following combination of characters: leptodactyliform body; adult SVL 6.9–9.0 mm for males and 9.2–9.6 mm for females; smooth skin lacking hyperossification; snout rounded in dorsal view; finger II with pointed tips (arrow-shaped); toe I externally absent (lacking phalanges), and toes II, III, IV and V distinct; toe II with rounded tips; brown background with few golden spots scattered aleatory in the general coloration of dorsal surfaces and cloacal region; presence of a dark brown stripe along the flanks and the thigh; yellow blotches on the ventral surfaces; presence of dark brown strips on the legs. The new species is also genetic distinct.

Holotype description. Body slender and leptodactyliform (Figure 2); head as wide as long (HL/HW = 1.02%), narrower than the body; head length approximately 34% of SVL; snout shorter than the eye diameter, rounded in dorsal and lateral views; nostrils oval and protuberant, directed anterolaterally; *canthus rostralis* distinct; loreal region slightly concave; mouth nearly sigmoid; eye slightly protruding in dorsal and lateral views, 38.5% (ED/HW) and 37.5% (ED/HL); tympanum indistinct; vocal sac not externally expanded; vocal slights present; tongue longer than wide, posterior half not adherent to the floor of mouth; choanae relatively small and round; vomerine teeth absent. Arm and forearm slender; arm slightly shorter than forearm (UAL 15.4% and FAL 30.5% of SVL); hands shorter than the forearm and longer than the upper arm; externally, finger I and IV small, vestigial; fingers II and III robust and distinct; relative lengths of fingers I ≤ IV < II < III; inner and outer metacarpal tubercles absent; subarticular tubercles undefined in hand (Figure 2C). Leg relatively long, total leg length 81% of SVL; shank slightly shorter than thigh (SHL/SVL = 35% and THL/SVL = 45.8%); foot longer than tarsus and the shank; toe I externally not visible; toes II, III, IV and V distinct; relative toe length V < II < III < IV; metatarsal tubercles present, both oval, outer slightly larger than inner; subarticular tubercles undefined in foot (Figure 2D). Skin smooth without dermal ossifications. Measurements of the holotype (and the type series) are available in Table 1.

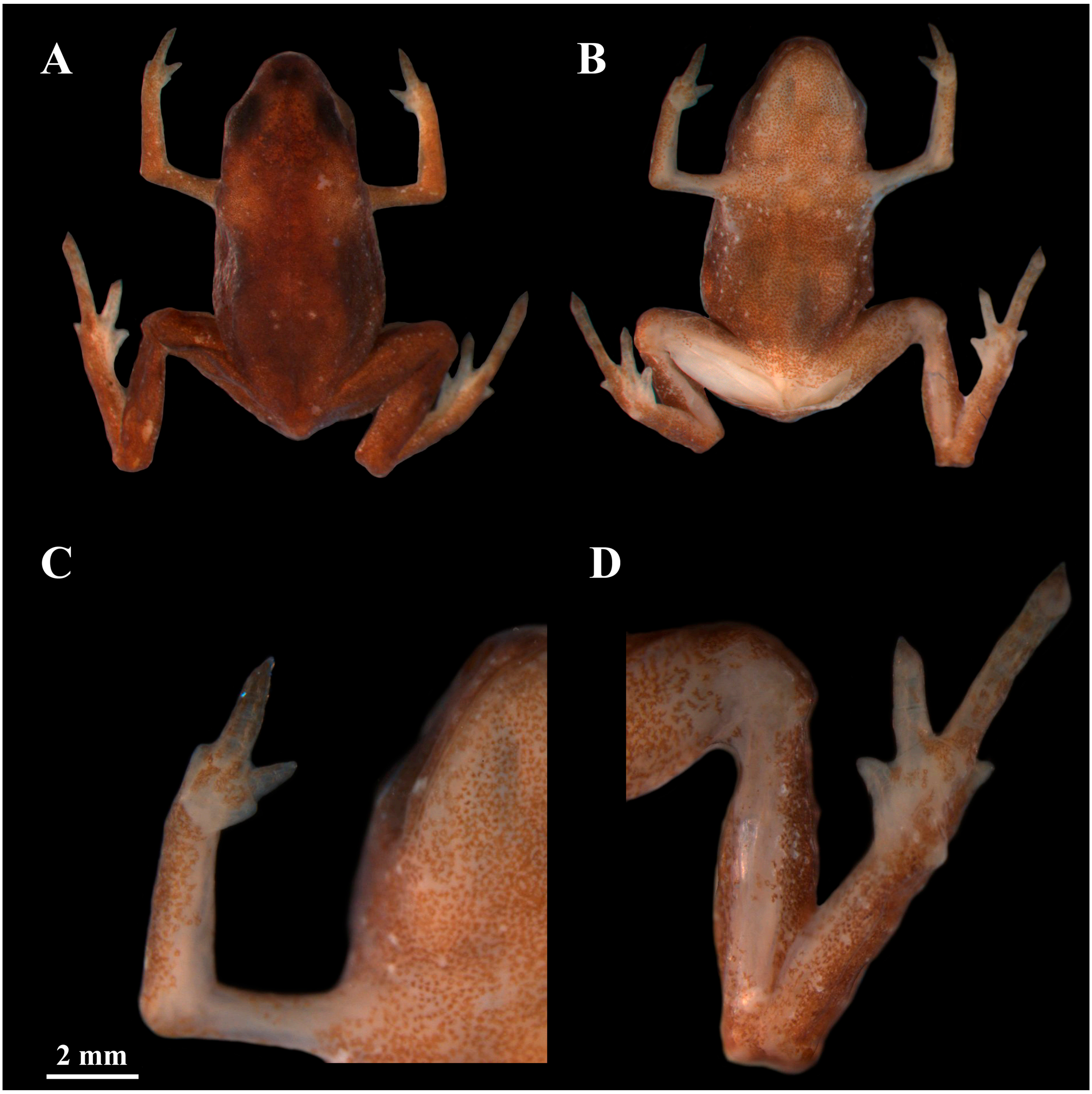

FIGURE 2. *Brachycephalus nanicus*, holotype (ZUEC-AMP 25581), adult male, SVL = 8.7 mm. (A) dorsal view, (B) ventral view, (C) ventral view of hand, and (D) ventral view of feet.

TABLE 1. Morphometric measurements of the holotype and five adult topotypes of *Brachycephalus nanicus*. Values are presented in millimeters (mm) as Range (mean ± standard deviation). See the Material and Methods section for the abbreviations.

Measurements	Holotype	Males (n = 4)	Females (n = 1)
SVL	8.7	7.25 – 8.16 (7.58 ± 0.41)	9.55
HL	2.96	2.85 – 3.00 (2.94 ± 0.07)	3.46
HW	2.88	2.69 – 3.19 (2.88 ± 0.22)	3.25
END	0.47	0.26 – 0.50 (0.41 ± 0.11)	0.51
IND	0.9	0.95 – 1.00 (0.99 ± 0.03)	1.17
ND	0.37	0.25 – 0.43 (0.36 ± 0.08)	0.50
ED	1.11	0.86 – 0.97 (0.90 ± 0.05)	0.98
IOD	1.77	1.62 – 1.66 (1.64 ± 0.02)	1.72
UAL	1.34	0.97 – 1.44 (1.19 ± 0.20)	1.08
FAL	1.66	1.15 – 1.67 (1.43 ± 0.22)	1.42
HAL	1.7	1.26 – 1.65 (1.38 ± 0.18)	1.79
THL	3.99	2.55 – 3.90 (3.23 ± 0.56)	3.72
SHL	3.05	2.42 – 3.00 (2.76 ± 0.25)	3.24
FL	5.43	4.60 –5.33 (4.96 ± 0.33)	5.13

Comparison with other species. The leptodactyliform body of *Brachycephalus nanicus* distinguishes it from *B. clarissae* and the species belonging to the groups of *B. ephippium*, *B. pernix*, and *B. vertebralis* which have bufoniform phenotype (Almeida-Silva *et al*. 2021; Condez *et al*. 2021; Folly *et al*. 2021, 2024; Lyra *et al*. 2021; Nunes *et al*. 2021; Mângia *et al*. 2023). *Brachycephalus nanicus* adult female size (SVL 9.55 mm) is smaller than that of *B. clarissae* (female SVL 10.1–10.9 mm; Folly *et al*. 2022), *B. didactylus* (female SVL 10.2 mm; Izecksohn 1971), *B. tabuleiro* (female SVL 10.9–12.7 mm; Mângia *et al*. 2023), and *B. puri* (female SVL 10.2 mm; Almeida-Silva *et al*. 2021). *Brachycephalus nanicus* can also be distinguished from *B. clarissae* by the smooth dorsal skin [dorsum with a median row of small light-colored (yellow in life, cream in preservative) tubercles; Folly *et al*. 2022], HL/SVL = 34%, SHL/SVL = 35%, and abdominal muscles transparent also can distinguish *Brachycephalus nanicus* from *B. dacnis* (HL/SVL = 23.6%, SHL/SVL = 40.6%, and muscles with a slight dark-brown coloration – melanocytes; Toledo *et al*. 2024). *Brachycephalus nanicus* has toe I externally not visible, and toes II, III, IV and V distinct. This condition distinguishes the new species from *B. didactylus* (toe I not visible, toes II and III distinct and functional, and toe IV and V vestigial; Izecksohn, 1971; Napoli *et al*. 2011), *B. hermogenesi* (toes IV [externally not visible] and V reduced; Napoli *et al*. 2011), *B. pulex* (toes I, II, and V not visible, and toes III and IV distinct and functional; Napoli *et al*. 2011), and *B. puri* (toe I not visible, II, III, and IV functional, and V vestigial; Almeida-Silva *et al*. 2021). The new species can be diagnosed from *B. dacnis*, *B. didactylus*, *B. hermogenesi*, *B. pulex*, *B. puri*, and *B. sulfuratus* by having brown background with few golden spots scattered randomly in the general coloration of dorsal surfaces and cloacal region [dark ornamentations (some with x-shaped mark) in these species; Izecksohn 1971; Giaretta & Sawaya 1998; Napoli *et al*. 2011; Condez *et al*. 2016; Almeida-Silva *et al*. 2021; Toledo *et al.* 2024)]. *Brachycephalus nanicus* can also be distinguished from *B. didactylus, B. hermogenesi, B. puri*, and *B. pulex* by having yellow blotches on the ventral surfaces (absent in these species; Izecksohn 1971; Giaretta & Sawaya 1998; Almeida-Silva *et al*. 2021). The new species can also be diagnosed from *B. puri* by the rounded snout in dorsal view, finger II with pointed tips (arrow-shaped), and the presence of a dark brown stripe along the flanks and of the thigh (mucronate snout, finger II with rounded tips, and dark brown stripe absent in *B. puri*; Pie & Ribeiro 2015; Almeida-Silva *et al*. 2021; Folly 2022). The new species can also be diagnosed from *B. didactylus* by toe II with pointed tips (toe II with rounded tips in *B. didactylus*; Izecksohn 1971). The new species can also be diagnosed from *B. pulex* by the dark brown strips on the legs (reddish brown stripes in *B. pulex*; Napoli *et al*. 2011).

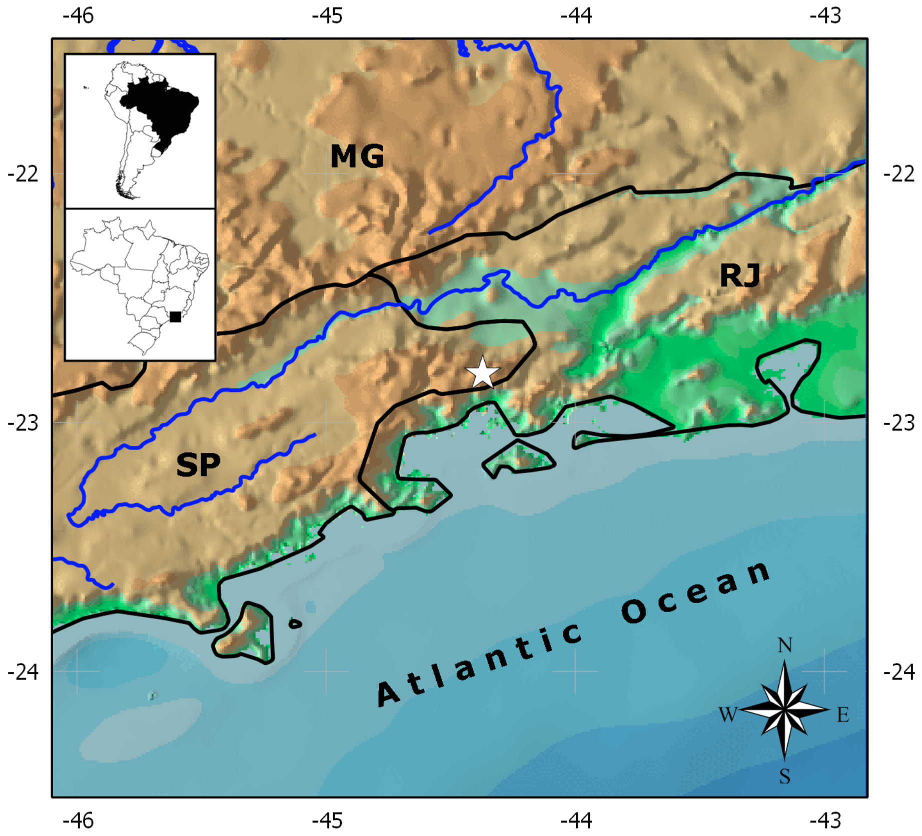

FIGURE 3. Locality where the specimens of the new species of *Brachycephalus* were collected (Star): Estação Ecológica de Bananal, municipality of Bananal, state of São Paulo, southeast Brazil. MG = Minas Gerais; RJ = Rio de Janeiro; SP = São Paulo.

The smooth skin lacking discernible osteoderms can diagnose *Brachycephalus nanicus* from *B. alipioi, B. bufonoides, B. crispus, B. ephippium, B. garbeanus, B. guarani, B. herculeus, B. margaritatus, B. nodoterga, B. pitanga, B. toby,* and *B. vertebralis* (which present hyperossification: Clemente-Carvalho *et al*. 2009; Haddad *et al*. 2010; Pombal 2010; Clemente-Carvalho *et al*. 2012; Condez *et al*. 2010, 2014; Folly *et al.* 2021, 2024). Though many flea-toads are often externally similar looking, there is substantial variation in the extent of reductions and patterns of co-ossification in their skeletons. In contrast to the extensively synostosed cranium of *B. nanicus*, the cranial bones are not coossified in *B. dacnis* or *B. sulfuratus*, but is similarly synostosed in *B. clarissae*, *B. didactylus*, and *B. hermogenesi*, (for comparisons, see Figure 7 of Toledo *et al*., 2024); in *B. hermogenesi*, the nasals are not synostosed to the composite cranium. Like *B. nanicus*, *B. clarissae* and *B. didactylus* are edentulous, whereas *B. dacnis* and *B. sulfuratus* have a handful of small teeth or odontoids on the maxillae and premaxillae, and teeth are well developed and more numerous in *B. hermogenesi*. The squamosal of *B. nanicus* has an anterior flange that incorporates a small zygomatic ramus and is like that of *B. dacnis*, *B. didactylus*, *B. hermogenesi*, and *B. sulfuratus*, whereas *B. clarissae* has only a small anterior flange along the body of the squamosal. The quadratojugal of *B. nanicus* is like that of *B. didactylus* in being small, restricted to the distal end of the squamosal, and lacking an anterior process, whereas there is a thin, rodlike anterior process in *B. clarissae*, *B. dacnis*, *B. hermogenesi*, and *B. sulfuratus*. The pterygoid of *B. nanicus* is less robust than in *B. dacnis*, and *B. hermogenesi*, but more robust than the thin rod-like pterygoid of *B. clarissae* and *B. didactylus*. In contrast to the vertebral fusions observed in a skeletally mature *B. nanicus* (ZUEC-AMP 25610; none observed in the smaller ZUEC-AMP 25580), there are no vertebral fusions in *B. clarissae*, *B. dacnis*, *B. hermogenesi*, or *B. sulfuratus*; in *B. didactylus*, presacral vertebrae VI, VII, and VIII are co-ossified. Among the flea-toads examined, the urostyle of *B. didactylus* is unique in having laterally expanded flanges extending along its length. In *B. nanicus*, the single phalanx of fingers I and IV are tiny elements (similar to *B. clarissae*), whereas these are slightly larger in *B. dacnis* and *B. sulfuratus*, even more robust in *B. hermogenesi*, and lacking completely in *B. didactylus*.

FIGURE 4. *Brachycephalus nanicus* (ZUEC-AMP 25617) in life. (A) On the leaf-litter, and (B) on top of a coin.

Osteology. Based on ZUEC-AMP 25610 (sex unknown). The skeleton of *B. nanicus* is 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) seems to be osteologically mature with ossified mesopodials and complete, ossified distal long bones.

The skull is compact, slightly longer than wide, and lacking ornamented dermal roofing bones. The frontoparietals, sphenethmoids, prootics, exoccipitals, parasphenoid, nasals, and ossified nasal cartilages are completely synostosed. There are well-defined and bony margins to the optic fenestrae and prootic foramina as well as within the sphenethmoid and nasal cartilages, including for the olfactory nerve. The premaxillae are broad, clearly separated from one another, and lack teeth; each has a robust and deep *pars dentalis*, and a robust club-like alary process that is taller than wide and widely separated from the nasal. In ventral view, the maxillae are nearly straight and lack teeth and odontoids. The quadratojugals are compact, restricted to the ventral end of the squamosal, and lack a rostrally projecting process; there is a small medially projecting process articulating with the squamosal. The pterygoids are slender, with a flattened subtriangular posterior ramus adjacent to but not articulating with the ventral ramus of the squamosal, and a short medial ramus that abuts the prootic each; in this specimen, the right anterior ramus is a short process whereas the right ramus is straight and long, approaching but not articulating with the adjacent maxilla. Distinct neopalatines are not observable and, if present, these might be synostosed to the sphenethmoid; in the other CT-scanned specimen (ZUEC-AMP 25580), which is skeletally immature and lacks cranial synostosis, there is no distinct neopalatine. If present, the vomers are synostosed to the ossified nasal cartilages and sphenethmoid. The septomaxillae appear to be synostosed to the anterior margin of the ossified n nasal cartilages. The parasphenoid is broad with a long, rectangular anterior ramus that is synostosed to the sphenethmoid and prootics. The squamosals are robust, and each has a prominent sheet-like flange along its anterior margin and a hook-like slender posterior ramus that is adjacent but not fused to the prootic. The prootic is well ossified and has a well-defined fenestra ovalis, but neither an operculum nor a columella is present. The posteromedial processes of the hyoid are ossified and slender. The arytenoid cartilages are not obviously mineralized.

There are eight procoelous, imbricating presacral vertebrae with well-developed and intercalating neural spines. The atlas lacks transverse processes and cotyles that are widely separated, a distance more than twice the posterior diameter of the atlas centrum. Transverse processes of presacral II are distinctly shorter than those of presacral III–IV, which are synostosed. The broad, fused transverse processes of III–IV have a large ventral foramen, presumably for the passage of spinal nerves. Presacral VIII is weakly synostosed to the sacrum; in dorsal view, these vertebrae appear continuous but in ventral view there are gaps between them. The sacrum has stout transverse processes that are approximately as long as those of III–IV. There is a prominent sesamoid at the distal transverse process of the sacrum at its articulation with the ilium. The urostyle is shorter than the presacral vertebral column and bears a dorsal ridge that decreases in height posteriorly along the anterior two-thirds of the urostyle.

The pectoral girdle is well ossified. The coracoids are expanded medially and appear to have met at the midline; they appear to be out of articulation from preservation and dissection. The coracoids are broad and articulated at the midline. The scapula is short with a well-developed anterior process and a prominent supraglenoid foramen. The epicoracoid and procoracoid cartilages are heavily mineralized and form solid elements that are synostosed to the coracoid and clavicle creating a larger composite pectoral girdle.

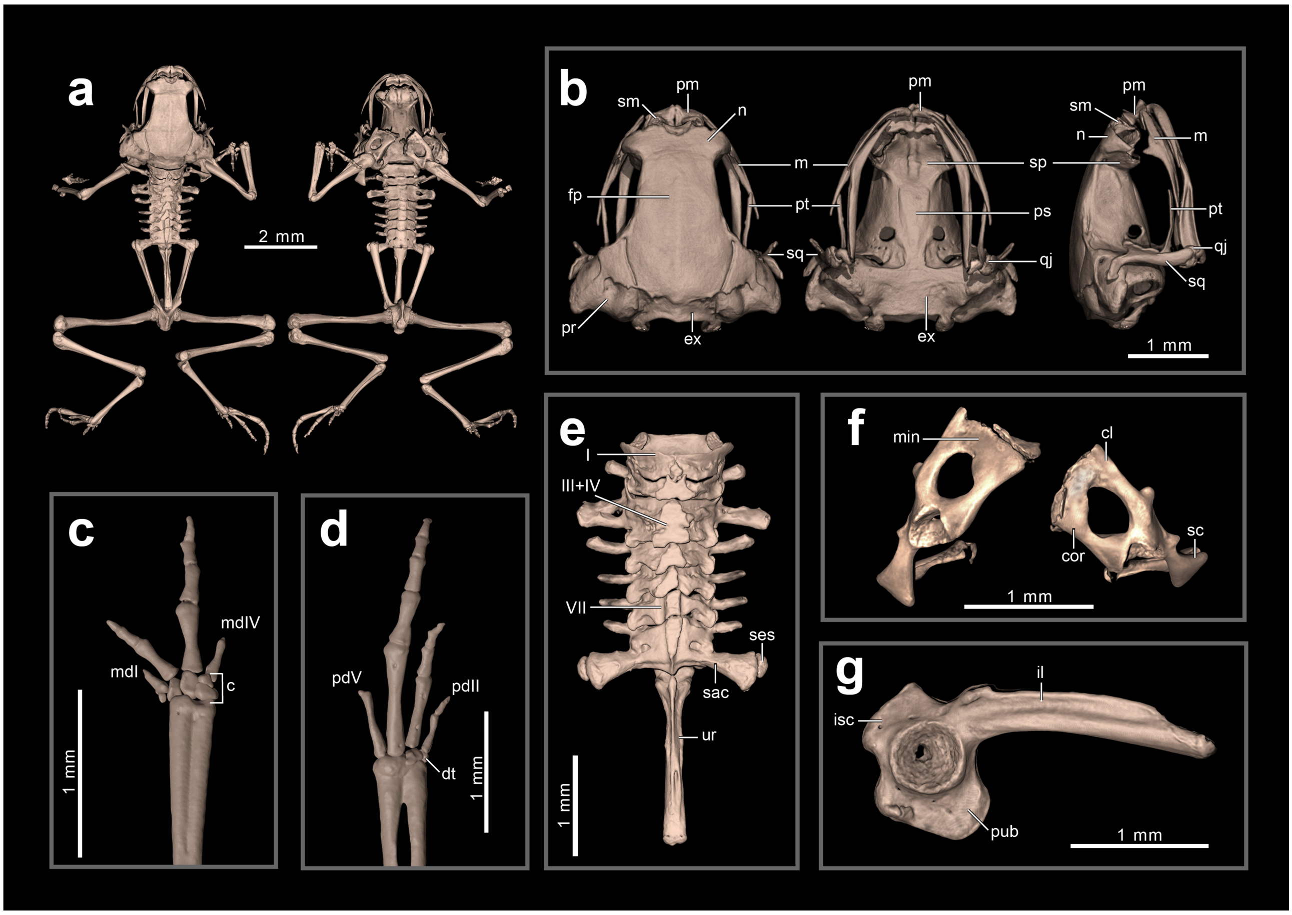

FIGURE 5. Skeletal anatomy of *Brachycephalus nanicus* (ZUEC-AMP 25610; undetermined sex) based on CT-scans. (A) The entire skeleton, with insets of (B) the skull (in dorsal, ventral, and right lateral views), (C) right hand and (D) left foot with pedal digit I not visible (both in dorsal view), (E) 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; mdIV, manual digit IV; min, mineralized cartilage; n, nasal; op, operculum; pd II, pedal digit II; 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.

The pelvic girdle is a robust element composed of synostosed ilium, pubis, and ischium; the pubis and ischium are completely ossified. The circular acetabulum is well ossified with 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 that is continuous with a prominent dorsal crest. There is a broad, plate-like ventral acetabular expansion comprising the ilium and pubis.

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, with the phalanges of I and IV being small and barely discernable. There appears to be a single, minute ossified prepollex. There is one palmar sesamoid, which is larger than the phalanges of I and IV. The tips of the terminal manual phalanges are arrow-shaped in digits II and III but small triangular blobs in I and IV. The tibiofibula is slightly shorter than the femur. There are two large distal tarsals that are shorter than the tibiofibular. There is no ossified fabellar or tarsal sesamoids. The phalangeal formula for the pes is 0–2–3–4–1 and there is a single small ossified prehallux. On the plantar surface, there are no ossified medial sesamoids, but laterally there are two large sesamoids. The tips of the terminal pedal phalanges are expanded and blunt (rounded) in in digits III–IV and rounded in digit II; the phalanx of digit V is small round ossification.

Color in life. The general coloration of dorsal surfaces is brown with a few golden spots scattered aleatory in the dorsum surface and in the cloacal region (Figure 4). A golden stripe extends laterally from the tip of the snout to the flanks. The dorsal surfaces of arms and legs have dark brown stripes. The lateral view of the body is dark brown; the maxilla is dark brown with distinct white spots. The ventral surface of the body is brown with small white blotches concentrated in the cloacal region. The ventral surface of the hand and feet are light brown with dark brown blotches in the fingers and toes. The pupil is black, and the iris is golden.

Color in preservative. The general coloration of the dorsal surfaces is brown, darkened in the nostril and eyes region; the golden spots became pale cream. In recently preserved individuals, the stripes in the arms and legs became less distinct and difficult to discern, becoming more visible in individuals that have been preserved for a long time. The extremities of fingers and toes became pale cream. The ventral background coloration is cream and tinged light brown; the *linea masculine* absent. There are white spots in the maxillae, ventral, and cloacal regions. Eyes are black.

Internal anatomy. An adult female (ZUEC-AMP 25259) and an adult male (ZUEC-AMP 25578) of *B. nanicus* were dissected. In the female, after removing the skin, we observed that the abdominal muscles were transparent, without pigmentation, and we could see through them the ventral abdominal vein, pelvic veins, liver, gallbladder, and heart (Figure 6A and B). After cutting the abdominal muscle, cutting, and separating the procoracoid bone, we observed the internal organs (Figure 6B). The liver was yellowish white (Figure 6A and B). The heart presented slight pigmentation on the left side of the ventricle in ventral view. The species presented pigmentation near the carotid artery. Similarly, dark pigmentation, probably melanocytes, was observed at the ventral abdominal vein, near the point of origin of the right and left branches of the abdominal vein (Figure 6C). Such dark pigmentation can also be observed in the external jugular vein. Behind the liver, we observed two large eggs in the oviduct, which (the oviduct) was darkly pigmented by melanocytes (Figure 6D). The white abdominal pigmentation was observed in the male of *B. nanicus*, the dorsal white musculature was pigmented in *B. nanicus* male. The parathyroid glands were ovoid, transparent, presented a minor blood supply, and were single on each side of the body, near the bifurcation of the external and internal carotid arteries (Figure 6C).

The male's abdominal musculature was triangular shaped, white pigmented, mainly covering the *rectus abdominalis* muscle. The *pars abdominalis* of *m. pectoralis* did not present such white coloration and was transparent (Figure 6E). In the female all the abdominal musculature was transparent. The liver was brown colored (Figure 6F), different from the female coloration. The left lobe of the liver was bilobed in males and females, but in males, the liver was relatively smaller than in females. In females, the left lobe covered the stomach almost completely (Figure 6B), whereas in males it slightly exceeded half the stomach size (Figure 6F). The right lobe was also smaller in the male than in the female. The dorsal musculature presented a slight dark pigmentation in the male (Figure 6G), not observed in the female. The heart and parathyroid glands were similar in male and female (Figure 6C and H).

Variation. The specimens are alike. We only have noticed some color variation among individuals with some frogs having a darker dorsal coloration.

Natural history. During the study we found six individuals of *B. nanicus* inhabiting a forested area with a deep leaf litter cover. Although the air humidity was over 98% in all samples, the individuals were always hidden under the leaf litter and were found among it or using roots and rotten trunks as shelter. The specimens displayed different defensive behaviors upon our approach: motionless, crouching down posture, or fleeing by jumping away. We also observed a congeneric syntopic species, *B. margaritatus* Pombal & Izecksohn, (2011) (ZUEC-AMP 25239), in the same area as *B. nanicus*. However, the *B. margaritatus* individuals were using the steepest areas of the ravines, whereas *B. nanicus* was all over the area.

We identified a total of 13 consumed prey items in the stomachs of one individual of *B. nanicus* (ZUEC-AMP 25610), belonging to five taxa divided between mites (Arachnida) and hexapods (Hexapoda) (Table 2; Figure 7). Mites were the most frequent (n = 12) prey item in the diet of *B. nanicus*, relative to the other prey categories found. Specifically, the order Mesostigmata was the most frequent item (n = 7), followed by the order Sarcoptiformes (n = 4), and Trombidiformes (n = 1). The Hexapoda prey species were from the order Psocoptera (n = 1; Table 2).

Geographic distribution. The new species is only known for its type-locality: Estação Ecológica de Bananal (22S 48’ 14”, 44W 22’ 17”, ~1.220 m a.s.l.; WGS84 Datum), municipality of Bananal, state of São Paulo, Brazil.

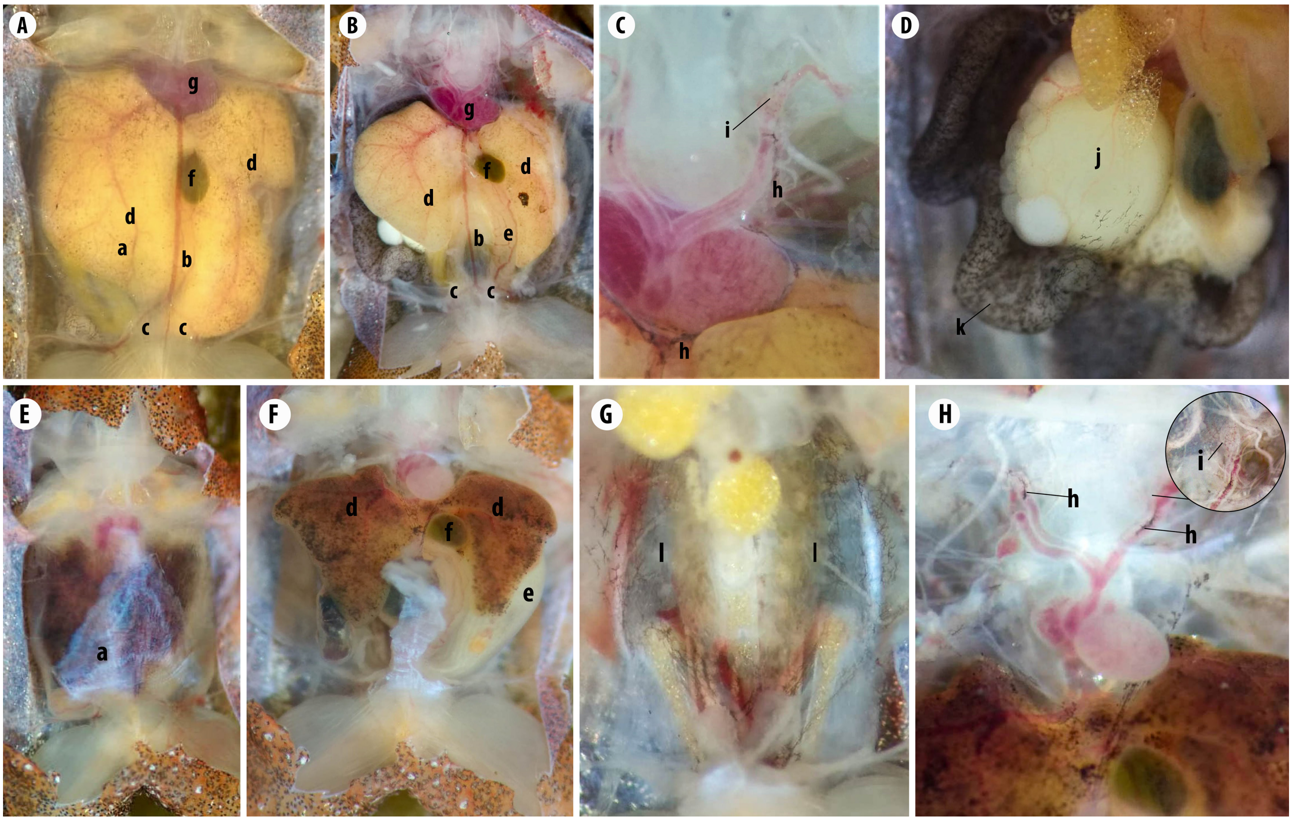

FIGURE 6. Ventral dissection of *Brachycephalus nanicus*. (A-D) Adult female (ZUEC-AMP 25259) and (E-H) adult male (ZUEC-AMP 25578). Structures label: a) abdominal muscles b) ventral abdominal vein, c) pelvic veins, d) livers, e) stomach f) gallbladder, g) heart, h) pigmentation of melanocytes, i) parathyroid gland, j) mature ovules, k) oviduct, and l) dorsal muscles.

TABLE 2. Prey items found in the stomach of one specimen of *Brachycephalus nanicus* (ZUEC-AMP 25610) from Bananal, São Paulo, Brazil. N = number of prey items.

Super Classes	Ordem	Subordem	N
Arachnida	Mesostigmata		7
Arachnida	Sarcoptiformes	Oribatida	3
Arachnida	Sarcoptiformes		1
Arachnida	Trombidiformes	Parasitengona	1
Hexapoda	Psocoptera		1

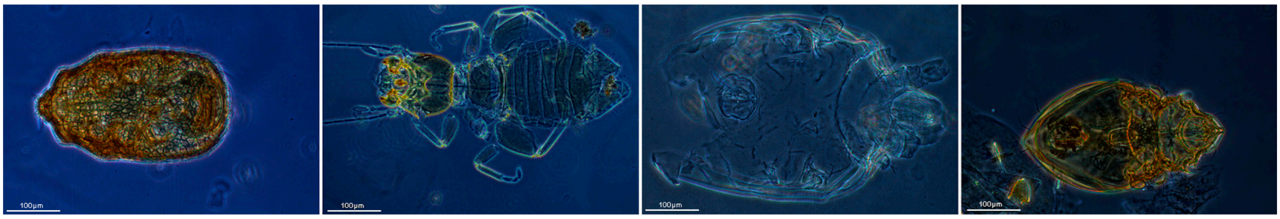

FIGURE 7. Prey items found in the stomach of *Brachycephalus nanicus* (ZUEC-AMP 25610). Mesostigmata (A); Psocoptera (B); Trombidiformes: Parasitengona (C); and Sacoptiformes: Oribatida (D).

Discussion

We found that *Brachycephalus nanicus* is associated with humid forest areas with a deep cover of leaf litter and fallen trunks that can be used as shelter. Our observations about the habitat are like the ones already documented for the genus (Pombal & Gasparini 2006; Clemente-Carvalho *et al.* 2011; Pombal & Izecksohn 2011; Nunes *et al*. 2021). Like other flea-toads' species (see Verdade *et al*. 2008; Almeida-Santos *et al*. 2011; Napoli *et al*. 2011; Condez *et al*. 2016), *B. nanicus* is difficult to find due to its extremely reduced body size, cryptic coloration and the habit of living among the leaf litter. The specimens were active only during the day, showing a tendency to prefer days with higher humidity. Species of the genus *Brachycephalus* find optimal temperature and humidity microclimatic conditions during the day (e.g., Verdade *et al.* 2008; Pombal & Izecksohn 2011; Oliveira & Haddad 2017; Pie *et al.* 2018; Nunes *et al.* 2021). The permeable skin of amphibians makes them susceptible to water loss and desiccation (Duellman & Trueb, 1994; Levy & Heald, 2016). As the water loss and desiccation is surface-area-to-volume ratio-dependent, *Brachycephalus* species are deeply affected due to their small sizes (Duellman & Trueb, 1994; Levy & Heald, 2016; Condez *et al.* 2019). For this reason, the leaf litter environment is important, because it provides not only shelter but also the moisture that protects the frogs from desiccation even in the driest or coldest conditions (Condez *et al.* 2019).

Brachycephalus nanicus presented different defensive strategies, but with similar functions. Being motionless or in a crouching posture, in addition to its cryptic coloration, provides individuals an advantage against visually oriented predators (Toledo *et al.* 2011). Fleeing by jumping, besides avoiding subjugation (Toledo *et al.* 2011), could be especially successful for the flea-toads, which could jump several times its body length, falling in the matching background leaf litter far from the predator.

The diet of the one adult *B. nanicus* that we examined (ZUEC-AMP 25610; Table 2) was similar to other limited existing records for congenerics. The presence of mites was also reported for *B. brunneus* Ribeiro, Haddad, Alves and Reis, 2005 (62% of the food items; Fontoura *et al.* 2011), *B. garbeanus* Miranda-Ribeiro, 1920 (Dorigo *et al.* 2012), *B. pernix* Pombal, Wistuba and Bornschein, 1998 (Wistuba 1998), and *B. pitanga* Alves, Sawaya, Reis, and Haddad, 2009 (~ 52–77%; Oliveira & Haddad 2015). However, the diet of *B. didactylus* and *B. ephippium* was composed mostly of collembolas, followed by mites (Almeida-Santos *et al.* 2011; Pombal 1992). *Brachycephalus garbeanus* had ants as the second most abundant stomach content (Dorigo *et al.* 2012) but was absent in the diet of *B. nanicus*. Anuran's diet is influenced by phenotypic traits such as body and mouth gape sizes (Simon & Toft 1991; Moroti *et al.* 2021; Ceron *et al.* 2023). Consequently, the remarkable miniaturization process that the species of *Brachycephalus* underwent (see Folly *et al.* 2022) must be influencing their diet.

Internally, the presence of melanocytes was found in *B. nanicus*, as well as in *B. dacnis* (Toledo *et al*. 2024). One of the hypotheses for the pigmentation of musculature and organs is due to the absorption of the tail from tadpoles to post-metamorphics (Divya *et al.* 2010). However, the species of the genus *Brachycephalus* have direct development and thus lack pigmented tails. Another hypothesis suggests that temperature is key in controlling the amount of internal melanin, and, among other possibilities, could protect temperature-sensitive organs (Franco-Belussi *et al.* 2017), especially in diurnal frogs as those of the genus *Brachycephalus*. The parathyroid gland of both individuals of *B. nanicus* was single (one on each side) and located in the same region, near the bifurcation of the external and internal carotid arteries. This could be a unique trait among species of *Brachycephalus*, or exclusive of flea-toads (A.C. Medina, personal observation). Thus, further studies may provide information that will clarify if this trait could be used as a diagnostic character among *Brachycephalus* species.

Out of the 42 known species of the genus *Brachycephalus*, 34 were described since the year 2000 (Frost 2024). Therefore, the use of an integrative approach has proven to be effective in solving taxonomic challenges owing to morphological similarity and genetic conservatism among *Brachycephalus* species (Firkowski *et al*., 2016; Condez *et al.* 2020; Nunes *et al.* 2021). This is the eighth flea-toad species described. *Brachycephalus nanicus* is closely related to *B. puri* and the candidate species *Brachycephalus* sp.8. *Brachycephalus nanicus* and *B. puri* are 370 km (straight-line distance) apart and in completely different mountain ranges (Serra da Bocaina and Serra do Caparaó) and thus separated by historical geography as well. In the other side, *Brachycephalus* sp.8 (*sensu* Condez *et al.* 2020), seems to occur in Ilha Grande, SP and Bananal, where it could be sympatric to *B. nanicus.* The occurrence of species in sympatry is not uncommon for flea-toads (Bornschein *et al* 2021), suggesting that additional information is needed to clarify the species status of these candidate species. *Brachycephalus* species recently described and with restricted distributions may be more susceptible to extinction due to habitat loss and lack of information about these species. Therefore, new natural history studies are essential to clarify the diversity of these genus and can provide data to serve as a basis for future conservation actions (Tapley 2019; Bornschein *et al.* 2019; Schulte *et al.* 2020).

Supporting Information. Online archives (Mendeley Data, V1, doi: 10.17632/pbcnwbwjt4.1)

S1 Figure. Bayesian Inference phylogenetic tree.
S2 Figure. Maximum Likelihood (ML) phylogenetic tree.
S3 File. Aligned data matrix of *Brachycephalus* based on the mitochondrially encoded 16S rRNA fragment (1375 pb).
S4 File. Raw morphometric data. Measurements of each individual of the type series of *Brachycephalus nanicus*.

Acknowledgments

We thank the museum curators J. P. Pombal Jr. and U. Caramaschi (MNRJ), T. Grant (MZUSP), R. N. Feio (MZUFV), P. C. A. Garcia (UFMG), I. A. Martins (CCLZU) for the loans or permissions to examine specimens under their care. E. L. Rotenberg provided logistics support. L. A. O. S. Ferreira, M. Moroti and J. P. B. Thomaz helped in the field sampling. Estação Ecológica de Bananal and its staff, T. Filete, L. Cabral, R. Da-Silva, D. Silva, P. Guimarães, R. Valente, W. Pereira, C. Da-Silva, T. Souza, L. Maia, and R. Nascimento, for their fieldwork support. Grants and fellowships were provided by São Paulo Research Foundation to M.L.L. (FAPESP # 2021/10639-5), F.S.A. (FAPESP # 2020/08291-8), T.K. (FAPESP # 2020/14780-1), and L.F.T. (FAPESP #2016/25358-3; #2019/18335-5); the National Council for Scientific and Technological Development to L.F.T. (CNPq #302834/2020-6), and by the Coordination for the Improvement of Higher Education Personnel to I.N, I.G.V. and T.M.M. (CAPES - Finance Code #001) and M.L.L. (CAPES CSF-PVE-S #88887.093994/2015-00).

References

Almeida-Santos, M., Siqueira, C.C., Van Sluys, M. & Rocha, C.F.D. (2011) Ecology of the Brazilian flea frog *Brachycephalus didactylus* (Terrarana: Brachycephalidae). *Journal of Herpetology*, 45, 251–255. https://doi.org/10.1670/10-015.1

Almeida-Silva, D., Silva-Soares, T., Rodrigues, M.T. & Verdade, V.K. (2021) New species of flea-toad, genus *Brachycephalus* (Anura: Brachycephalidae) from the Atlantic Forest of Espírito Santo, Brazil. *Zootaxa*, 5068(4), 517–532. https://doi.org/10.11646/zootaxa.5068.4.3

Alves, A.C.R., Ribeiro, L.F., Haddad, C.F.B. & Reis, S.F. (2006) Two new species of *Brachycephalus* (Anura: Brachycephalidae) from the Atlantic Forest in Paraná state, Southern Brazil. *Herpetologica*, 62, 221–233. https://doi.org/10.1655/05-41.1

Alves, A.C.R., Sawaya, R.J., Reis, S.F. & Haddad, C.F.B. (2009) New species of *Brachycephalus* (Anura: Brachycephalidae) from the Atlantic rain forest in São Paulo state, southeastern Brazil. *Journal of Herpetology*, 43, 212–219. https://doi.org/10.1670/0022-1511-43.2.212

Bornschein, M.R., Ribeiro, L.F., Blackburn, D.C., Stanley, E.L. & Pie, M.R. (2016) A new species of *Brachycephalus* (Anura: Brachycephalidae) from Santa Catarina, southern Brazil. *PeerJ*, 4, e2629.
https://doi.org/10.7717/peerj.2629

Bornschein, M.R., Ribeiro, L.F., Teixeira, L., Belmonte-Lopes, R., de Moraes, L.A., Corrêa, L., Maurício, G.N., Nadaline, J. & Pie, M.R. (2021) A review of the diagnosis and geographical distribution of the recently described flea toad *Brachycephalus sulfuratus* in relation to *B. hermogenesi* (Anura: Brachycephalidae). *PeerJ*, 9, e10983.
https://doi.org/10.7717/peerj.10983

Bouckaert, R., Vaughan, T.G., Barido-Sottani, J., Duchêne, S., Fourment, M., Gavryushkina, A., Heled, J., Jones, G., Kühnert, D., De Maio, N., Matschiner, M., Mendes, F.K., Müller, N.F., Ogilvie, H.A., du Plessis, L., Popinga, A., Rambaut, A., Rasmussen, D., Siveroni, I., Suchard, M.A., Wu, C.H., Xie, D., Zhang, C., Stadler, T. & Drummond, A.J. (2019) BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *Plos Computational Biology*, 15 (4), e1006650.
https://doi.org/10.1371/journal.pcbi.1006650

Ceron, K., Paula, T., Scheibler, P., Fadel, R.M., Guimaraes, C.S., Silva, L.A. & Santana, D.J. (2023) Trophic ecology of small to large hylids from an Amazonia-Cerrado transitional zone in Brazil. *Food Webs*, 36, e00295.
https://doi.org/10.1016/j.fooweb.2023.e00295

Clemente-Carvalho, R.B.G., Giaretta, A.A., Condez, T.H., Haddad, C.F.B. & Reis, S.F. (2012) A new species of miniaturized toadlet, genus *Brachycephalus* (Anura: Brachycephalidae), from the Atlantic Forest of southeastern Brazil. *Herpetologica*, 68 (3), 365–374.
https://doi.org/10.1655/HERPETOLOGICA-D-11-00085.1

Condez, T.H., Clemente-Carvalho, R.B.G., Haddad, C.F.B. & Reis, S.F. (2014) A new species of *Brachycephalus* (Anura: Brachycephalidae) from the highlands of the Atlantic Forest, southeastern Brazil. *Herpetologica*, 70 (1), 89–99.
https://doi.org/10.1655/HERPETOLOGICA-D-13-00044

Condez, T.H., Monteiro, J.P.C., Comitti, E.J., Garcia, P.C.A., Amaral, I.B. & Haddad, C.F.B. (2016) A new species of flea-toad (Anura: Brachycephalidae) from southern Atlantic Forest, Brazil. *Zootaxa*, 4083 (1), 40–56.
https://doi.org/10.11646/zootaxa.4083.1.2

Condez, T.H., Haddad, C.F.B. & Zamudio, K.R. (2021) Historical biogeography and multi-trait evolution in miniature toadlets of the genus *Brachycephalus* (Anura: Brachycephalidae). *Biological Journal of the Linnean Society*, 129 (3), 664–686.
https://doi.org/10.1093/biolinnean/blz200

de Queiroz, K. (1998) The general lineage concept of species, species criteria, and the process of speciation and terminological recommendations. *In*: Howard, D.J. & Berlocher, S.H. (Eds.), *Endless Forms: Species and Speciation*. University Press, Oxford, pp. 57–75.

de Queiroz, K. (2007) Species concepts and species delimitation. *Systematic Biology*, 56 (6), 879–886.
https://doi.org/10.1080/10635150701701083

Dorigo, T.A., Siqueira, C.C., Vrcibradic, D., Maia-Carneiro, T., Almeida-Santos, M. & Rocha, C.F.D. (2012) Ecological aspects of the pumpkin toadlet, *Brachycephalus garbeanus* Miranda-Ribeiro, 1920 (Anura: Neobatrachia: Brachycephalidae), in a highland forest of southeastern Brazil. *Journal of Natural History*, 46 (39–40), 2497–2507.
https://doi.org/10.1080/00222933.2012.713525

Feller, A.E. & Hedges, S.B. (1998) Molecular evidence for the early history of living amphibians. *Molecular Phylogenetics and Evolution*, 9, 509–516.
https://doi.org/10.1006/mpev.1998.0500

Firkowski, C.R., Bornschein, M.R., Ribeiro, L.F. & Pie, M.R. (2016) Species delimitation, phylogeny and evolutionary demography of co-distributed, montane frogs in the southern Brazilian Atlantic Forest. *Molecular Phylogenetics and Evolution*, 100, 345–360.
https://doi.org/10.1016/j.ympev.2016.04.023

Folly, M., Amaral, L.C., Carvalho-E-Silva, S.P. & Pombal, J.P. (2020) Rediscovery of the toadlet *Brachycephalus bufonoides* Miranda-Ribeiro, 1920 (Anura: Brachycephalidae) with osteological and acoustic descriptions. *Zootaxa*, 4819 (2), 265–294.
https://doi.org/10.11646/zootaxa.4819.2.3

Folly, M., Luna-Dias, C., Miguel, I.R., Ferreira, Jr, J.C., Machado, A., Lopes, R.T. & Pombal, J.P., Jr. (2021) Tiny steps towards greater knowledge: An osteological review with novel data on the Atlantic Forest toadlet of the *Brachycephalus ephippium* species group. *Acta Zoologica*, 104, 71–105.
https://doi.org/10.1111/azo.12398

Folly, M., Costa, B.B., Carmo, L.F., Martins, A. & Pombal, J.P., Jr. (2022) Untangling the morphological contradiction: First ontogenetic description of the post-hatching skeleton of the direct-developing frog *Brachycephalus garbeanus* Miranda-Ribeiro, 1920 (Amphibia: Anura: Brachycephalidae) with comments on the genus miniaturization. *Zoologischer Anzeiger*, 298 (2), 39–54.
https://doi.org/10.1016/j.jcz.2022.02.005

Folly, M., Condez, T.H., Vrcibradic, D., Rocha, C.F.D., Machado, A.S., Lopes, R.T. & Pombal, J.P., Jr. (2024) A new species of *Brachycephalus* (Anura: Brachycephalidae) from the northern portion of the state of Rio de Janeiro, southeastern Brazil. *Vertebrate Zoology. Senckenberg*, 74, 1–21.
https://doi.org/10.3897/vz.74.e103573

Fontoura, P.L., Ribeiro, L.F. & Pie, M.R. (2011) Diet of *Brachycephalus brunneus* (Anura: Brachycephalidae) in the Atlantic rainforest of Paraná, southern Brazil. *Zoologia*, 28 (5), 687–68.
https://doi.org/10.1590/S1984-46702011000500019

Fouquet, A., Vences, M., Salducci, M.D., Meyer, A., Marty, C., Blanc, M. & Gilles, A. (2007) Revealing cryptic diversity using molecular phylogenetics and phylogeography in frogs of the *Scinax ruber* and *Rhinella margaritifera* species groups. *Molecular Phylogenetics and Evolution*, 43, 567–582.
https://doi.org/10.1016/j.ympev.2006.12.006

Frost, D.R. (2024) Amphibian Species of the World: an online reference. Version 6.2. 6 February 2025. American Museum of Natural History, New York, New York. Available from: https://amphibiansoftheworld.amnh.org/index.php (accessed 28 July 2025)
https://doi.org/10.5531/db.vz.0001

Garey, M., Provete, D., Martins, I., Haddad, C.F.B. & Rossa-Feres, D. (2014) Anurans from the Serra da Bocaina National Park and surrounding buffer area, southeastern Brazil. *Check List*, 10 (2), 308–316.
https://doi.org/10.15560/10.2.308

Garey, M.V., Lima, A.M.X., Hartmann, M.T. & Haddad, C.F.B. (2012) A new species of miniaturized toadlet, genus *Brachycephalus* (Anura: Brachycephalidae), from southern Brazil. *Herpetologica*, 68, 266–271.
https://doi.org/10.1655/HERPETOLOGICA-D-11-00074.1

Giaretta, A.A. & Sawaya, R.J. (1998) Second species of *Psyllophryne* (Anura: Brachycephalidae). *Copeia*, 1998 (4), 985–987.
https://doi.org/10.2307/1447345

Guimarães, C.S., Luz, S., Rocha, P.C. & Feio, R.N. (2017) The dark side of pumpkin toadlet: A new species of *Brachycephalus* (Anura: Brachycephalidae) from Serra do Brigadeiro, southeastern Brazil. *Zootaxa*, 4258 (4), 327–344.
https://doi.org/10.11646/zootaxa.4258.4.2

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the Performance of PhyML 3.0. *Systematic Biology*, 59 (3), 307–321.
https://doi.org/10.1093/sysbio/syq010

Haddad, C.F.B., Alves, A.C.R., Clemente-Carvalho, R.B.G. & Reis, S.F. (2010) A new species of *Brachycephalus* from the Atlantic rain forest in São Paulo State, Southeastern Brazil (Amphibia: Anura: Brachycephalidae). *Copeia*, 2010, 410–420.
https://doi.org/10.1643/CH-09-102

Hedges, S.B. (1994) Molecular evidence for the origin of birds. *Proceedings of the Natural Academy of Science*, 91, 2621–2624.
https://doi.org/10.1073/pnas.91.7.2621

Heled, J. & Bouckaert, R.R. (2013) Looking for trees in the forest: summary tree from posterior samples. *BMC Evol Biol*, 13, 221.
https://doi.org/10.1186/1471-2148-13-221

Iuliis, G. & Pulerà, D. (2006) *The Dissection of Vertebrates. 2nd Edition*. Academic Press, San Diego, California,812 pp.

Izecksohn, E. (1971) Nôvo gênero e nova espécie de Brachycephalidae do Estado do Rio de Janeiro, Brasil (Amphibia, Anura). *Boletim do Museu Nacional*, 280, 1–12.

Katoh, K. & Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, 30 (4), 772–780.
https://doi.org/10.1093/molbev/mst010

Kohlsdorf, T. & Wagner, G.P. (2006) Evidence for the reversibility of digit loss: a phylogenetic study of limb evolution in *Bachia* (Gymnophthalmidae: Squamata). *Evolution*, 60 (9), 1896–1912.
https://doi.org/10.1111/j.0014-3820.2006.tb00533.x

Lefort, V., Longueville, J.E. & Gascuel, O. (2017) SMS: Smart Model Selection in PhyML. *Molecular Biology and Evolution*, 34(9), 2422–2424.
https://doi.org/10.1093/molbev/msx149

Levy, D.L. & Heald, R. (2016) Biological Scaling Problems and Solutions in Amphibians. In: Heald, R., Hariharan, I.K. & Wake, D.B. (Eds), *Additional Perspectives on Size Controlin Biology: From Organelles to Organisms*. Cold Spring Harbor Laboratory Press, Long Island, 8(1), pp. a019166.
https://doi.org/10.1101/cshperspect.a019166

Lindquist, E.E., Krantz, G.W. & Walter, D.E. (2009) Chapter 8. Classification. *In*: Krantz, G.W. & Walter, D.E. (Eds.), *A Manual of Acarology. 3rd Edition*. Texas Tech University Press, Lubbock, Texas, pp. 97–103.

Lyra, M.L., Haddad, C.F.B. & Azeredo-Espin, A.M.L. (2017) Meeting the challenge of DNA barcoding Neotropical amphibians: polymerase chain reaction optimization and new COI primers. *Molecular Ecology Resources*, 17 (5), 966–980.
https://doi.org/10.1111/1755-0998.12648

Lyra, M.L., Monteiro, J.P.C., Rancilhac, L., Irisarri, I., Kunzel, S., Sanchez, E., Condez, T.H., Rojas-Padilla, O., Solé, M., Toledo, L.F., Haddad, C.F.B. & Vences, M. (2021) Initial phylotranscriptomic confirmation of homoplastic evolution of the conspicuous coloration and bufoniform morphology of pumpkin-toadlets in the genus *Brachycephalus*. *Toxins*, 13 (11), 816.
https://doi.org/10.3390/toxins13110816

Mângia, S., Santana, D.S., de Oliveira Drummond, L., Sabagh, L.T., Ugioni, L., Nogueira Costa, P. & Wachlevski, M. (2023) A new species of *Brachycephalus* (Anura: Brachycephalidae) from Serra do Tabuleiro, Southern Brazil. *Vertebrate Zoology*, 73, 575–597.
https://doi.org/10.3897/vz.73.e102098

Monteiro, J.P.C., Condez, T.H., Garcia, P.C.A., Comitti, E.J., Amaral, I.B. & Haddad, C.F.B. (2018) A new species of *Brachycephalus* (Anura, Brachycephalidae) from the coast of Santa Catarina State, Southern Atlantic Forest, Brazil. *Zootaxa*, 4407 (4), 483–505.
https://doi.org/10.11646/zootaxa.4407.4.2

Moroti, M.T., Soares, P.T., Pedrozo, M., Provete, D.B. & Santana, D.J. (2021) The effects of morphology, phylogeny and prey availability on trophic resource partitioning in an anuran community. *Basic and Applied Ecology*, 50, 181–191.
https://doi.org/10.1016/j.baae.2020.11.005

Napoli, M.F., Caramaschi, U., Cruz, C.A.G. & Dias, I.R. (2011) A new species of flea-toad, genus *Brachycephalus* Fitzinger (Amphibia: Anura: Brachycephalidae), from the Atlantic rainforest of southern Bahia, Brazil. *Zootaxa*, 2739 (1), 33–40.
https://doi.org/10.11646/zootaxa.2739.1.3

Nunes, I., Guimaraes, C.S., Moura, P.H.A.G., Pedrozo, M., Moroti, M.T., Castro, L.M., Stuginski, D.R. & Muscat, E. (2021) Hidden by the name: a new fluorescent pumpkin toadlet from the *Brachycephalus ephippium* group (Anura: Brachycephalidae). *PLoS One*, 16, e0244812.
https://doi.org/10.1371/journal.pone.0244812

Oliveira, E.G. & Haddad, C.F.B. (2015) Diet Seasonality and Feeding Preferences of *Brachycephalus pitanga* (Anura: Brachycephalidae). *Journal of Herpetology*, 49, 252–256.
https://doi.org/10.1670/13-211

Oliveira E.G. & Haddad C.F.B. (2017) Activity, acoustic repertoire and social interactions of the Red Toadlet, *Brachycephalus pitanga* (Anura: Brachycephalidae). *Salamandra*, 53, 501–506

Padial, J.M., Aurélien, M., De La Riva, I. & Vences, M. (2010) Integrative future of taxonomy. *Frontiers in Zoology*, 7(1), 16.
https://doi.org/10.1186/1742-9994-7-16

Padial, J.M., Grant, T. & Frost, D.R. (2014) Molecular systematics of terraranas (Anura: Brachycephaloidea) with an assessment of the effects of alignment and optimality criteria. *Zootaxa*, 3825 (1), 1–132.
https://doi.org/10.11646/zootaxa.3825.1.1

Palumbi, S.R., Martins, A., Romano, S., Mcmillan, W.O., Stice, L. & Grabawski, G. (1991) *The Simple Fool'S Guide to PCR. Version 2.0*. University of Hawaii, Honolulu. [program]

Pie, M.R. & Ribeiro, L.F. (2015) A new species of *Brachycephalus* (Anura: Brachycephalidae) fromthe Quiriri mountain range of southern Brazil. *PeerJ*, 3, e1179.
https://doi.org/10.7717/peerj.1179

Pie, M.R., Ribeiro, L.F., Confetti, A.E., Nadaline, M.J. & Bornschein, M.R. (2018) A new species of *Brachycephalus* (Anura: Brachycephalidae) from southern Brazil. *PeerJ*, 6, e5683.
https://doi.org/10.7717/peerj.5683

Pombal, J.P., Jr. (2001) A new species of *Brachycephalus* (Anura: Brachycephalidae) from Atlantic Rain Forest of southeastern Brazil. *Amphibia-Reptilia*, 22 (2), 179–185.
https://doi.org/10.1163/15685380152030409

Pombal, J.P., Jr. (2010) A posição taxonômica das "variedades" de *Brachycephalus ephippium* (Spix, 1824) descritas por Miranda-Ribeiro, 1920 (Amphibia, Anura, Brachycephalidae). *Boletim do Museu Nacional, Nova Série, Zoologia*, 526, 1–12.

Pombal, J.P. & Gasparini, J.L. (2006) A new *Brachycephalus* (Anura: Brachycephalidae) From the Atlantic rainforest of Espírito Santo, southeastern Brazil. *South American Journal of Herpetology*, 1 (2), 87–93.
https://doi.org/10.2994/1808-9798(2006)1[87:ANBABF]2.0.CO;2

Pombal, J.P., Jr. & Izecksohn, E. (2011) Uma nova espécie de *Brachycephalus* (Anura, Brachycephalidae) do Estado do Rio de Janeiro. *Papéis Avulsos de Zoologia*, 51 (28), 443–451.
https://doi.org/10.1590/s0031-10492011002800001

Pombal, J.P., Jr., Wistuba, E.M. & Bornschein, M.R. (1998) A new species of brachycephalid (Anura) from the Atlantic Rain Forest of Brazil. *Journal of Herpetology*, 32 (1), 70–74.
https://doi.org/10.2307/1565481

Rambaut, A., Drummond, A.J., Xie, D., Baele, G. & Suchard, M.A. (2018) Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, 67 (5), 901–904.
https://doi.org/10.1093/sysbio/syy032

Rebouças, R., Carollo, A.B., Freitas, M.O., Lambertini, C., Santos, R.N.M. & Toledo, L.F. (2019) Is the conspicuous dorsal coloration of the Atlantic forest pumpkin toadlets aposematic? *Salamandra*, 55 (1), 39–47.

Ribeiro, L.F., Alves, A., Haddad, C.F.B. & Reis, S.F. (2005) Two new species of *Brachycephalus* Günther, 1985 from the state of Paraná, southern Brazil (Amphibia, Anura, Brachycephalidae). *Boletim do Museu Nacional*, 519, 1–18.

Ribeiro, L.F., Bornschein, M.R., Belmonte-Lopes, R., Firkowski, C.R., Morato, S.A.A. & Pie, M.R. (2015) Seven new microendemic species of *Brachycephalus* (Anura: Brachycephalidae) from southern Brazil. *PeerJ*, 3, e1011.
https://doi.org/10.7717/peerj.1011

Ribeiro, L.F., Blackburn, D.C., Stanley, E.L., Pie, M.R. & Bornschein, M.R. (2017) Two new species of the *Brachycephalus pernix* group (Anura: Brachycephalidae) from the state of Paraná, southern Brazil. *PeerJ*, 5, e3603. https://doi.org/10.7717/peerj.3603

Schulte, L.M., Ringler, E., Rojas, B. & Stynoski, J.L. (2020). Developments in amphibian parental care research: history,present advances, and future perspectives. *Herpetol. Monogr*. 34, 71–97. https://doi.org/10.1655/HERPMONOGRAPHS-D-19-00002.1

Sebben, A. (2007) Microdissecação fisiológica a fresco: uma nova visão sobre a anatomia de anfíbios e répteis. *In*: Nascimento, L.B. & Oliveira, M.E. (Eds.), *Herpetologia no Brasil II. 1ed*. Sociedade Brasileira de Herpetologia, Belo Horizonte, pp. 311–325.

Simon, M.P. & Toft, C.A. (1991) Diet specialization in small vertebrates: mite-eating in frogs. *Oikos*, 61(2), 263–278. https://doi.org/10.2307/3545344

Stecher, G., Tamura, K. & Kumar, S. (2020) Molecular Evolutionary Genetics Analysis (MEGA) for macOS. *Molecular Biology and Evolution*, 37(4), 1237–1239. https://doi.org/10.1093/molbev/msz312

Tamura, K., Stecher, G. & Kumar, S. (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7), 3022–3027. https://doi.org/10.1093/molbev/msab120

Tapley, B., Michaels, C.J., Gumbs, R., Böhm, M., Luedtke, J., Pearce-Kelly, P. & Rowley, J.J.L. (2019) The disparity between species description and conservation assessment: a case study in taxa with high rates of species discovery. *Biological Conservation*, 220, 209–214. https://doi.org/10.1016/j.biocon.2018.01.022.

Timothy, J.G. & Oseto, C. (2020) Chapter 4 - Classification of insects and mites. *In*: Timothy, J.G. & Oseto, C. (Eds.), *Insect Collection and Identification. 2nd Edition*. Academic Press, San Diego, California, pp. 129–145. https://doi.org/10.1016/B978-0-12-816570-6.00004-6

Thyssen, P.J. (2009) Keys for Identification of Immature Insects. *In*: Amendt, J., Goff, M., Campobasso, C. & Grassberger, M. (Eds.), *Current Concepts in Forensic Entomology*. Springer, Dordrecht, pp. 25–42. https://doi.org/10.1007/978-1-4020-9684-6_2

Toledo, L.F., Botelho, L.M., Carrasco-Medina, A.S., Gray, J.A., Ernetti, J.R., Gama, J.M., Lyra, M.L., Blackburn, D.C., Nunes, I. & Muscat, E. (2024) Among the world's smallest vertebrates: a new miniaturized flea-toad (Brachycephalidae) from the Atlantic rainforest. *PeerJ*, 12, e18265. https://doi.org/10.7717/peerj.18265

Verdade, V.K, Rodrigues, M.T., Cassimiro, J., Pavan, D., Liou, N. & Lange, M.C. (2008) Advertisement Call, Vocal Activity, and Geographic Distribution of *Brachycephalus hermogenesi* (Giaretta and Sawaya, 1998) (Anura, Brachycephalidae). *Journal of Herpetology*, 42(3), 542–549. https://doi.org/10.1670/07-287.1

Vieites, D.R., Wollenberg, K.C., Andreone, F., Köhler, J., Glaw, F. & Vences, M. (2009) Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences of the United States of America, PNAS*, 106 (20), 8267–8272. https://doi.org/10.1073/pnas.0810821106

Wistuba, E.M. (1998) *História Natural de Brachycephalus pernix Pombal Jr., Wistuba, Bornschein, 1998 (Anura) no Morro Anhangava, Município de Quatro Barras, Estado do Paraná*. Unpublished MSc Thesis UFPR, Curitiba. [unknown pagination]

APPENDIX I. Additional specimens examined.

Brachycephalus alipioi: Brazil, Espírito Santo, Castelo (MZUFV 5877–5882).

Brachycephalus brunneus: Brazil, Paraná, Campina Grande do Sul (MNRJ 40289–91, paratypes).

Brachycephalus bufonoides: Rio de Janeiro, Nova Friburgo (MZUSP 1459 lectotype).

Brachycephalus darkside: Brazil, Minas Gerais, Ervália (MZUFV 16636, holotype, MZUFV 15557–15561, 15565–15571, 15716–15721, 16491, 16579, 16627–16628,16631–16633, 16634–16635, 16780 paratypes, UFMG 19522 paratype, MNRJ 91327 paratype); Miradouro (MZUFV 2897, 6658–6660 paratypes).

Brachycephalus didactylus: Brazil, Rio de Janeiro, Paulo de Frontim (MNRJ 4067–73 paratypes).

Brachycephalus ephippium: Brazil, Rio de Janeiro, Itatiaia (MZUFV 4161, MNRJ 52427–40); Nova Friburgo (MNRJ 39342–43); Rio de Janeiro (MNRJ 30919–29, 40782–807); Teresópolis (MNRJ 51580–83).

Brachycephalus garbeanus: Brazil, Rio de Janeiro, Nova Friburgo (MZUSP 0811 lectotype, MNRJ 17440–41, 25390–400, 67498).

Brachycephalus hermogenesi: Brazil, São Paulo, Picinguaba (CFBH 29793–94).

Brachycephalus izecksohni: Brazil, Paraná, Quaratuba (MNRJ 76259–60 paratypes).

Brachycephalus margaritatus: Brazil, Rio de Janeiro, Petrópolis (MNRJ 85300–396).

Brachycephalus nodoterga: Brazil, São Paulo, São Paulo (MZUSP 0975 holotype).

Brachycephalus pernix: Brazil, Paraná, Quatro Barras (MNRJ 17343 holotype, MNRJ 17328–42, 17427–28, paratypes).

Brachycephalus pitanga: Brazil, São Paulo, São Luís do Paraitinga (MNRJ 60790–93 paratypes).

Brachycephalus rotenbergae: Brazil, São Paulo, São José dos Campos (HCLP-A 260 holotype, HCLP-A 78–83, 284–287 paratypes, CCLZU 1274, 1278–1279 paratypes, ZUFMS-AMP 13647–13649 paratypes, HCLP-A 281–283).

Brachycephalus sulfuratus: Brazil, Paraná, Guaraqueçaba (MNRJ 87912).

Brachycephalus toby: Brazil, São Paulo, Ubatuba (MNRJ 76382–83 paratypes).

Brachycephalus tridactylus: Brazil, Paraná, Guaraqueçaba (MNRJ 87908–910).

Brachycephalus vertebralis: Brazil, Rio de Janeiro, Paraty (MNRJ 11098 holotype, MNRJ 89199–201).

APPENDIX II. Primers and PCR conditions used to amplify the 16S rRNA fragment in the molecular studies of the genus *Brachycephalus*. Each PCR reaction was carried out in a final volume of 25ul and contained 2 uL of genomic DNA, 2X PCR Master Mix Taq Pol (Cellco), 0.5 uL (10 pmol) of each primer. The fragment amplified by primers 16SAForward+16SAReverse is homologous to the “Palumbi” fragment amplified with primers 16Sar-L and 16Sbr-H (Palumbi *et al*. 1991). Fragment “UP” is upstream to “Palumbi” fragment. Amplification conditions: UP [3 min at 95 °C; 36x (20 s at 95 °C, 20s at 50 °C; 1:20 min 68 °C); 3 min at 68 °C]; Palumbi [2 min at 94 °C; 45x (25 s at 94 °C, 1 min at 52°C; 1 min 72 °C); 5 min at 72 °C].

Fragment	Primer name	Sequence (5'→3')	Reference
UP	12S-L13	TTAGAAGAGGCAAGTCGTAACATGGTA	Feller & Hedges (1998)
	16S-H10	TGCTTACGCTACCTTTGCACGGT	Hedges (1994)
Palumbi	16SAFoward	GCAAAGGTAGCATAATCACT	Palumbi *et al*. (1991)
	16SAReverse	CTGTTATCCCTGGGGTAGCTTG	Palumbi *et al*. (1991)

Appendix III. Molecular data of the genus *Brachycephalus* used in the present study. Samples included in the phylogenetic inferences, 16S sequence accession number and sample metadata.* Holotype.

Specie	Specimen-Voucher	GenBank Acc. number	Body plan	Species group	Local
***Brachycephalus nanicus* sp. nov**	**ZUEC-AMP25581**	**OR250404**	**leptodactyliform**	***B. puri***	**Bananal, SP**
***Brachycephalus nanicus* sp. nov**	**ZUEC-AMP25578**	**OR250405**	**leptodactyliform**	***B. puri***	**Bananal, SP**
***Brachycephalus nanicus* sp. nov**	**ZUEC-AMP25577**	**OR399156**	**leptodactyliform**	***B. puri***	**Bananal, SP**
***Brachycephalus nanicus* sp. nov**	**ZUEC-AMP25580**	**OR399157**	**leptodactyliform**	***B. puri***	**Bananal, SP**
Brachycephalus puri	MZUSP139152	OK298954	leptodactyliform	*B. puri*	Ibitirama, ES
Brachycephalus puri	MZUSP139154	OK298955	leptodactyliform	*B. puri*	Ibitirama, ES
Brachycephalus sp8	CTMZ 01522/ MZUSP133731	MK697410	leptodactyliform	*B. puri*	Bananal, SP

......continued on the next page

Appendix III. (Continued)

Specie	Specimen-Voucher	GenBank Acc. number	Body plan	Species group	Local
Brachycephalus sp8		HQ435692	leptodactyliform	*B. puri*	Ilha Grande, Angra dos Reis, RJ
Brachycephalus pulex	PU65	MT929509	leptodactyliform		Camacan, BA
Brachycephalus pulex		MZ770742	leptodactyliform		Arataca, BA
Brachycephalus dacnis	ZUEC-AMP 24982	OR250414	leptodactyliform		Reserva Dacnis, Ubatuba, SP
Brachycephalus dacnis	ZUEC-AMP 25270	OR250415	leptodactyliform		Reserva Dacnis, Ubatuba, SP
Brachycephalus hermogenesi	TC129	MT929506	leptodactyliform		Salesópolis, SP
Brachycephalus hermogenesi	TC133	MT929507	leptodactyliform		Salesópolis, SP
Brachycephalus hermogenesi	MVTIS 3210	MZ770740	leptodactyliform		São Luiz do Paraitinga, SP
Brachycephalus hermogenesi	ZUEC-AMP 24983	OR250409	leptodactyliform		Reserva Dacnis, Ubatuba, SP
Brachycephalus hermogenesi	ZUEC-AMP 24980	OR250410	leptodactyliform		Reserva Dacnis, Ubatuba, SP
Brachycephalus didactylus	CFBH11506	MK697365/ JX267467	leptodactyliform		Terespolis, RJ
Brachycephalus didactylus	MNRJ37327	MT929504	leptodactyliform		Terespolis, RJ
Brachycephalus sulfuratus	CFBH39141	MK697392	leptodactyliform		São Francisco do Sul, SC
Brachycephalus sulfuratus		MZ770751	leptodactyliform		São Francisco do Sul, SC
Brachycephalus clarissae	MNRJ 56504	MK697394	intermediate		Nova Friburgo, RJ
Brachycephalus clarissae	MNRJ 56516	MK697395	intermediate		Nova Friburgo, RJ
Brachycephalus darkside	PPGT600	MT734440	bufoniform	*B. ephippium*	Ervalia, MG
Brachycephalus ephippium	MNRJ 56517	MK697366	bufoniform	*B. ephippium*	Cachoeiras de Macacu, RJ
Brachycephalus ephippium	CFBH 12355	MK697367	bufoniform	*B. ephippium*	Terespolis, RJ
Brachycephalus garbeanus	MNRJ51472	MT929466	bufoniform	*B. ephippium*	Macaé de Cima, RJ
Brachycephalus garbeanus	CFBH16800	MT929467	bufoniform	*B. ephippium*	Macaé de Cima, RJ
Brachycephalus ibitinga	CFBH 19348	MK697399	bufoniform	*B. ephippium*	Itanhaem, SP

......continued on the next page

Appendix III. (Continued)

Specie	Specimen-Voucher	GenBank Acc. number	Body plan	Species group	Local
Brachycephalus ibitinga	CFBH 2978	MK697401	bufoniform	*B. ephippium*	São Paulo, SP
Brachycephalus margaritatus	MNRJ73702	MT929478	bufoniform	*B. ephippium*	Engenheiro Paulo de Frontin, RJ
Brachycephalus margaritatus	MNRJ76100	MT929479	bufoniform	*B. ephippium*	Engenheiro Paulo de Frontin, RJ
Brachycephalus rotenbergae	CFBH16828	MT929457	bufoniform	*B. ephippium*	São José dos Campos, SP
Brachycephalus rotenbergae	MVTIS 3213	MZ770745	bufoniform	*B. ephippium*	Mogi das Cruzes, SP
Brachycephalus sp6	CFBH 44422	MK697406	bufoniform	*B. ephippium*	Bananal, SP
Brachycephalus sp6	CFBH 44424	MK697407	bufoniform	*B. ephippium*	Cunha, SP
Brachycephalus alipioi		HQ435690	bufoniform	*B. vertebralis*	Vargem Alta, ES
Brachycephalus alipioi	AL1	MT929481	bufoniform	*B. vertebralis*	Vargem Alta, ES
Brachycephalus bufunoides	ZUFRJ 15428	MH259788	bufoniform	*B. vertebralis*	Nova Friburgo, RJ
Brachycephalus bufunoides	ZUFRJ 15426	MH259791	bufoniform	*B. vertebralis*	Nova Friburgo, RJ
Brachycephalus crispus	TC34	MT929483	bufoniform	*B. vertebralis*	Cunha, SP
Brachycephalus crispus	TC36	MT929484	bufoniform	*B. vertebralis*	Cunha, SP
Brachycephalus guarani	PRU1	MT929485	bufoniform	*B. vertebralis*	Ubatuba, SP
Brachycephalus guarani	PRU3	MT929487	bufoniform	*B. vertebralis*	Ubatuba, SP
Brachycephalus herculeus	CFBH13729	OQ629303	bufoniform	*B. ephippium*	Santa Maria Madalena, RJ
Brachycephalus herculeus	CFBH13730	OQ629304	bufoniform	*B. ephippium*	Santa Maria Madalena, RJ
Brachycephalus nodoterga	BO130	KJ649772	bufoniform	*B. vertebralis*	Salespolis, SP
Brachycephalus nodoterga	CA2226	KJ649779	bufoniform	*B. vertebralis*	Serra Cantareira, São Paulo, SP
Brachycephalus nodoterga	IB634	KJ649781	bufoniform	*B. vertebralis*	São Sebastiao, SP
Brachycephalus pitanga		HQ435699	bufoniform	*B. vertebralis*	São Luiz do Paraitinga, SP
Brachycephalus pitanga	MVTIS 3205	MZ770741	bufoniform	*B. vertebralis*	São Luiz do Paraitinga, SP

......continued on the next page

Appendix III. (Continued)

Specie	**Specimen-Voucher**	**GenBank Acc. number**	**Body plan**	**Species group**	**Local**
Brachycephalus sp3	CFBH 44409	MK697397	bufoniform	*B. vertebralis*	Ubatuba, SP
Brachycephalus toby		HQ435701	bufoniform	*B. vertebralis*	Ubatuba, SP
Brachycephalus toby	TO1	MT929499	bufoniform	*B. vertebralis*	Ubatuba, SP
Brachycephalus vertebralis		HQ435702	bufoniform	*B. vertebralis*	Ubatuba, SP
Brachycephalus vertebralis	CFBH1388	MT929502	bufoniform	*B. vertebralis*	Ubatuba, SP
Brachycephalus actaeus		MZ770736	bufoniform	*B. pernix*	São Francisco do Sul, SC
Brachycephalus actaeus		MZ770737	bufoniform	*B. pernix*	São Francisco do Sul, SC
Brachycephalus albolineatus		MZ770738	bufoniform	*B. pernix*	Jaraguá do Sul, SC
Brachycephalus auroguttatus	Bauroguttatus2	KX025355	bufoniform	*B. pernix*	Garuva, SC
Brachycephalus auroguttatus		MZ770739	bufoniform	*B. pernix*	Garuva, SC
Brachycephalus boticario	Bboticario1	KX025370	bufoniform	*B. pernix*	Blumenau, SC
Brachycephalus boticario	Bboticario2	KX025371	bufoniform	*B. pernix*	Blumenau, SC
Brachycephalus brunneus		HQ435691	bufoniform	*B. pernix*	Campina Grande do Sul PR
Brachycephalus brunneus	CAPR1	MT929511	bufoniform	*B. pernix*	Campina Grande do Sul PR
Brachycephalus coloratus	Ind1	MT928335	bufoniform	*B. pernix*	Piraquara, PR
Brachycephalus coloratus	Ind3	MT928336	bufoniform	*B. pernix*	Piraquara, PR
Brachycephalus curupira	BSalto4	KX025381	bufoniform	*B. pernix*	São José dos Pinhais, PR
Brachycephalus curupira	BSalto5	KX025382	bufoniform	*B. pernix*	São José dos Pinhais, PR
Brachycephalus ferruginus		HQ435695	bufoniform	*B. pernix*	Morretes, PR
Brachycephalus ferruginus		MT929515	bufoniform	*B. pernix*	Morretes, PR
Brachycephalus fuscolineatus	Bfuscolineatus1	KX025296	bufoniform	*B. pernix*	Ilhota, SC
Brachycephalus fuscolineatus	Bfuscolineatus2	KX025297	bufoniform	*B. pernix*	Ilhota, SC

......continued on the next page

Appendix III. (Continued)

Specie	**Specimen-Voucher**	**GenBank Acc. number**	**Body plan**	**Species group**	**Local**
Brachycephalus izecksohni		HQ435696	bufoniform	*B. pernix*	Morretes, PR
Brachycephalus izecksohni	TPPR1	MT929518	bufoniform	*B. pernix*	Morretes, PR
Brachycephalus leopardus	Bleopardus2	KX025240	bufoniform	*B. pernix*	Tijucas do Sul, PR
Brachycephalus leopardus	Bleopardus3	KX025241	bufoniform	*B. pernix*	Tijucas do Sul, PR
Brachycephalus mariaeterezae	Bmariaterezae2	KX025344	bufoniform	*B. pernix*	Joinville, SC
Brachycephalus mariaeterezae	Bmariaterezae3	KX025345	bufoniform	*B. pernix*	Joinville, SC
Brachycephalus mirissimus		MH136570	bufoniform	*B. pernix*	Massaranduba, SC
Brachycephalus olivaceus	Bolivaceus1	KX025300	bufoniform	*B. pernix*	Joinville, SC
Brachycephalus olivaceus	TC236	MG889435	bufoniform	*B. pernix*	Joinville, SC
Brachycephalus pernix		HQ435698	bufoniform	*B. pernix*	Quatro Barras, PR
Brachycephalus pernix	AGPR2	MT929522	bufoniform	*B. pernix*	Quatro Barras, PR
Brachycephalus pombali		HQ435700	bufoniform	*B. pernix*	Guaratuba, PR
Brachycephalus pombali	SIPR1	MT929525	bufoniform	*B. pernix*	Guaratuba, PR
Brachycephalus quiririensis	Bquiririensis2	KX025318	bufoniform	*B. pernix*	Campo Alegre, SC
Brachycephalus quiririensis		MZ770743	bufoniform	*B. pernix*	CampoAlegre SC
Brachycephalus sp9	CFBH 42815	MK697411	bufoniform	*B. pernix*	Corupá, SC
Brachycephalus tabuleiro	MNRJ93858	OR116969	bufoniform	*B. pernix*	São Bonifácio, SC
Brachycephalus tabuleiro	UNIFESSPA112	OR116970	bufoniform	*B. pernix*	São Bonifácio, SC
Brachycephalus tridactylus	Btridactylus2	KX025388	bufoniform	*B. pernix*	Guaraqueçaba, PR
Brachycephalus tridactylus	TRID-5	MG889432	bufoniform	*B. pernix*	Guaraqueçaba, PR
Brachycephalus verrucosus	Bverrucosus9	KX025234	bufoniform	*B. pernix*	Joinville, SC
Brachycephalus verrucosus	Bverrucosus10	KX025235	bufoniform	*B. pernix*	Joinville, SC
Ischnocnema henseli		MZ770752			Jaraguá do Sul, SC

Appendix IV. Uncorrected p-distances (%) between individuals of leptodactyliform and intermediate body plan in the genus *Brachycephalus*. Lower diagonal: Mitochondrially encoded 16S rRNA mitochondrial - Palumbi fragment (518 bp). Upper diagonal: 16S rRNA mitochondrial - UP fragment (727 bp). See Mains text for additional information on sequences. NA: fragment is not available for the individual analyzed. Cells highlighted in gray indicate intraspecific variation.

	Individuals	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
1	*B. nanicus* OR250404*		0.0	0.0	0.0	NA	NA	16.0	14.7	26.4	26.3	NA	NA	NA	NA	25.7	25.6	25.6	23.1	22.5	26.3	24.4	24.7	24.7
2	*B. nanicus* OR250405	0.0		0.0	0.0	NA	NA	16.0	14.7	26.4	26.3	NA	NA	NA	NA	25.7	25.6	25.6	23.1	22.5	26.3	24.4	24.7	24.7
3	*B. nanicus* OR399156	0.0	0.0		0.0	NA	NA	16.0	14.7	26.4	26.3	NA	NA	NA	NA	25.7	25.6	25.6	23.1	22.5	26.3	24.4	24.7	24.7
4	*B. nanicus* OR399157	0.0	0.0	0.0		NA	NA	16.0	14.7	26.4	26.3	NA	NA	NA	NA	25.7	25.6	25.6	23.1	22.5	26.3	24.4	24.7	24.7
5	*B. puri* OK298955	16.0	16.0	16.0	16.0		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	*B. puri* OK298954	15.8	15.8	15.8	15.8	0.2		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	*B.* sp 8 HQ435692	12.7	12.7	12.7	12.7	16.4	16.2		9.2	26.8	26.7	NA	NA	NA	NA	25.6	24.4	24.4	25.5	24.9	27.1	25.6	24.4	24.4
8	*B.* sp 8 MK697410	NA	NA	NA	NA	NA	NA	NA		26.3	26.2	NA	NA	NA	NA	24.4	24.1	24.1	23.3	22.7	26.0	24.5	23.6	23.6
9	*B. didactylus* MK697365/ JX267467	15.5	15.5	15.5	15.5	16.7	16.5	NA	18.1		0.2	NA	NA	NA	NA	19.7	19.2	19.2	21.9	21.1	24.4	22.5	15.2	15.2
10	*B. didactylus* MT929504	15.5	15.5	15.5	15.5	16.7	16.5	NA	18.1	0.0		0.0	NA	NA	NA	19.6	19.1	19.1	21.6	20.9	24.3	22.4	14.9	14.9
11	*B. dacnis* OR250415	16.4	16.4	16.4	16.4	18.5	18.3	NA	18.2	17.3	17.3		0.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
12	*B. dacnis* OR250414	16.8	16.8	16.8	16.8	18.7	18.4	NA	18.7	17.8	17.8	0.0		0.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	*B. hermogenesi* OR250409	17.9	17.9	17.9	17.9	17.1	16.9	NA	19.7	16.9	16.9	7.0	7.0		0.0	NA	NA	NA	NA	NA	NA	NA	NA	NA

......continued on the next page

Appendix IV. (Continued)

	Individuals	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
14	*B. hermogenesi* OR250410	17.9	17.9	17.9	17.9	17.1	16.9	NA	19.7	16.9	16.9	7.0	7.0	0.0		0.0	NA	NA	NA	NA	NA	NA	NA	NA
15	*B. hermogenesi* MZ770740	16.6	16.6	16.6	16.6	16.3	16.1	NA	18.1	16.0	16.0	7.5	7.8	2.5	2.5		5.6	5.6	18.8	18.3	22.4	21.1	21.0	21.0
16	*B. hermogenesi* MT929506	17.0	17.0	17.0	17.0	17.6	17.4	NA	18.1	16.4	16.4	6.5	6.8	2.5	2.5	2.4		0.0	19.2	18.6	21.6	20.6	20.3	20.3
17	*B. hermogenesi* MT929507	17.0	17.0	17.0	17.0	17.6	17.4	NA	18.1	16.4	16.4	6.5	6.8	2.5	2.5	2.4	0.0		19.2	18.6	21.6	20.6	20.3	20.3
18	*B. sulfuratus* MZ770751	17.7	17.7	17.7	17.7	19.2	19.0	NA	19.5	16.4	16.4	11.4	11.8	12.0	12.0	11.6	12.0	12.0		0.6	22.3	22.1	20.9	20.9
19	*B. sulfuratus* MK697392	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		21.7	21.5	20.3	20.3
20	*B. pulex* MZ770742	20.4	20.4	20.4	20.4	23.1	22.9	NA	22.2	19.9	19.9	16.0	16.6	17.9	17.9	17.2	16.6	16.6	NA	18.8		10.3	22.0	22.0
21	*B. pulex* MT929509	21.7	21.7	21.7	21.7	24.6	24.4	NA	22.8	21.3	21.3	15.5	16.2	18.7	18.7	17.2	16.6	16.6	NA	19.4	6.9		21.7	21.7
22	*B. clarissae* MK697395	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		0.0
23	*B. clarissae* MK697394	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	