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The marsupial frog *Gastrotheca microdiscus* (Anura: Hemiphractidae) in South Brazil: distribution, natural history, advertisement call and molecular genetics

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Two species groups of marsupial frogs, genus *Gastrotheca* (Anura: Hemiphractidae), occur in the Atlantic Forest of Brazil, the endemic *G. fissipes* group and the *G. microdiscus* group (sensu Castroviejo-Fisher et al. 2015). They correspond to the subgenera *Eotheca* and *Australotheca*, respectively (Duellman 2015). The *G. fissipes* group is represented by six species: *G. fissipes* (Boulenger, 1888); *G. flamma* Juncá & Nunes, 2008; *G. megacephala* Izecksohn, Carvalho-e-Silva & Peixoto, 2009; *G. prasina* Teixeira, Vechio, Recoder, Carnaval, Strangas, Damasceno, Sena & Rodrigues, 2012; *G. pulchra* Caramaschi & Rodrigues, 2007; *G. recava* Teixeira, Vechio, Recoder, Carnaval, Strangas, Damasceno, Sena & Rodrigues, 2012. The *G. microdiscus* group is represented by four species: *G. albolineata* (Lutz & Lutz, 1939); *G. ernestoi* Miranda-Ribeiro, 1920; *G. fulvorufa* (Andersson, 1911); *G. microdiscus* (Andersson, 1910). Recent molecular studies have recovered each one of these species groups as monophyletic, though for *G. fissipes* group only potential morphological synapomorphies were mentioned (Castroviejo-Fisher et al. 2015, Duellman 2015).

The original description of *G. microdiscus* was based on two adult specimens collected by P. Dusén in 1908 (Andersson 1910). The syntypes, female and male, are held by the Naturhistoriska Riksmuseet, Stockholm, with the collection number NHRM 1480 (Frost 2018). According to the original publication, the type-material was collected at Forest Desiro Ribas, Brazil, which is currently associated with the Desvio Ribas train station in the municipality of Ponta Grossa, PR (Straube 2015). The species is known to occur from northeastern São Paulo State to northeastern Santa Catarina State, Brazil (Frost 2018). The southernmost locality of *G. microdiscus* is based on two historical records from Santa Catarina State. This species has not been observed or collected in Santa Catarina State since 1978 (Duellman 1984), despite recent intensive herpetological field surveys conducted by the National Action Plan for the conservation of endangered amphibians and reptiles of southern Brazil (Ministério do Meio Ambiente 2019).

The first record of *G. microdiscus* from Santa Catarina State is represented by a single specimen collected by E. Nahderer in 1946 at Serra Alta, municipality of São Bento do Sul (MNRJ 19322, designated *a posteriori* by B. Lutz). Duellman (1984) mentioned a second record, from an unknown collector, based on a specimen collected in 1978, i.e. more than three decades later and suggesting that *G. microdiscus* is rare. The locality for this specimen was reported as 16–17 km west of Pirabeiraba, along the federal route BR 280 to Campo Alegre (which actually refers to the interstate route SC 301; Smithsonian US-ANIMALIA 235735). Another 40 years later, we report new records of *G. microdiscus* and provide new information on the species' natural history and formal description of its advertisement call.

Fieldwork was conducted on 29 August 2017 at Castelo dos Bugres (26°13'59" S, 49°03'14" W; 770 m above sea level; Datum WGS 84), and on 30–31 August 2017 at Morro da Tromba (26°12'37" S, 48°57'29" W; 910 m a.s.l.; Datum WGS 84), both in the municipality of Joinville, SC. A single specimen, collected at Morro da Tromba, was euthanized with 5% lidocaine; muscle tissue was extracted and stored in 99.5% ethanol, while the specimen was fixed in 10% formalin and preserved in 70% ethanol. The muscle tissue and specimen were deposited in the Célio F. B. Haddad amphibian collection (CFBHT 22444, CFBH 42069), Depar-

tamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, SP, Brazil.

We compared the external morphology of the collected specimen with other specimens of *Gastrotheca* housed at CFBH and the following Brazilian collections: the Adolpho Lutz collection (AL-MN) at Museu Nacional Rio de Janeiro, RJ; the general collection of the Museu Nacional Rio de Janeiro (MNRJ), Rio de Janeiro, RJ; and the collection of Museu de Zoologia da Universidade de São Paulo, São Paulo, SP (MZUSP) (Appendix 1).

For taxonomic identification, we extracted total genomic DNA from the muscle sample following a standard salt extraction protocol adapted from Maniatis et al. (1982). For species comparisons we chose a 514 bp fragment of the mitochondrial 16S rRNA gene, which is currently available for several species of the family Hemiphractidae in the GenBank database and has been widely used as a barcoding marker for amphibians (Vences et al. 2005). We carried out DNA amplification in a 21 µl volume reaction using master mix Fermentas Taq Polymerase and reagents (MBI Fermentas), and the primers 16Sar-L (CGCCTGTTTATCAAAAACAT) and 16Sbr-H (CCGGTCTGAACTCAGATCACGT) (Palumbi et al. 1991). PCR reactions were amplified with initial hold at 95°C (3 min), followed by 35 cycles of denaturation at 95°C (15 s), annealing at 50°C (15 s), extension at 60°C (50 s), and then by a final hold at 60°C (3 min). PCR products were purified using Exonuclease and Shrimp Alkaline Phosphatase (Affimetrix/USB), and sequenced in both directions by Macrogen Inc. (Seoul, South Korea). Consensus sequences were assembled with CodonCode Aligner v3.

Molecular comparisons were made with eight species of the genus *Gastrotheca* (Appendix 2), which encompassed all available sequences for the genus from the Atlantic Forest deposited in GenBank (*G. albolineata*, *G. ernestoi*, *G. fulvorufa*, *G. megacephala*, *G. microdiscus*, *G. prasina*, *G. pulchra*, *G. recava*). We aligned sequences using Muscle with default parameters (Edgar 2004) and estimated uncorrected *p* distances among all samples using pairwise deletion in Mega v7.0 (Kumar et al. 2016).

We recorded advertisement calls of male individuals with a Marantz PMD-661 digital recorder coupled to an external unidirectional Sennheiser ME-66 microphone. One calling male was recorded on 29 August 2017 at Castelo dos Bugres (JPCM 007 unvouchered recording; 14:15 h, air temperature 21 °C, air relative humidity 80%). A second calling male was recorded on 30 August 2017 at Morro da Tromba (recording JPCM 008 unvouchered recording; 18:00 h, air temperature 20 °C, air relative humidity 90%), in the location where the single voucher specimen of *G microdiscus* was collected. We confirmed that the advertisement call belonged to *G. microdiscus* by observing the collected specimen vocalizing in a plastic bag. The advertisement calls were recorded at approximately 3 m from the calling individuals. We analyzed 10 calls of a single individual (JPCM 008) using a sampling frequency rate of 44.1 kHz and 16-bit resolution in the mono pattern. Spectral and temporal parameters were measured using Raven Pro v1.5 (http://www.birds.cornell.edu/raven). Spectrograms were produced with the following settings: Hann window type, window size of 512 samples, overlap 90% (locked), hop size 51 samples, DFT 1024 samples (locked), grid spacing 46.9 Hz, brightness and contrast at default, and color map Cool. We used a note-centered approach and obtained measurements for nine advertisement call parameters following the definitions provided by Köhler et al. (2017): (1) call duration (s); (2) number of notes per call (notes/call); (3) note duration (s); (4) inter-note interval (s); (5) number of pulses per note (pulses/note); (6) dominant frequency (Hz; defined as peak frequency in Raven Pro 1.5); (7) lower and (8) upper frequencies (Hz; defined as frequency 5% and frequency 95% in Raven Pro 1.5, respectively); and (9) rise time (%; defined as peak time relative in Raven Pro 1.5). Sound figures were prepared in R using the package seewave 2.0.5, with the following settings: Hann window, 75% overlap, 512 point resolution, color scale defined by 36 relative dB (Sueur et al. 2008).

During our survey at Castelo dos Bugres and Morro da Tromba, more than 15 calling males of *G. microdiscus* were heard at the same time in the forest canopy. Both areas are well-preserved remnants of dense sub-montane and montane ombrophilous Atlantic Forest represented by secondary forests in medium to advanced stages of regeneration. Mature trees covered by lianas and bromeliads characterize the forest canopy. Males were active during the hottest time of the day as well as during the night. We recorded the advertisement calls of *G. microdiscus* at both localities but were unable to find specimens due to difficulties with accessing calling sites, which were generally two or three meters above the ground. The single specimen collected at Morro da Tromba (CFBH 42069, Fig. 1) was found following its vocalization during the end of the day on a large bromeliad that was two meters above the ground, adhered to a tree branch. The weather was drizzly during the afternoon, and males of *G. microdiscus* stopped calling activity at around 02:00–05:00 h. Our observations were made near the end of the winter season (that is unusually rainy in this region), suggesting that this might be the breeding season for this species. The lowest elevation recorded for *G. microdiscus* (based on heard vocalizations) was around 530 m a.s.l. (26°12'24" S, 48°57'20" W), in the same area.

The adult male collected at Morro da Tromba was promptly identified as *G. microdiscus* based on external morphology. The specimen has 36.4 mm and possessed the following characteristics proposed as diagnostic characters by Caramaschi & Rodrigues (2007) and Duellman (2015): tympanum circular, about 71% of eye diameter; snout rounded in dorsal view, obtuse in profile; upper eyelid lacking fleshy appendage or tubercle; interorbital distance is slightly less than twice the width of the upper eyelid; skin on skull adhered to the frontoparietals; skin on dorsum with small scattered tubercles; first finger is longer than the second; fingers II–III and III–IV are webbed basally; crescent order of length of fingers is II<I≅IV<III; tibia length is more than 50% of the snout–vent length, and is notably longer than the foot; webbing formula I1 – 2^{-}II1^{-}

– 2III1- – 2+IV2- – 1-V; heel lacks a calcar or tubercles. The coloration in dorsum is green, with brown spots or blotches bordered by black lines; in the head the markings consist of a brown canthal stripe bordered above by a pale yellow stripe, a pale labial stripe, the upper eyelid and tympanum are brown bordered by a black line; the flanks are tan, with diagonal brown bars bordered by black lines; in venter is pale.

The taxonomic identity was corroborated by the genetic distances within the Atlantic Forest species of *Gastrotheca* based on a single fragment of the 16S gene. Our sample differed from two of *G. microdiscus* from Mananciais da Serra, Piraquara, PR, by only 0.2%, yet from the *G. microdiscus* sample from Pilar do Sul, SP, by 0.9%. The previously available samples of *G. microdiscus* (Mananciais da Serra, Piraquara, PR, and Pilar do Sul, SP) differed by 1.1%. Surprisingly, when compared to different samples of *G. microdiscus* from Pilar do Sul, Mananciais da Serra and Morro da Tromba (SC, our sample), and the sample of *G. fulvorufa* from Paranapiacaba, SP, differed by 0.7%, 1.2%, and 1.4%, respectively. Within the same species group, *G. ernestoi* and *G. albolineata* differed from *G. microdiscus* by 5.8–6.8% and 13.7–14.9%, respectively. Our data also revealed differences of 13.7–17.5% between *G. microdiscus* and species of the *G. fissipes* group (*G. megacephala*, *G. prasina*, *G. pulchra*, and *G. recava*).

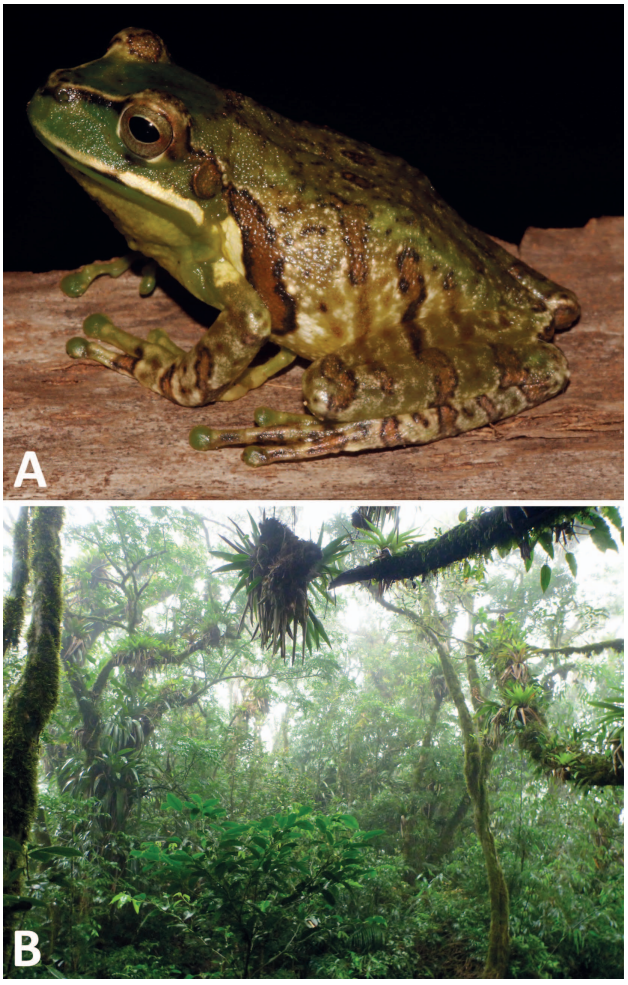

Figure 1. (A) Adult male *Gastrotheca microdiscus* collected at Morro da Tromba, municipality of Joinville, SC (CFBH 42069). (B) General view of the forest canopy and calling sites where the specimen was collected.

In order to compare calls, and to provide a detailed description of the advertisement call of *G. microdiscus*, we used our recordings from Morro da Tromba and considered the ranges (minimum and maximum values) of the parameters of call notes 1, 2, and 3, as published in Antunes & Haddad (2009). The recordings from Castelo dos Bugres match the recordings from Morro da Tromba reported herein, and both match the recordings from Pilar do Sul (Antunes & Haddad 2009). However, the recording from Castelo dos Bugres was not included in further call analysis due to the lower quality; the recording from Pilar do Sul was not deposited in any collection and is apparently lost. In our study, we refer to the advertisement call following the definition proposed by Köhler et al. (2017). The sound was regularly heard in the reproductive sites for long periods and is similar to the advertisement call available for other congeners (Izecksohn & Carvalho-e-Silva 2008). We did not observe aggressive calls and defensive calls, as reported in some *Gastrotheca* species (Sinsch & Joermann 1989, Lourenço-de-Moraes et al. 2016).

The advertisement call of *G. microdiscus* from Morro da Tromba comprised a series of 2–3 notes (mean 3 ± 0.5; n = 10) and lasted 0.4–0.8 s (0.7 ± 0.2; n = 10; Fig. 2). Ten advertisement calls were emitted over the course of 16 min at intervals of 28.2–226.3 s (79.4 ± 69.9; n = 9). We classified three note types. Note A lasted 0.2 s (0.2 ± 0; n = 10) and had a range of lower frequency of 1265.6–1406.2 Hz (1350.0 ± 43.1; n = 10), a range of upper frequency of 1593.8–1640.6 Hz (1631.2 ± 19.7; n = 10), and a dominant frequency range of 1453.1–1546.9 Hz (1500.0 ± 31.3; n = 10). Note B lasted 0.1–0.2 s (0.1 ± 0; n = 10) and had a range of lower frequency of 1406.2–1500.0 Hz (1448.4 ± 26.6; n = 10), a range of upper frequency of 1640.6–1687.5 Hz (1645.3 ± 14.8; n = 10), and a dominant frequency range of 1500.0–1593.8 Hz (1551.6 ± 26.6; n = 10). Note C was not always emitted (n = 3; Fig. 2A), but when it was, it had a variable pattern that was similar to either Note A (n = 2; Fig. 2B) or Note B (n = 5; Fig. 2C). In general, Note C lasted 0.1–0.2 s (0.1 ± 0; n = 7), had a range lower frequency of 1031.2–1453.1 Hz (1325.9 ± 161.7; n = 7), a range upper frequency of 1500.0–1640.6 Hz (1607.1 ± 52.1; n = 7), and a dominant frequency range of 1125.0–1546.9 Hz (1466.5 ± 152.4; n = 7). The A–B inter-note interval was 0.1 s (0.1 ± 0; n = 10) while the B–C inter-note interval was 0.2–0.3 s (0.2 ± 0; n = 7). The amplitude modulation throughout Note A and Note C (when it was similar to Note A) was highly variable, making it impossible to reliably determine the number of pulses, and the number of pulses/note was disregarded in these cases. In contrast, the number of pulses/note for Note B ranged 3–12 (7 ± 4, n = 8). The highest energy concentra-

tion throughout the entire call was also variable, with rise time ranging 13–89% of call length (51 ± 30; n = 10).

The low number of marsupial frog specimens in museum collections (cf. Izecksohn & Carvalho-e-Silva 2008, Teixeira et al. 2012) and the limited associated natural history information reflects the difficulty involved in finding individuals in the field. The habits of species of *Gastrotheca* are poorly known, mainly because their activities take place above 2 or 3 m from the ground in the forest canopy (Izecksohn & Carvalho-e-Silva 2008, Teixeira et al. 2012). This lack of information clearly constrains taxonomic knowledge of the genus, although some recent collection efforts have proven productive, such as the recent description of new taxa (Teixeira et al. 2012, Duellman 2015).

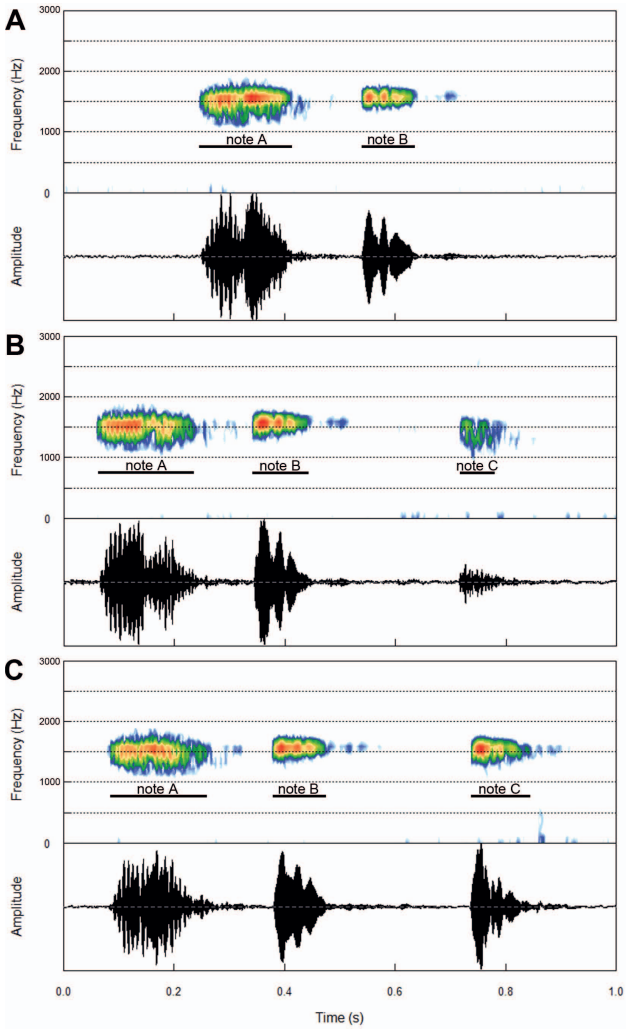

Figure 2. Advertisement call of *Gastrotheca microdiscus* (unvouchered recording JPCM 008), recorded at Morro da Tromba, municipality of Joinville, SC (30 August 2017, 18:00 h, air temperature 20 °C, air relative humidity 90%): Spectrogram and oscillogram of (A) a single call with two notes; (B) a single call with three notes where Note C is similar to Note A; (C) a single call with three notes where Note C is similar to Note B. Time scale bar = 1 s.

Co-ossification of the skin of the head has been discussed in literature and was used as a diagnostic character to separate *G. microdiscus* (absent) from *G. fulvorufa* and *G. ernestoi* (present; Caramaschi & Rodrigues 2007). However, the specimens of *G. microdiscus* in the present study, as well as the specimens examined by Izecksohn et al. (2009), have the skin adhered to the region of the frontoparietals. We also observed that this character is variable among the eight examined adult specimens of *G. microdiscus*, however the majority has skin adhered to frontoparietals (n = 6).

Large genetic divergences were reported within the *G. fissipes* group, except for the sister species *G. megacephala* and *G. prasina* (Teixeira et al. 2012). Our analysis of a small fragment of the 16S mt rRNA gene corroborated these results and add information for *G. microdiscus* group. We found genetic divergence for the sister species *G. microdiscus* and *G. fulvorufa* in the same magnitude of the divergences within *G. microdiscus* populations. The genetic divergence of populations identified as *G. microdiscus* occurs over its latitudinal distribution, with higher genetic divergence between pairs of populations that are separated by greater geographic distances. Delimitation of species within *Gastrotheca*, and assessment of their phylogenetic relationships, should be further investigated.

Advertisement calls of species of the *G. microdiscus* group have been described by Izecksohn & Carvalho-e-Silva (2008) and informally by Antunes & Haddad (2009). The number of notes, dominant frequency, and note duration parameters for our recordings completely overlap with the original description of the advertisement call of *G. microdiscus* (Antunes & Haddad 2009). The advertisement call of *G. microdiscus* exhibits fewer notes and a greater call duration than the call of *G. albolineata* (5–14 notes and a call duration of 200–400 ms; Izecksohn & Carvalho-e-Silva 2008). The advertisement calls of *G. ernestoi*, *G. fulvorufa*, and *G. microdiscus* are similar in duration and number of notes per call, however, the advertisement call of *G. microdiscus* has a higher dominant frequency and lower duration of Note A than *G. ernestoi* (1119 Hz and 110 ms for the first note, as described in Izecksohn & Carvalho-e-Silva 2008). *Gastrotheca fulvorufa* has a higher dominant frequency for notes A and B (1785 Hz for the first and second notes, as described in Izecksohn & Carvalho-e-Silva 2008) than *G. microdiscus*.

It is possible that the occurrence of *G. microdiscus* in the Santa Catarina State has been underestimated, as well as in other localities throughout its distribution. The species was observed during a season of low amphibian activity (Wells 2007), when herpetological field surveys are rare. In this region, winter season is commonly rainy. The geographic distance between municipality of Joinville and the type locality in the municipality of Ponta Grossa is approximately 150 km. The new records and information we provide here will contribute to a better understanding of the geographical distribution, natural history, and biology of *G. microdiscus*, and thus will be valuable for its conservation.

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Appendix 1

Brazilian specimens of *Gastrotheca* examined for morphological comparisons in scientific collections. For abbreviations see main text.

Gastrotheca albolineata: **RJ**: municipality of Cachoeiras de Macacu, Reserva Ecológica Guapiaçu (MNRJ 59537, 65496); between municipalities of Cachoeiras de Macacu and Nova Friburgo (MNRJ 56528); municipality of Cachoeiras de Macacu, Parque Estadual dos Três Picos (MNRJ 54401); municipality of Paraty, Parque Nacional Serra da Bocaina (CFBH 35946); municipality of Saquarema (MNRJ 75915, 75917); municipality of Teresópolis (MNRJ 1778); municipality of Teresópolis, Parque Nacional da Serra dos Órgãos (MNRJ 3223). *G. ernestoi*: **RJ**: municipality of Itatiaia, Maromba, Parque Nacional do Itatiaia (MNRJ 19328); municipality of Macaé, Serra de Macaé (MZUSP 238 – holo-

type); municipality of Nova Friburgo, Baixo Caledônia (MNRJ 57129); municipality of Nova Friburgo, Estrada da Torre Repetidora (MNRJ 64000); municipality of Teresópolis, Parque Nacional da Serra dos Órgãos (MNRJ 19327, 19329–19331); municipality of Teresópolis (MNRJ 19325, 19326). **SP**: municipality of São José do Barreiro, Campo de Fruticultura da Bocaina (MZUSP 139365); municipality of São José do Barreiro, Bonito, Serra da Bocaina (AL-MN 969). *G. fissipes*: **PE**: municipality of Paulista, Reserva Ecológica Caetés (CFBH 2551). *G. fulvorufa*: **RJ**: municipality of Nova Friburgo, Macaé de Cima (MNRJ 22114); municipality of Teresópolis, Alto do Soberbo (MZUSP 53394); municipality of Teresópolis, Parque Nacional da Serra dos Órgãos (MNRJ 19324); municipality of Teresópolis (MNRJ 19323). **SP**: municipality of Santo André, Parque Natural Municipal das Nascentes de Paranapiacaba (MZUSP 143700). *G. megacephala*: **BA**: municipality of Cumuruxatiba, Fazenda Imbaçuaba (MZUSP 63078). **ES**: municipality of Guarapari, Restinga de Setiba (CFBH 582, 1240, 22800, 35260); municipality of Vila Velha, Ponta da Fruta (CFBH 35532). *G. microdiscus*: **PR**: municipality of Guaraqueçaba, Reserva Natural Salto Morato (MNRJ 85788); municipality of Morretes, Parque Estadual do Marumbi MNRJ 47955; municipality of Piraquara, Banhados (MNRJ 19321); municipality of Piraquara, Mananciais da Serra (CFBH 11044–45). **SC**: municipality of São Bento do Sul, Serra Alta (MNRJ 19322). **SP**: municipality of Pilar do Sul (CFBH 7553, 11541); municipality of São Luiz do Paraitinga, Parque Estadual da Serra do Mar – Núcleo Santa Virgínia (CFBH 8920); municipality of São Miguel Arcanjo, Parque Estadual Carlos Botelho (CFBH 40773). *G. pulchra*: **BA**: municipality of Ilhéus, Fazenda Provisão (CFBH 37984).

Appendix 2

Brazilian voucher specimens included in the molecular analysis. Locality data, accession numbers, and reference for each sample was also provided, according to GenBank. Acronyms CTMZ refers to tissue collection at MZUSP (see abbreviation for scientific collections in main text).

Gastrotheca albolineata (MNRJ 54401): **RJ**: municipality of Cachoeiras de Macacu: Parque Estadual Três Picos: KC844924 (Blackburn & Duellman 2013). *Gastrotheca ernestoi* (MNRJ 64000): **RJ**: municipality of Nova Friburgo: KC844927 (Blackburn and Duellman 2013). *G. fulvorufa* (CTMZ 07467): **SP**: municipality of Santo André: Parque Natural Municipal Nascentes de Paranapiacaba: KC844929 (Blackburn & Duellman 2013). *G. megacephala* (CFBH T377): **ES**: municipality of Guarapari: Restinga de Setiba: KC844928 (as *G. fissipes* in Blackburn & Duellman 2013). *G. microdiscus* (CFBH T3068 and CFBH T3069): **PR**: municipality of Piraquara: Mananciais da Serra: KC844932 and KC844933 (Blackburn & Duellman 2013). *G. microdiscus* (CFBH T1250): **SP**: municipality of Pilar do Sul: KC844957 (Blackburn & Duellman 2013). *G. microdiscus* (CFBH T22444): **SC**: municipality of Joinville: Morro da Tromba: MK433548 (present study). *G. prasina* (MZUSP 17460): **MG**: municipality of Jequitinhonha: Reserva Biológica da Mata Escura: KJ489476 (Duellman 2015). *G. pulchra* (MZUSP 16228): **BA**: municipality of Camacan: Reserva Particular do Patrimônio Natural Serra Bonita: KJ489495 (Duellman 2015). *G. recava* (MZUSP 22049): **BA**: municipality of Wenceslau Guimarães: Estação Ecológica Estadual de Wenceslau Guimarães: KJ489497 (Duellman 2015).