



Rediscovery of the rare *Phrynomedusa appendiculata* (Lutz, 1925) (Anura: Phyllomedusidae) from the Atlantic Forest of southeastern Brazil

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Abstract

The genus *Phrynomedusa* Miranda-Ribeiro, 1923 comprises rare and little known phyllomedusid species from southern Atlantic Forest, Brazil. *Phrynomedusa appendiculata* (Lutz, 1925) is known from three localities since its description and considered a “lost species” because it was last sighted 51 years ago. This pervasive lack of knowledge raised a significant concern about its threat status. Here, we present the rediscovery of *P. appendiculata* from a breeding population in the Atlantic Plateau forests of the state of São Paulo. This new record allowed the gathering of novel ecological, acoustic and morphological data for this species. Most of the novel data agreed with the variation historically reported for the species, but we found subtle divergences that we interpret as intraspecific variation. Moreover, this record also allowed a reassessment of geographic distribution of the species, and the first inference of its phylogenetic relationships based on molecular data (mitochondrial and nuclear DNA). The resulting phylogeny corroborated the generic placement and evolutionary distinctiveness of *P. appendiculata*, evidencing the species as sister to the clade *P. marginata* + *P. dryade*. Based on novel and historical data, we discuss some putative factors influencing the rarity of *P. appendiculata* and its congeners, and provide conservation perspectives. We expect that the novel data can support further assessments of threat status for this rare species, as well as initiatives aiming its conservation.

Key words: Amphibia, Alto Paranapanema, Continuum of Paranapiacaba, lost species, Parque Estadual Nascentes do Paranapanema—PENAP, protected area

Resumo

O gênero *Phrynomedusa* Miranda-Ribeiro, 1923 compreende espécies raras e pouco conhecidas de filomedusídeos do sul da Floresta Atlântica, no Brasil. *Phrynomedusa appendiculata* (Lutz, 1925), é somente conhecida de três localidades desde a sua descrição, e é considerada uma “espécie desaparecida” pois foi avistada pela última vez há 51 anos. Tamanha escassez generalizada de conhecimento levantou grandes preocupações quanto ao status de ameaça desta espécie. Neste estudo, relatamos a redescoberta de *P. appendiculata* com base em uma população em atividade reprodutiva nas florestas do Planalto Atlântico do estado de São Paulo. Este novo registro permitiu a coleta de novos dados ecológicos, acústicos e morfológicos para esta espécie. A maior parte dos novos dados estão de acordo com a variação historicamente reportada para a espécie, mas nós também notamos divergências sutis que interpretamos como variação intraespecífica. Este registro também permitiu uma revisão da distribuição geográfica da espécie, e a primeira inferência de suas relações filogenéticas com base em dados moleculares (DNA mitocondrial e nuclear). A filogenia resultante corroborou o posicionamento

genérico e a distinção evolutiva de *P. appendiculata*, evidenciando a espécie como irmã do clado *P. marginata* + *P. dryade*. Baseado nas informações novas e históricas, nós discutimos sobre os possíveis fatores influenciando a raridade de *P. appendiculata* e seus congêneres, e provemos perspectivas conservacionistas. Nós esperamos que tais dados inéditos possam amparar novas avaliações do status de ameaça desta espécie rara, bem como iniciativas objetivando a sua conservação.

Palavras-chave: Alto Paranapanema, Amphibia, área protegida, Contínuo de Paranapiacaba, espécie desaparecida, Floresta Atlântica, Parque Estadual Nascentes do Paranapanema—PENAP

Introduction

Adolpho Lutz described *Phyllomedusa appendiculata* based on a single specimen from São Bento do Sul, state of Santa Catarina, Brazil, sent to him by J. Nahderer (Lutz 1925). The taxonomic history of *P. appendiculata* was intrinsically associated with *Phrynomedusa fimbriata* Miranda-Ribeiro, 1923, type species of the genus *Phrynomedusa* (Miranda-Ribeiro, 1923). *Phyllomedusa appendiculata* was considered a junior synonym of *P. fimbriata* until the revision of Cruz (1985), which removed this species from its synonymy, redescribed both taxa and allocated them in the previously erected *Phyllomedusa fimbriata* group (Cruz 1982).

A subsequent taxonomic revision of phyllomedusid species of the Atlantic Forest (Cruz 1990) revalidated the genus *Phrynomedusa* to include three species of the former *P. fimbriata* group sensu Cruz (1985): *P. fimbriata*, *P. appendiculata* and *Phrynomedusa marginata* (Izecksohn & Cruz, 1976). Later, two new species were described for the genus: *Phrynomedusa vanzolinii* Cruz, 1991 and *Phrynomedusa bokermanni* Cruz, 1991. A recent and extensive review of the genus (Baêta *et al.* 2016), provided relevant new phenotypic and natural history data, updated the known geographic distribution of species, and described the sixth species of the genus: *Phrynomedusa dryade* Baêta, Giasson, Pombal, & Haddad, 2016. Therefore, currently six species of *Phrynomedusa* are recognized: *P. appendiculata*, *P. bokermanni*, *P. dryade*, *P. fimbriata*, *P. marginata*, and *P. vanzolinii*.

The taxonomy of *Phrynomedusa* species has relied heavily on external morphology that underestimated variation, culminating in historical misidentifications of museum specimens (Baêta *et al.* 2016). In addition, as a result of the overall difficulty in locating new living individuals, knowledge scarcity on the ecological and phenotypic data remains for most of the *Phrynomedusa* species (Baêta *et al.* 2016). According to molecular-based phylogenetic analyses, *Phrynomedusa* is an early diverging phyllomedusid lineage (e.g. Wiens *et al.* 2006; Faivovich *et al.* 2010; Duellmann *et al.* 2016). However, due to low comprehensiveness of species from this genus in these phylogenetic analyses, relationships among *Phrynomedusa* species remain largely unknown. To date, only *Phrynomedusa marginata* and *P. dryade* have been included in phylogenetic analyses (Baêta *et al.* 2016). Regarding the geographic distributions, the six species of *Phrynomedusa* are known from the Atlantic Forest of southeastern and southern Brazil (Baêta *et al.* 2016). Nevertheless, complete geographic range is irresolute for most *Phrynomedusa* species, as they are known only from 12 localities (Baêta *et al.* 2016; Frost 2021).

There is an overall perception that *Phrynomedusa* species are all suffering population declines and are near threatened to extinction (Stuart *et al.* 2008; Garcia *et al.* 2013) given that last sightings of most species spans decades or even more than a century—for *P. fimbriata*, now considered as extinct—(see Stuart *et al.* 2008 and Baêta *et al.* 2016 for details). However, conservation assessments seem conservative when assigning threat status to *Phrynomedusa* species. For instance, *P. appendiculata* is currently considered near threatened to extinction (NT) according to the IUCN Red List (Garcia *et al.* 2004), but as “data deficient” (DD) according to a most recent assessment for the Brazilian Red List (ICMBio 2018).

We recorded breeding individuals of *P. appendiculata* during a short-term (14 days) amphibian survey in a region of pristine Atlantic Forest in southern Brazil. This represents the first documented sighting of this species since 1970, and fills a relevant gap in its geographic distribution. Based on this record, we describe additional data on ecological, acoustic and morphological variation of *P. appendiculata*. We also inferred, for the first time, the phylogenetic relationships of this species based on molecular data. We expect that these novel data support further assessments of the threat status of this species.

Materials and methods

Study site and species sampling. The new locality of occurrence for *P. appendiculata* lies within the limits of a recently created protected area (Parque Estadual Nascentes do Paranapanema—PENAP), in Capão Bonito municipality, state of São Paulo, Brazil (24°12' S, 48°12' W datum = WGS84; ca. 800 m above sea level). The regional climate is highly seasonal, with a cold and dry period between June and August and a rainy and warm period between December and February (Alvares *et al.* 2013). The average annual temperature is 18.8° C and total annual rainfall is ca. 1,400 mm (Alvares *et al.* 2013).

Amphibians were surveyed by LJCLM and DP for 14 days, between December 2011 and January 2012. This sampling covered distinct habitats of the landscape such as humid primary upland and highland forests, riparian forests, natural open habitats and anthropic habitats (see Instituto Amigos da Reserva da Biosfera da Mata Atlântica 2012). Diurnal and nocturnal vocal and active surveys (Crump & Scott Jr. 1994) were conducted by sampling all visually accessible microhabitats. We photographed individuals in life, and given the rarity and unknown threat status of the species, we only collected a single specimen. This specimen was euthanized with a topic solution of 5% xylocaine, fixed with 10% formalin for 24 h, and preserved in 70% ethanol. Before this process, we extracted a liver tissue sample for molecular analysis and stored it in 100% ethanol. The specimen was deposited in the Museu de Zoologia, Universidade de São Paulo, state of São Paulo, Brazil (voucher number MZUSP 159234).

Morphological data collection and comparisons. We obtained qualitative and quantitative morphological data of the collected specimen, representing data traditionally used in the genus taxonomy (Baêta *et al.* 2016). The following 13 morphometric measurements were taken using a caliper to the nearest 0.1 mm: snout–vent length from tip of snout to posterior margin of vent (SVL); head length from angle of jaw to tip of snout (HL); head width at level of angle of jaw (HW); internarial distance between inner margins of nostrils (IN); interorbital distance between the anterior corners of orbits (IO); eye diameter from anterior to posterior corner of eye (ED); eye–nostril distance from anterior corner of eye to posterior margin of nostril (EN); tympanum diameter from anterior to posterior corner of tympanum (TD); forearm length from edge of hand to the outer edge of flexed elbow (FA); hand length from proximal edge of palmar tubercle to tip of finger III (HA); thigh length from the posterior extremity of the vent to knee (TH); tibia length from outer edge of flexed knee to heel (TL); and foot length from proximal edge of outer metatarsal tubercle to the tip of Toe IV (FL). Fingers and toes were numbered preaxially to postaxially from I–IV and I–V, respectively. Toe webbing formula followed Savage & Heyer (1997). Sex was determined from calling activity and presence of secondary sexual characteristics in males (nuptial pads and vocal slits). Color patterns of living specimens were described using photographs and field notes. Morphological data were compared with published information on *Phrynomedusa* (compiled in Baêta *et al.* 2016) as well as through direct examination of 54 museum specimens of *Phrynomedusa*, including 24 specimens of *P. appendiculata*. Examined specimens are listed in the Appendix 1, and institutional abbreviations followed Sabaj (2020).

Acoustic data collection and comparisons. Advertisement calls were recorded about 1–2 m distant from an uncollected calling male with a Zoom H4n Handy Recorder (Zoom Corporation, Tokyo, Japan) with internal microphone, at sampling frequency of 44 kHz and 16-bit resolution, and stored in uncompressed WAV format. Due to an intense background noise and the presence of other frog species concomitantly vocalizing, we obtained the acoustic parameters only from *P. appendiculata* calls fully isolated from interference in the acoustic space. Spectral and temporal parameters obtained were also considered in the description of *P. appendiculata* advertisement call (Forti *et al.* 2019), and include: number of notes; note duration (s); harmonic structure; minimum frequency (Hz); maximum frequency (Hz); peak frequency (Hz); and call rate (calls/min). Acoustic analyses were conducted in Raven Pro (v1.5, Cornell Laboratory of Ornithology, USA), and graphic representations were generated in R environment (R Core Team 2019), using the packages ‘seewave’ (Sueur *et al.* 2008) and ‘TuneR’ (Ligges *et al.* 2018). Acoustic data were summarized in range and/or mean ± standard deviation.

Molecular data sampling and analyses. We generated sequences for *P. appendiculata* from fragments of two mitochondrial DNA loci (*12S rRNA* and *16S rRNA*), and two complete nuclear DNA loci (*Recombination activating gene 1*; *Rag1*, and *Rhodopsin*; *Rhod*). Genomic DNA was extracted following the protocols of Fetzner Jr. (1999). The target loci were amplified via Polymerase Chain Reaction (PCR) using primers and standard protocols described in Amaro *et al.* (2013). PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (Applied Biosystems, Waltham, MA, USA) and sequenced using Big Dye Terminator sequencing kit (Applied Biosystems, Waltham, MA, USA) on automated sequencers ABI PRISM 310, 3700 or 3170 (Applied Biosystems,

Waltham, MA, USA). Chromatograms were inspected, embedded primer sequences were deleted, and forward and reverse strands were compared before assembling consensus sequences with CodonCode Aligner 3.5 (Codon Code Corporation, MA, USA).

We included the novel sequences of *P. appendiculata* in a matrix with sequences of phyllomedusids, hylids and pelodyadids downloaded from GenBank (Clark *et al.* 2016), which allowed testing the phylogenetic relationships of the *Phrynomedusa* species. This matrix includes the complete rRNAs and their intervening tRNA (tRNA Valine) (*12S-tRNA^{Val}-16S*; 2,577 base pairs—bp; only fragments of the *12S* and *16S* are available for four species), and the complete nuclear loci *Rag1* (428 bp) and *Rhod* (316 bp). Taxon sampling includes representatives of each phyllomedusid genera (number of samples in parenthesis): *Agalychnis* Cope, 1864 (9), *Callimedusa* Duellman, Marion, & Hedges, 2016 (4), *Cruziohyala* Faivovich, Haddad, Garcia, Frost, Campbell, & Wheeler, 2005 (1), *Hylomantis* Peters, 1873 (2), *Phasmahyla* Cruz, 1991 (5), *Phyllomedusa* Wagler, 1830 (13), and *Pithecopus* Cope, 1866 (10). Representatives of closely related genera from families Pelodyadidae (*Ranoidea* Tschudi, 1838, *Nyctimystes* Stejneger, 1916 and *Litoria* Tschudi, 1838) and Hylidae (*Acris* Duméril & Bibron, 1841, *Boana* Gray, 1825, *Dendropsophus* Fitzinger, 1843, *Dryophytes* Fitzinger, 1843, *Nesorohyla* Pinheiro, Kok, Noonan, Means, Haddad, & Faivovich, 2018, *Phyllodytes* Wagler, 1830, *Pseudis* Wagler, 1830, *Scinax* Wagler, 1830, and *Trachycephalus* Tschudi, 1838) were included as outgroups. Newly generated sequences were deposited at GenBank, and the accession numbers of all sequences included in this dataset can be found in Appendix 2.

Each fragment was independently aligned using the online software MAFFT version 7 (Katoh *et al.* 2019) under the strategy Q-INS-i for *12S-tRNA^{Val}-16S*, and G-INS-i for *Rag1* and *Rhod*, with default parameters for gap opening and extension, and subsequently concatenated in Mesquite version 3.61 (Madison & Madison 2019), leading to a final alignment with 3,321 nucleotide sites. Using this alignment, we inferred phylogenetic relationships among individuals under the Parsimony optimality criterion in TNT version 1.5 (Goloboff & Catalano 2016). Internal gaps were considered as a fifth state and all classes of transformation events were equally weighted. Nodal support was assessed by 1,000 Jackknife replicates. Uncorrected pairwise genetic distances among *Phrynomedusa* samples were computed in Mega 7 (Kumar *et al.* 2016), with gaps removed using a pairwise deletion option.

Results

Species account

Phyllomedusidae Günther, 1858

Phrynomedusa Miranda-Ribeiro, 1923

Phrynomedusa appendiculata (Lutz, 1925)

(Figs. 1–3)

Description of the specimen. Adult male (MZUSP 159234; field number DT 2969, Figs. 1, 2), collected by D. Pavan on 04 December 2011. Slender body; head wider than long (33% of SVL); snout acuminate in dorsal view, oblique in lateral view, internarial distance 55% of interorbital distance; nostrils lateral and slightly protuberant; *canthus rostralis* curved (concave); loreal region oblique; upper lip dermal fringe absent; interorbital region flat; eye large; palpebral membrane not reticulated; eye diameter 82% of interorbital distance; supratympanic fold well defined, extending posteroventrally to above insertion of arm; tympanum visible; tympanic membrane weakly differentiated from surrounding skin; tympanic annulus round, with its uppermost portion obscured by the supratympanic fold, tympanum diameter 47.5% of eye diameter, separated from eye by 21% of tympanum diameter; tongue cordiform, anteriorly attached; dentigerous process of vomer absent; choanae large and elliptical widely separated from each other; vocal sac single, subgular, not externally evident.

Forearm more robust than upper arm; forearm length 80% of hand length; inner metacarpal tubercle single and elliptical; outer metacarpal tubercle absent; subarticular tubercles rounded; supernumerary tubercles absent; dermal folds smooth on postaxial margin of forearm; webbing formula: I 2⁺ – 2^{1/2} II 2 – 3 – III 2^{2/3} – 2 IV; finger length I < II < IV < III; adhesive discs rounded, more distinct in fingers III and IV; keratinized nuptial pad present at dorsal and preaxial surface of finger I, not extending ventrally.

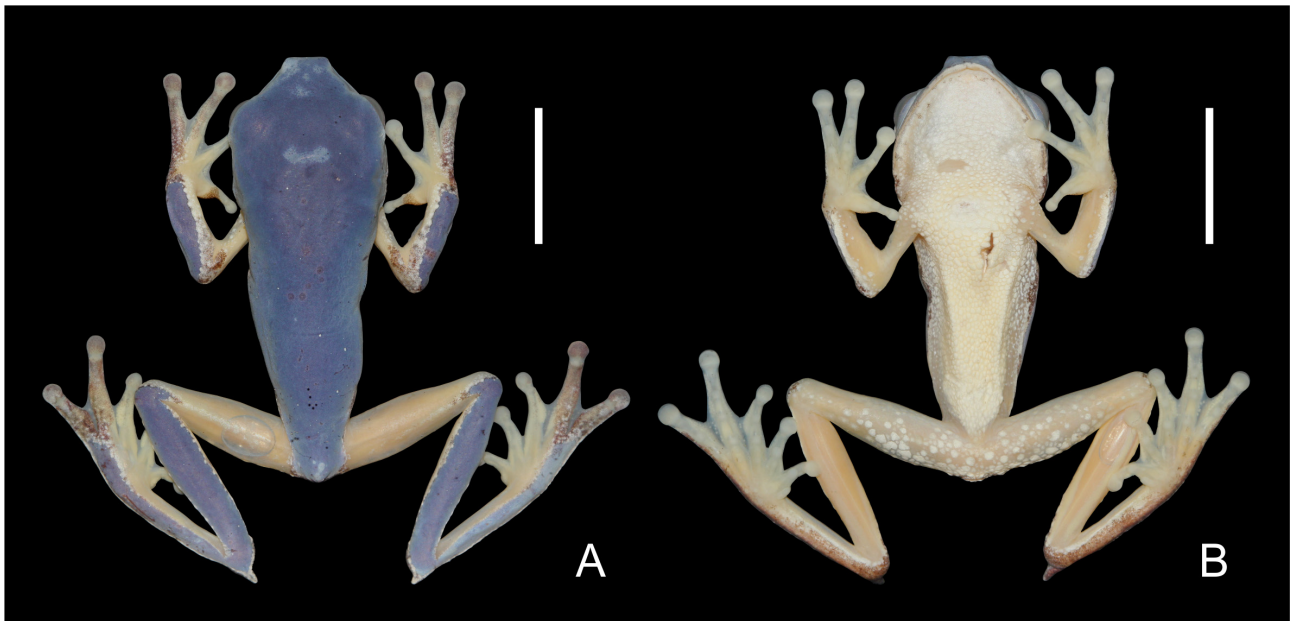


FIGURE 1. Dorsal (A) and ventral (B) views of the preserved adult male of *Phrynomedusa appendiculata* (Lutz, 1925) from Capão Bonito, state of São Paulo, Brazil (MZUSP 159234; 31.4 mm SVL). Scale bar = 1.0 mm. Photographs by L.J.C.L. Moraes.

Hind limbs robust; tibia length 51% of SVL; foot length 72.5% of tibia length; triangular calcar appendix present, slender and long, parallel with tarsus sagittal axis and occupying approximately half of tibio-tarsal articulation; smooth lateral fringes present on external face of tibia and feet, less developed at the toe V; inner metatarsal tubercle single and elliptical; outer metatarsal tubercle absent; subarticular tubercles single and rounded; supernumerary tubercles absent; webbing formula I 2⁺ – 2 II 2⁻ – 2⁺ III 1^{1/2} – 2 IV 2⁻ – 1 V; toe length I < II < III < V < IV; adhesive discs rounded, more distinct on toes IV and V.

Skin on the dorsal surfaces of head, body and limbs smooth; skin on ventral surfaces smooth to areolate on throat, areolate in chest and belly and almost smooth with few areoles in the posterior region of thigh and arm; cloaca directed posteriorly above dorsal plane of thighs; osteoderms parotoid and dorsolateral glands absent.

Measurements of the specimen. SVL 31.4; HL 10.5; HW 11.6; IN 2.7; IO 4.9; ED 4.0; EN 3.3; TD 1.9; FA 7.9; HA 9.9; TH 15.4; TL 16.0; FL 11.6.

Coloration. The color in life for *P. appendiculata* has been described based on a sketch (Cochran, 1955) and recently redescribed with the aid of old photographs from an individual recorded in Santo André, state of São Paulo (Baêta *et al.* 2016). Here we complement the description of color in life variation for this species based on the newly recorded individuals (Figs. 2, 3). Dorsal surface of the head and body, lateral surface of head and loreal region bright green. Upper and lower lips grayish-white, often delimited above by a thin darker stripe. Grey bordered nostrils. Lateral surface of the body bright green up to its medial portion, abruptly changing to orange. A marbled black and white stripe borders this green color. Lateral surface of the body includes a thin pale orange stripe upon an areolate skin, ventrally changing to bright orange upon a smooth skin (Figs. 2, 3). Throat, chest and abdomen pale yellow (Fig. 2).

In forelimbs, the bright green is present on the dorsal surface of the forearm, bordered by a marble black and white stripe that gradually changes to bright orange in the upper arm and forearm, reaching the finger IV. Remaining dorsal and ventral surfaces of hands pale orange; dorsal surfaces of the adhesive discs in fingers brownish-orange (Figs. 2, 3). Nuptial pad brown. On hindlimbs, bright green covers most of the dorsal surface of tibia and reaches the toe V. The bright green is bordered in feet by a marbled black and white stripe, which reaches the dorsal surfaces of toes IV and V and the adhesive disc of toe V. This marble stripe is gradually replaced by bright orange, which covers the remaining dorsal and ventral surfaces of thighs (along with sparse pale yellow over the areolate skin), ventral surface of tibia, and dorsal surface of feet. Dorsal surfaces of adhesive discs of toes brownish-orange. Ventral surface of feet, toes and adhesive discs of toes pale orange. The calcar retains part of the bright green from dorsal surfaces of tibia, but is marble in most of its dorsal and ventral surfaces (Figs. 2, 3).



FIGURE 2. Dorsal (A), ventral (B) and lateral (C) views of an adult male of *Phrynomedusa appendiculata* (Lutz, 1925) in life, from Capão Bonito, state of São Paulo, Brazil (MZUSP 159234; 31.4 mm SVL). Photographs by D. Pavan.

Transparent eyelid, bordered by a thin grayish-white stripe. A black bordered eye with a black pupil, a bicolored iris (golden-yellow in its upper third and light-grey in the remaining lower portion), dark vermiculations and a diffuse dark stripe in the medial region (Figs. 2, 3). Newly metamorphosed individuals have a similar coloration pattern, except by an olive green color instead of bright green, and by less pronounced grey borders between the olive green and bright orange areas (Fig. 3).

In preservative (based on the collected specimen after 10 years of its preservation), green colored areas became light blue, orange areas (at the limbs and the lateral surface of the body) became almost transparent cream, pale yellow areas (the venter and other parts with areolate skin) became creamy-white, as well as the marble stripe delimiting the division of green and orange colored areas; and the pale grey color at dorsal surfaces of hands and feet became greyish-purple (Fig. 1).

Advertisement call. The call of *P. appendiculata* in the new locality is composed by a single short note, followed by silence intervals with variable duration (Fig. 4). Call duration is 0.03 ± 0.00 s ($n = 15$ calls), and is emitted at a variable rate of 2.98 ± 1.66 calls/min ($n = 5$ males). Notes are composed of highly fused pulses, the first one more evidently segregated from the others (Fig. 4). Minimum frequency ranges 1,236–1,538 Hz ($1,435 \pm 86$ Hz; $n = 15$ calls), peak frequency ranges 1,781–2,531 Hz ($2,118 \pm 301$ Hz; $n = 15$ calls), and maximum frequency ranges

2,824–3,257 Hz ($3,047 \pm 109$ Hz; $n = 15$ calls). The recorded advertisement call is very similar to the call from Santo André, state of São Paulo (recorded in 1963) (Forti *et al.* 2019). The only subtle divergences are more evident note pulses, slightly higher maximum frequency, and a lower call rate.

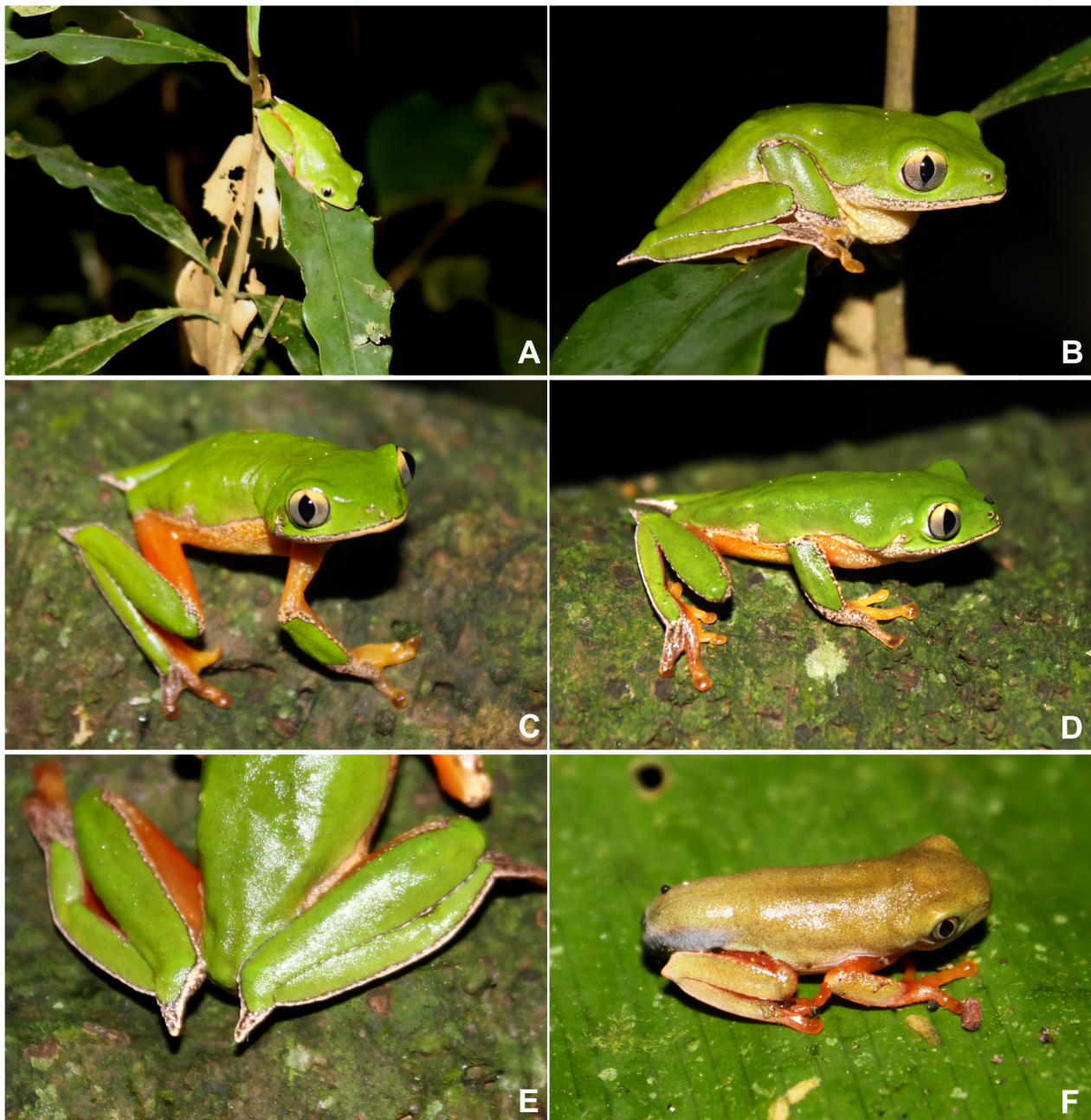


FIGURE 3. Unvouchered individuals of *Phrynomedusa appendiculata* (Lutz, 1925), recorded in Capão Bonito, state of São Paulo, Brazil, including adult males (A–D), a detailed view of the calcar (D) from the male in (C, D), and a newly metamorphosed individual (F). Photographs by L.J.C.L. Moraes.

Geographic distribution. *Phrynomedusa appendiculata* is only known from four localities in montane forests of the Atlantic Plateau at southern Atlantic Forest (Baêta *et al.* 2016, Fig. 5). These localities are within the limits of the municipalities of São Bento do Sul (type locality) and Lauro Müller in the state of Santa Catarina, and Santo André in the state of São Paulo. Our new record represents the second known locality for the species' occurrence in the state of São Paulo, also filling a range gap (Fig. 5). This locality is ca. 250 km northwards from the type locality and ca. 200 km southwards from the first record in the state of São Paulo (Fig. 5). It is noteworthy that the species has not been recorded since 1924–1970 in the other three localities, despite being extensively inventoried (Verdade *et al.* 2009; Trevine *et al.* 2014).

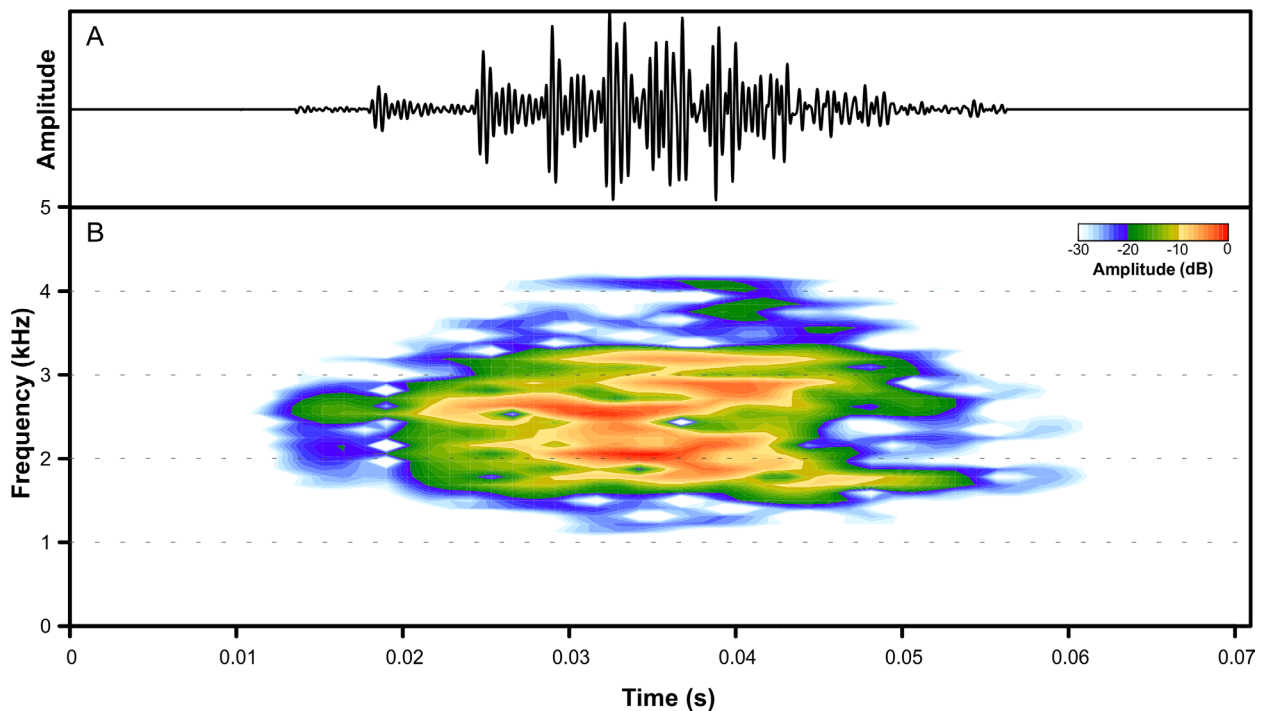


FIGURE 4. Waveform (A) and spectrogram (B) of the advertisement call of an unvouchered individual of *Phrynomedusa appendiculata* (Lutz, 1925) from Capão Bonito, state of São Paulo, Brazil.

Natural history. Despite the diurnal and nocturnal sampling effort in distinct habitats of the regional landscape, we only recorded *P. appendiculata* during night surveys and in a single collecting site. The species was recorded in this locality (Figs. 5, 6) during three distinct days, on 04 December 2011 at ca. 22:00 h, on 24 January 2012 at ca. 23:00 h, and on 25 January 2012 at ca. 01:00 h. In late January (rainy season), individuals of *P. appendiculata* were recorded in breeding activity, based on males emitting advertisement calls and newly metamorphosed individuals (Fig. 3). The species was breeding in two near black-water ponds, part of oxbow lakes of an adjacent river (Fig. 6B), corroborating the known habitat condition for breeding events of *Phrynomedusa* species (Haddad & Prado 2005; Faivovich *et al.* 2010, Baêta *et al.* 2016). About 10 adult individuals of *P. appendiculata* were assembled at this breeding event based on direct observations and vocalizations. Individuals were spotted perched at leaf petioles, herbaceous vegetation, and thin branches of understory trees, always near or above the water (Fig. 3A, B, 6C). No egg-clutch and tadpole were observed. The species was not recorded in lentic rivulets with clean water near these ponds.

Regarding antipredator mechanisms (following the classification system of Ferreira *et al.* 2019), we observed that *P. appendiculata* avoid detection through a combination of camouflage (background matching with vegetation), immobility and call interruption. To prevent attack, the species performed a series of mechanisms widely employed by phyllomedusids (Ferreira *et al.* 2019): showed hidden orange coloration (supposedly aposematic) in movement; retreated when disturbed, hiding the orange parts of the body (contraction posture); or escaped, by climbing or jumping away (in most cases). Besides the secretory products of cutaneous glands (usual for amphibians; Ferreira *et al.* 2019), no additional counterattack mechanism were noted. Other syntopic breeding species included *Boana bischoffi* (Boulenger, 1887), *Dendropsophus giesleri* (Mertens, 1950) and *Physalaemus olfersii* (Lichtenstein & Martens, 1856).

Molecular-based phylogenetic relationships. Our phylogenetic inference mirrored the results of previous phylogenies (e.g. Wiens *et al.* 2006; Faivovich *et al.* 2010; Duellman *et al.* 2016) by evidencing a monophyletic *Phrynomedusa* as an early divergent lineage within the phyllomedusid radiation (Fig. 7). The generic placement and evolutionary distinctiveness of *P. appendiculata* was corroborated, as the species is recovered with high support as sister to the clade *P. marginata* + *P. dryade* (Fig. 7). Genetic distances between *P. appendiculata* and *P. marginata* + *P. dryade* clade range from 8.9–9.1% (*12S*), 8.0% (*16S*), 1.7% (*Rag1*) and 1.3% (*Rhod*), while genetic distance between *P. marginata* and *P. dryade* reached 3.0% at the single locus sampled for *P. marginata* (*12S*).

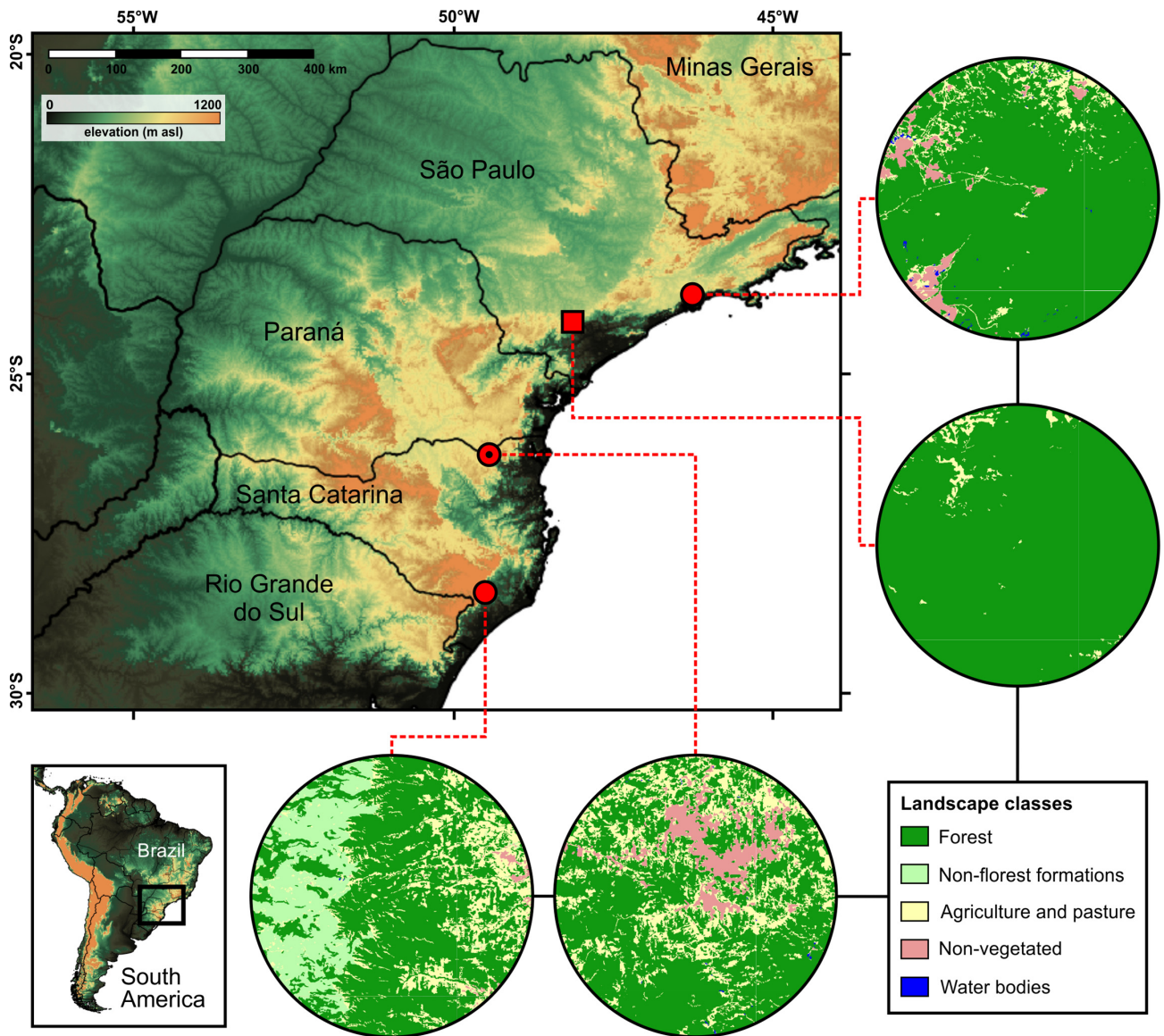


FIGURE 5. Known localities of *Phrynomedusa appendiculata* (Lutz, 1925) at southern Brazil (red symbols) upon an elevation background, and the regional landscape characteristics in a 10 km buffer around each locality (data from 2019; MapBiomas 2020). Landscape classes are detailed in the inset legend. The square represent the new locality, in Capão Bonito, state of São Paulo; the dotted circle represent the type locality, in São Bento do Sul, state of Santa Catarina, and the hollow circles represent additional localities, in Lauro Müller, state of Santa Catarina and Santo André, state of São Paulo.

Discussion

By sampling three of the six species of the genus, our molecular-based phylogenetic inference is the most complete for *Phrynomedusa* to date. As a consequence, this is the first evidence of polarization of phenotypic diversification in the genus. This result indicates that the prevalence of orange coloration on the lateral surface of the body and head is secondarily developed across *Phrynomedusa* diversification. Based on the phylogenetic relationships, the similarity of color patterns, and taxonomic history (Baêta *et al.* 2016), it is possible to predict that the other congeners not yet molecularly sampled are more closely related to *P. appendiculata*, and therefore, early diverging lineages.

Our study also bring novelties at an intraspecific level, by showing subtle divergences on the external morphology, vocalization and ecological preferences of *P. appendiculata*. Nevertheless, we interpret that such dissimilarities

should be part of intraspecific variation given the broad geographic distance among the known populations, and distinct preservation conditions (Baêta *et al.* 2016). Some knowledge gaps still remain mostly on natural history of *P. appendiculata*. Those gaps largely hamper our understanding of its current distribution range and threat status. In fact, it is difficult to estimate threat status for *Phrynomedusa* species because the long time lapse between sightings may not necessarily indicate their decline and disappearance (Solow 2005). However, we expect that such a rediscovery can provide important guidelines for further assessments of the threat status of this seldom recorded species.

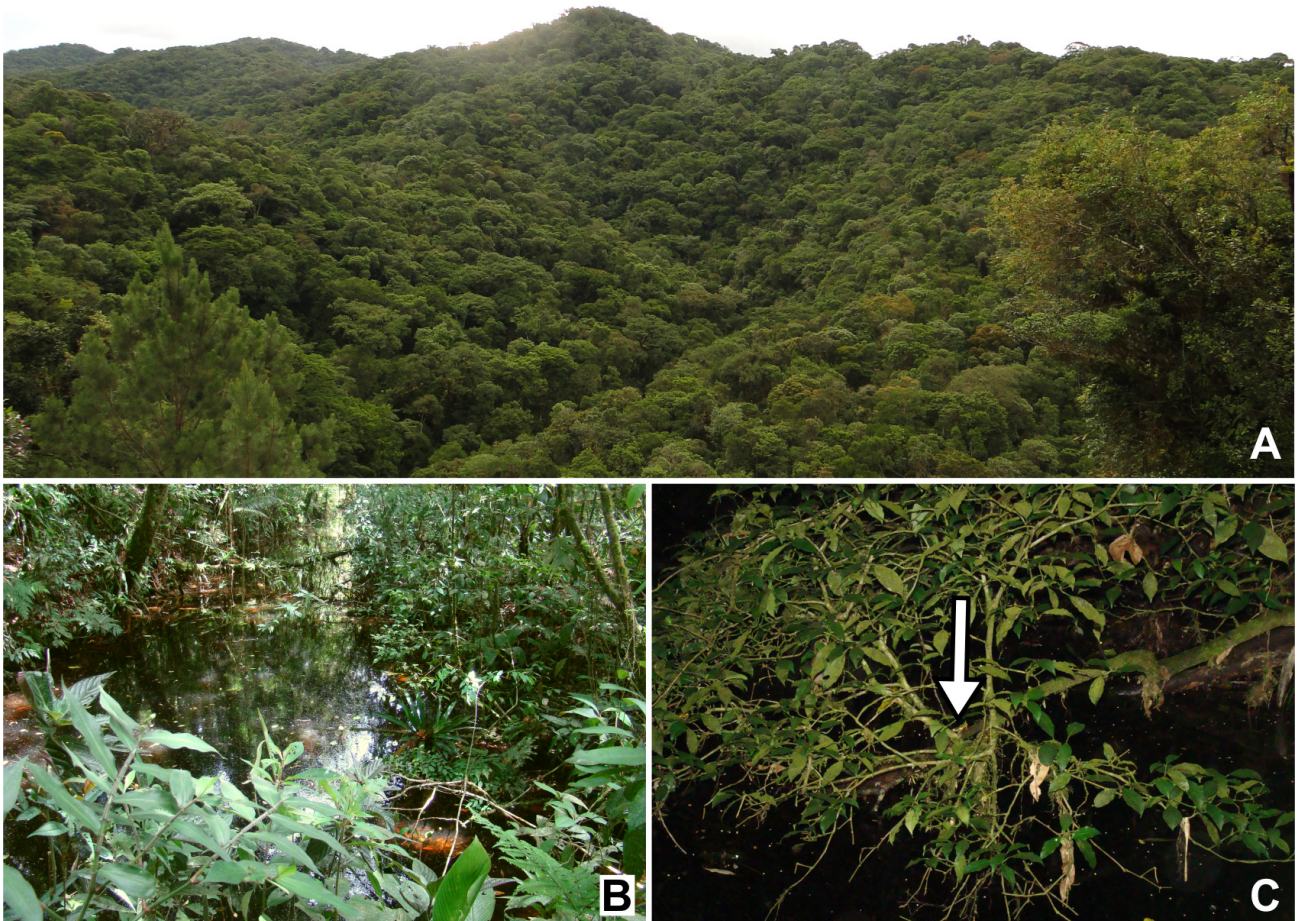


FIGURE 6. Habitat of *Phrynomedusa appendiculata* (Lutz, 1925) in Capão Bonito, state of São Paulo, Brazil. Panoramic view of highland pristine Atlantic Forest (A), a blackwater pond where breeding individuals were recorded (B); and an individual (indicated by the arrow) perched on branches above this pond (C). Photographs by D. Pavan (A) and D.L. Ingui (B, C).

To the best of our knowledge, *P. appendiculata* has not been recorded elsewhere since our sampling about 10 years ago. This is noteworthy considering that many “lost species” have been recently recorded due to the astonishing growth on the global biodiversity data (Scheffers *et al.* 2011; Heberling *et al.* 2021). There are many factors that can be directly or indirectly attributed to the scarcity of information related to *P. appendiculata*, as well as to its congeners. One of these putative factors is the alarming advance of infectious chytridiomycosis, which has been linked to several amphibian populations to decline over the globe, and is considered as especially threatening to montane amphibians from tropical regions (Daszak *et al.* 2003). In fact, there are records of this pathogen in the same localities where *Phrynomedusa* disappearances are known (Jenkinson *et al.* 2016). Therefore, such a threat may be contributing to the suggested population declines, rarity, and extinctions within the genus (Baêta *et al.* 2016).

Another putative influential factor is the human-induced habitat modifications. The Atlantic Forest has lost ca. 75–90% of its original distribution due to human occupation (Tabarelli *et al.* 2005; Ribeiro *et al.* 2009, 2011; Rezende *et al.* 2018). *Phrynomedusa appendiculata* is especially sensitive to this loss as it seems related to specific environmental conditions along the Atlantic Plateau. As a pond-breeder that exclusively reproduces in ponds inserted in pristine highland forests (Baêta *et al.* 2016), this species are geographically and physiologically confined

to an environmental condition in which these reproductive sites are naturally scattered. This is because lentic water microhabitats with a greater volume and long-term persistence are considerably less frequent in highlands when compared to the lowland sedimentary basins (Schumm 1985). In highlands, such microhabitats are typically restricted to isolated alveolar alluvial plains, where rivers acquire meandering characteristics. Under these conditions, meanders are eventually cut from the main river channel (Schumm 1985), forming oxbow lakes that can be used by highland pond-breeders such as *P. appendiculata*. However, due to the ease of human occupation in these flat terrains, such areas were also the primarily impacted within the Atlantic Plateau highland, being historically converted into population nucleus and access roads (e.g. Lima 2015). Conversely, in the more inaccessible and therefore more preserved forest slopes (Silva *et al.* 2007), breeding sites for highland pond-breeder species typically do not occur. Therefore, considering these habitat restrictions and that large tracks of Atlantic Plateau forests are currently rare (Ribeiro *et al.* 2011), we argue that Atlantic Forest remnants of the region we recorded *P. appendiculata* is vital for the species viability. These remnants are large enough to encompass not only a large extension of continuous suitable habitat for this species (Fig. 5), but also a high prevalence of environmental conditions that enables the formation of their breeding sites.

Finally, another putative influential factor for the rarity of *P. appendiculata* and its congeners is that usual methods employed for anuran sampling may be ineffective to record them. In fact, some “lost species” still have a large amount of knowledge gaps in their ecological preferences, are highly cryptic in behavior, have low densities, or typically occupy microhabitats seldom sampled (Scheffers *et al.* 2011; Black 2020). For instance, during the lifecycle of another closely related early-divergent phyllomedusid genus (*Cruziohyla* Faivovich *et al.* 2005), adults typically occupy the higher forest strata, only descending to ground level to lay their eggs (Hoogmoed & Cadle 1991; Turrell *et al.* 2016). Such life history has always influenced the knowledge associated with its occurrence and natural history, since such unpredictable reproductive events are rarely recorded (Turrell *et al.* 2016; Fraga & Torralvo 2019). Our observations do not discard that the *P. appendiculata* may inhabit the canopy during most of its lifecycle and remains at the understory only during the reproductive period. It is also noteworthy that the presence of most vocally active males around 23:00 h to 01:00 h indicate that the peak of the species’ reproductive activity is from that period until dawn, a timeframe that may be neglected in anuran inventories. Therefore, we argue that alternative sampling methods need to be employed to maximize the chance of new records of this species. One of the main alternatives that has been useful to mitigate sampling biases and detect Atlantic Forest “lost species” is the analysis of environmental DNA (eDNA) (Lopes *et al.* 2021). Based on our novel data, more finely targeted surveys can be conducted to obtain eDNA samples aiming to record *P. appendiculata*.

The new locality of *P. appendiculata* lies within one recently created Conservation Unit (PENAP; Instituto Amigos da Reserva da Biosfera da Mata Atlântica 2012), just recently integrated into the largest corridor of Atlantic Forest protected areas (Continuum of Paranapiacaba, Ribeiro *et al.* 2009). Many other localities within this corridor have historically been extensively inventoried for amphibians, and no sightings of *P. appendiculata* were reported (e.g. Bertoluci & Rodrigues 2002; Forlani *et al.* 2010; Trevine *et al.* 2014; Bertoluci *et al.* 2021). However, these localities are predominantly composed of steep mountainous forests, which may be one of the reasons for the lack of *P. appendiculata* sightings. In addition, the other three localities for this species have historically been subjected to anthropic impacts (Fig. 5). Such a scenario emphasizes the relevance of the PENAP creation for the species’ conservation. Considering that *P. appendiculata* is a charismatic and rare species, we highlight its potential as a flagship species (Home *et al.* 2009) for biodiversity conservation in the PENAP region. In fact, biodiversity is currently seen by the local inhabitants as an impediment to regional development (A. C. Martensen, pers. obs.), and we expect that this study will increase its overall appreciation.

Therefore, to ensure long-term viability of *P. appendiculata*, we encourage initiatives involving: (1) gathering of additional occurrence and natural history data for the species in key hotspots, as in the newly created protected area and nearby unprotected areas; (2) expansion of Atlantic Forest protected areas or creation of new ones targeting highland forests encompassing lentic habitats; (3) a monitoring program to investigate the long-term viability of the recorded population; (4) *ex-situ* conservation strategies, if feasible considering the status of the recorded population after *in-situ* monitoring; and (5) greater involvement of local inhabitants in conservation strategies, through environmental education and citizen science projects.

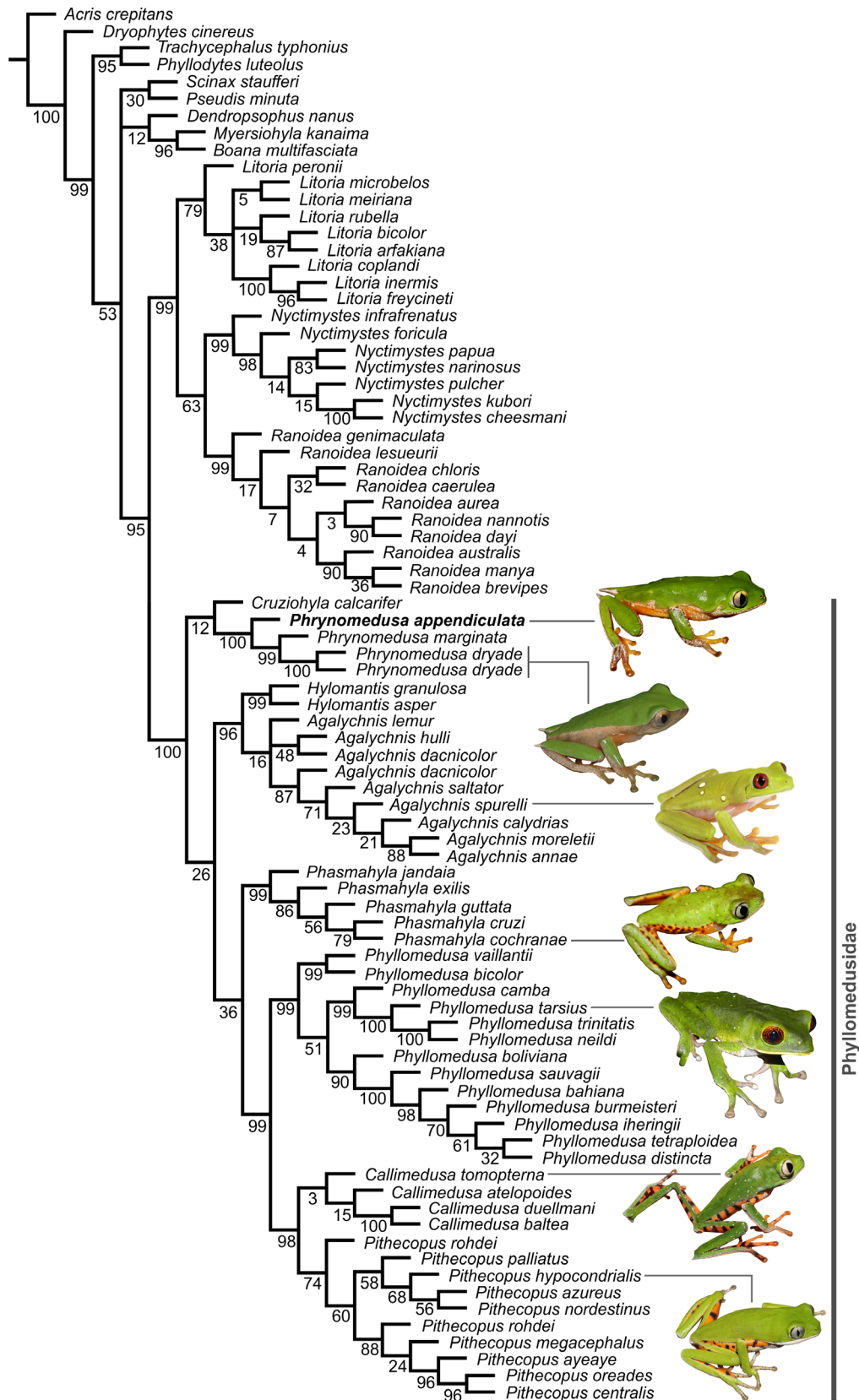


FIGURE 7. Phylogenetic tree of Phyllomedusidae inferred under Parsimony criterion, based on the variation of four loci (two mitochondrial DNA, *12S* and *16S*, and two nuclear DNA, *Rag1* and *Rhod*). Nodal support values (Jackknife) are shown below branches, and outgroups were omitted. The generic placement and evolutionary distinctiveness of *Phrynomedusa appendiculata* (Lutz, 1925) (in bold) was corroborated, based on a newly collected sample from Capão Bonito, state of São Paulo, Brazil. Photos by L.J.C.L. Moraes, except for *P. dryade* (C.F.B. Haddad), and *A. spurelli* (S. Ron; BioWeb Ecuador, <https://bioweb.bio>).

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APPENDIX 1. Specimens examined.

- Phrynomedusa appendiculata*.—BRAZIL: SANTA CATARINA: Lauro Müller, Novo Horizonte (MZUSP 35182–35194); São Bento do Sul (holotype; AL-MN 770); SÃO PAULO: Santo André, Paranapiacaba (MZUSP 4094, 9678, 81423–81424, 81426–81431).
- Phrynomedusa bokermanni*.—BRAZIL: SÃO PAULO: Mongaguá (holotype: MZUSP 81340).
- Phrynomedusa dryade*.—BRAZIL: RIO DE JANEIRO: Paraty (paratype: MNRJ 32861) SÃO PAULO: São Luiz do Paraitinga (holotype: CFBH 16026; paratopotypes: CFBH 7613, 7684, 7716, 7722, 16025, 38749); Salesópolis, Estação Ecológica de Boracéia (paratype: MZUSP 137423); Cananéia, Ilha do Cardoso (paratype: CFBH 22576).
- Phrynomedusa fimbriata*.—BRAZIL: SÃO PAULO: Santo André, alto da Serra de Cubatão (holotype: MZUSP 316).
- Phrynomedusa marginata*.—BRAZIL: ESPÍRITO SANTO: Santa Teresa (paratypes: MNRJ 4100–4101, 60139; MZUSP 74149; topotypes: MNRJ 46881, 57954, USNM 217823–217829).
- Phrynomedusa vanzolinii*.—BRAZIL: RIO DE JANEIRO: município de Teresópolis (paratype: AL-MN 1983), Parque Nacional da Serra dos Órgãos (topotype: MZUSP 81421); SÃO PAULO: Salesópolis, Estação Ecológica de Boracéia (holotype: MZUSP 37669; paratypes: MZUSP 3998, 9387).

APPENDIX 2. Samples included in molecular analyses, with respective GenBank accession numbers. The newly generated sequences are highlighted in bold.

Family	Species	<i>12S-rRNA^{mt}-16S or 12S/16S</i>	<i>Rhod</i>	<i>Ragl</i>
Hylidae	<i>Acris crepitans</i>	AY843559	AY844533	AY844358
Hylidae	<i>Boana multifasciata</i>	AY843648	AY844633	AY844436
Hylidae	<i>Dendropsophus nanus</i>	AY549346	AY844634	AY844437
Hylidae	<i>Nesorohyla kanaima</i>	AY843634	AY844617	AY844422
Hylidae	<i>Pseudis minuta</i>	AY843739	—	AY844505
Hylidae	<i>Phyllodytes luteolus</i>	AY843721	AY844708	AY844494
Hylidae	<i>Scinax staufferi</i>	AY843761	AY844748	AY844523
Hylidae	<i>Trachycephalus typhonius</i>	AY549362	AY844707	AY844493
Pelodyadidae	<i>Litoria arfakiana</i>	AY326039	—	—
Pelodyadidae	<i>Litoria coplandi</i>	DQ116841 / DQ116865	—	—
Pelodyadidae	<i>Litoria freycineti</i>	AY843693	AY844686	AY844473
Pelodyadidae	<i>Litoria rubella</i>	AY819407	—	—
Pelodyadidae	<i>Litoria meiriana</i>	AY843695	AY844688	AY844475
Pelodyadidae	<i>Litoria microbelos</i>	DQ116831 / DQ116831	—	—
Pelodyadidae	<i>Litoria inermis</i>	DQ283211 / DQ283212	DQ283892	—
Pelodyadidae	<i>Litoria peronii</i>	AY819408	—	—
Pelodyadidae	<i>Nyctimystes cheesmani</i>	AY819409	—	—
Pelodyadidae	<i>Nyctimystes forficula</i>	AY819400	—	—
Pelodyadidae	<i>Nyctimystes infrafronatus</i>	AY843694	AY844687	AY844474
Pelodyadidae	<i>Nyctimystes kubori</i>	AY843702	AY844693	AY844479
Pelodyadidae	<i>Nyctimystes narinusus</i>	AY843703	AY844694	—
Pelodyadidae	<i>Nyctimystes papua</i>	AY819410	—	—
Pelodyadidae	<i>Nyctimystes pulcher</i>	AY843701	AY844692	—
Pelodyadidae	<i>Ranoidea aurea</i>	AY843691	AY844684	—
Pelodyadidae	<i>Ranoidea australis</i>	AY843580	AY844553	AY844376
Pelodyadidae	<i>Ranoidea brevipes</i>	AY819411	—	—
Pelodyadidae	<i>Ranoidea caerulea</i>	AY843692	AY844685	—
Pelodyadidae	<i>Ranoidea chloris</i>	DQ116851 / DQ11687	—	—
Pelodyadidae	<i>Ranoidea dayi</i>	DQ283220	DQ283921	—

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APPENDIX 2. (Continued)

Family	Species	12S- <i>tRNA^{Asp}</i> -16S or 12S/16S	Rhod	Ragl
Pelodyridae	<i>Ranoidea genimaculata</i>	DQ283222	DQ283899	—
Pelodyridae	<i>Ranoidea lesueurii</i>	DQ283204	DQ283887	—
Pelodyridae	<i>Ranoidea manya</i>	AY819397	—	—
Pelodyridae	<i>Ranoidea nannotis</i>	DQ283218	DQ283896	—
Phyllomedusidae	<i>Agalychnis annae</i>	GQ366221	—	—
Phyllomedusidae	<i>Agalychnis callidryas</i>	GQ366223	AY844537	—
Phyllomedusidae	<i>Agalychnis dacnicolor</i>	AY843714	AY844702	AY844488
Phyllomedusidae	<i>Agalychnis hulli</i>	GQ366226	GQ366101	GQ366073
Phyllomedusidae	<i>Agalychnis lemur</i>	AY843725	AY844712	GQ366074
Phyllomedusidae	<i>Agalychnis moreletii</i>	GQ366227	GQ366102	—
Phyllomedusidae	<i>Agalychnis saltator</i>	GQ366228	—	—
Phyllomedusidae	<i>Agalychnis spurrelli</i>	AY326043	—	—
Phyllomedusidae	<i>Callimedusa atelopoides</i>	AY819413	—	—
Phyllomedusidae	<i>Callimedusa baltea</i>	GQ366252	GQ366127	GQ366085
Phyllomedusidae	<i>Callimedusa duellmani</i>	AY819414	—	—
Phyllomedusidae	<i>Callimedusa tomoderna</i>	AY843728	AY844715	AY844497
Phyllomedusidae	<i>Cruziohyala calcarifer</i>	GQ366230	GQ366104	—
Phyllomedusidae	<i>Hylomantis aspera</i>	GQ366222	GQ366098	—
Phyllomedusidae	<i>Hylomantis granulosa</i>	AY843687	AY844680	AY844469
Phyllomedusidae	<i>Phasmahyla cochranae</i>	AY843715	GQ366105	GQ366076
Phyllomedusidae	<i>Phasmahyla cruzi</i>	AY843716	AY844703	AY844489
Phyllomedusidae	<i>Phasmahyla exilis</i>	GQ366231	GQ366106	GQ366077
Phyllomedusidae	<i>Phasmahyla guttata</i>	GQ366232	GQ366107	—
Phyllomedusidae	<i>Phasmahyla jandaia</i>	GQ366233	GQ366108	—
Phyllomedusidae	<i>Phrynomedusa appendiculata</i>	OK352270 / OK350365	OK357602	OK357603
Phyllomedusidae	<i>Phrynomedusa dryade</i>	GQ366234	GQ366109	GQ366078
Phyllomedusidae	<i>Phrynomedusa dryade</i>	GQ366235	GQ366110	—
Phyllomedusidae	<i>Phrynomedusa marginata</i>	AY819417	—	—

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APPENDIX 2. (Continued)

Family	Species	12S- <i>tRNA^{Asp}</i> -16S or 12S/16S	Rhod	Ragl
Phyllomedusidae	<i>Pithecopus ayeaye</i>	GQ366245	GQ366120	—
Phyllomedusidae	<i>Pithecopus azureus</i>	GQ366249	GQ366124	GQ366084
Phyllomedusidae	<i>Phyllomedusa centralis</i>	GQ366260	—	—
Phyllomedusidae	<i>Pithecopus hypochondrialis</i>	AY843724	AY844711	—
Phyllomedusidae	<i>Pithecopus megacephalus</i>	GQ366268	GQ366140	—
Phyllomedusidae	<i>Pithecopus nordestinus</i>	GQ366273	GQ366145	—
Phyllomedusidae	<i>Pithecopus oreades</i>	GQ366276	GQ366148	—
Phyllomedusidae	<i>Pithecopus palliatus</i>	GQ366280	—	GQ366092
Phyllomedusidae	<i>Pithecopus rohdei</i>	GQ366237	GQ366112	GQ366080
Phyllomedusidae	<i>Phyllomedusa bahiana</i>	GQ366251	GQ366126	—
Phyllomedusidae	<i>Phyllomedusa bicolor</i>	AY843723	AY844710	AY844495
Phyllomedusidae	<i>Phyllomedusa boliviana</i>	GQ366254	GQ366129	—
Phyllomedusidae	<i>Phyllomedusa burmeisteri</i>	GQ366255	GQ366130	GQ366087
Phyllomedusidae	<i>Phyllomedusa camba</i>	GQ366258	GQ366133	GQ366088
Phyllomedusidae	<i>Phyllomedusa distincta</i>	GQ366263	GQ366135	—
Phyllomedusidae	<i>Phyllomedusa iheringii</i>	GQ366264	GQ366136	—
Phyllomedusidae	<i>Phyllomedusa neildi</i>	GQ366270	GQ366142	—
Phyllomedusidae	<i>Phyllomedusa sauvagii</i>	GQ366282	GQ366153	GQ366094
Phyllomedusidae	<i>Phyllomedusa tarsius</i>	AY843726	AY844713	GQ366095
Phyllomedusidae	<i>Phyllomedusa tetraploidea</i>	GQ366284	GQ366155	GQ366096
Phyllomedusidae	<i>Phyllomedusa trinitatis</i>	GQ366287	GQ366158	GQ366097
Phyllomedusidae	<i>Phyllomedusa vaillanti</i>	AY549363	AY844716	AY844498