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Geographic distributions of *Pseudopaludicola boliviana* and congeneric long-legged species (Anura: Leiuperinae)

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ABSTRACT

In recent years, the taxonomic knowledge of *Pseudopaludicola* has increased and it is currently possible to elucidate aspects of the species within this genus such as their geographic distributions. Here we accessed new data on geographic distribution of *P. boliviana* and three long-legged species of the *P. saltica* group (*P. saltica*, *P. murundu*, and *P. jaredi*). Based on morphometric, acoustic and genetic data, we accessed the specific status of long-legged specimens from Chapada dos Veadeiros highlands and attributed them to the *P. murundu*. We also performed a phylogenetic analysis including several populations of the three species. Based on this novel dataset, we discussed the geographical distributions of *P. saltica*, *P. murundu*, and *P. jaredi*, providing new records for them. *Pseudopaludicola boliviana* is one of the five species of the *P. pusilla* group and it is thought to be a widely distributed species based on its morphology. Hence, we evaluated the geographic distribution of *P. boliviana*, using novel acoustic data of specimens from Argentina, Northern Brazil, and Bolivia.

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Introduction

The genus *Pseudopaludicola* Miranda-Ribeiro, 1926 includes 25 species (Andrade et al. 2020) that occur throughout South America (Lynch 1989). *Pseudopaludicola* is treated as a monophyletic genus, supported by a hypertrophied antibrachial tubercle (Lynch 1989) and osteological features (Lobo 1995) as well as by mitochondrial DNA evidence (Veiga-Menoncello et al. 2014). Most of the known species were described in the last ten years by integrative approaches using multiple sources of evidence, considering that the external morphology of closely-related species is conserved and insufficient for a refined taxonomy (e.g. Pansonato et al. 2016; Cardozo et al. 2018; Andrade et al. 2018a, 2018b, 2019, 2020). This advance in taxonomic knowledge makes it possible, henceforth, to investigate the distribution patterns of these dwarf swamp frogs, such as species from the *P. saltica* group, all occurring in the Brazilian Cerrado (Andrade et al. 2016a), as well as of *P. boliviana*, considered to be

widely distributed in South America (Pansonato et al. 2016).

Toledo (2010) was the first to suggest the *P. saltica* (Cope, 1887) species group. It was later confirmed by Veiga-Menoncello et al. (2014), recognizing a sub-clade within their Clade I, which nowadays includes three species with long hindlimbs: *P. saltica*, *P. murundu* Toledo et al., 2010, and *P. jaredi* Andrade et al. 2016 (Pansonato et al. 2014; Andrade et al. 2016a). Andrade et al. (2016a), Andrade et al. (2018a, 2018b, 2019, 2020) also included the *P. saltica* species group in their phylogenetic hypothesis. *Pseudopaludicola saltica* is the species with more distinct external morphology compared with the other two long-legged species, *P. murundu* and *P. jaredi* (Toledo et al. 2010; Pansonato et al. 2014; Andrade et al. 2016a). *Pseudopaludicola murundu* and *P. jaredi* have a darker vocal sac with longitudinal folds, whereas *P. saltica* has a brighter vocal sac with no longitudinal folds (Toledo et al. 2010). Toledo et al. (2010) also pointed out as a diagnostic feature between *P. murundu* and *P. saltica*

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the aspect of the nuptial pads in adult males, being larger and brighter in *P. murundu*, and smaller and darker in *P. saltica*, with both species collected during the peak of the breeding season. *Pseudopaludicola jaredi* is morphologically indistinguishable from *P. murundu* (Andrade et al. 2016a). However, acoustic and genetic evidence was useful to elucidate the specific delimitations among these three species (Andrade et al. 2016a). *Pseudopaludicola jaredi* is known from the Brazilian states of Ceará, Rio Grande do Norte, and Maranhão (Andrade et al. 2016a, 2016b). *Pseudopaludicola murundu* is known from the Brazilian states of São Paulo and Minas Gerais (Toledo 2010; Toledo et al. 2010; Pansonato et al. 2014). *Pseudopaludicola saltica* has already been registered in the states of Mato Grosso, Minas Gerais, and Goiás (Haddad & Cardoso 1987; Pansonato et al. 2013; Andrade et al. 2016a; Vaz-Silva et al. 2020). Based on external morphology, Santoro and Brandão (2014) reported the occurrence of *P. saltica* in the Chapada dos Veadeiros highlands, in the State of Goiás, Brazil.

Lynch (1989) proposed the *Pseudopaludicola pusilla* (Ruthven, 1916) species group, which currently includes *P. boliviana* Parker, 1927, *P. ceratophyes* Rivero & Serna, 1985, *P. llanera* Lynch, 1989, *P. motorzinho* Pansonato et al. 2016, and *P. pusilla*, all sharing the presence of T-shaped terminal phalanges. Later, two phylogenetic studies corroborated the monophyly of the *P. pusilla* group, namely the morphology-based study conducted by Lobo (1995) and the molecular approach performed by Veiga-Menoncello et al. (2014). *Pseudopaludicola boliviana* was described from Santa Cruz, Bolivia and, based on its morphology, it is thought to be widely distributed, occurring in Eastern Colombia, Northern Brazil (States of Roraima, Amapá, and Pará), and in the Amazonian Venezuela through Southern Guyana, Surinam, Western non-Andean Bolivia, Paraguay, and Argentina (Parker 1935; Hoogmoed 1979; Lynch 1989; Lobo 1990, 1992, 1994; Márquez et al. 1995; Reichle 1997; Reichle & Köhler 1998; De la Riva et al. 2000; Duré et al. 2004; Alcalde & Williams 2004; Brusquetti & Lavilla 2006; Cardozo & Lobo 2009; Cole et al. 2013; Pansonato et al. 2016; Cardozo et al. 2016; Lavilla et al. 2016; Costa-Campos et al. 2016). De la Riva et al. (2000) suggested that the Amazon basin separates two sets of populations of *P. boliviana*, indicating that further research is necessary to elucidate their taxonomic status, emphasizing that, perhaps, the Northern populations might represent an undescribed species (De la Riva et al. 2000).

We collected long-legged specimens from the Chapada dos Veadeiros highlands, which raised

questions about their specific status. In order to clarify this issue, we combined acoustic, morphological, and genetic evidence. This assessment led us to analyze novel acoustic and distributional data on *P. saltica*, *P. murundu*, and *P. jaredi*. Therefore, our objectives in relation to the *P. saltica* species group were (i) to clarify the specific status of the long-legged specimens from Chapada dos Veadeiros highlands, and (ii) to elucidate the geographical distributions of *P. saltica*, *P. murundu*, and *P. jaredi*. In addition, although acoustic data on *P. boliviana* are available in the literature, there is no acoustic comparison among populations. Considering the literature data and representative new samples, we also aimed (iii) to compare the acoustic data of specimens of *P. boliviana* from Argentina, Bolivia, and Northern Brazil.

Materials and methods

Reference specimens

We collected 18 long-legged specimens from Chapada dos Veadeiros highlands at Fazenda São Bento, municipality of Alto Paraíso de Goiás (14°11'21.26" S, 47°37'27.06" W; 1153 m above sea level [a.s.l.]; datum = WGS84), and at Pousada Cachoeira Poço Encantado, municipality of Teresina de Goiás (13°52'33" S, 47°15'43" W; 799 m a.s.l.; datum = WGS84), both in the State of Goiás (GO), Brazil (Figure 1). These specimens were deposited in the collection of frogs of the *Museu de Biodiversidade do Cerrado* [Museum of Cerrado Biodiversity] (AAG-UFU), Universidade Federal de Uberlândia (UFU), in the municipality of Uberlândia, State of Minas Gerais, and in the amphibian collection of *Museu de Zoologia 'Prof. Adão José Cardoso'* [Museum of Zoology] (ZUEC), Universidade Estadual de Campinas (Unicamp), in the municipality of Campinas, State of São Paulo, both in Brazil. Specimens of this study were collected under authorization no. SISBio #30059 issued by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio). Specimens were killed by applying 5 % lidocaine to the skin. We fixed all specimens at 10% formalin and transferred them to 70% ethanol for permanent storage. We collected muscle tissue from the thigh of some long-legged specimens right after death, and stored samples in absolute ethanol for genetic analysis.

Morphometry

We measured 18 adult long-legged males from Alto Paraíso de Goiás and 5 adult males from Teresina de

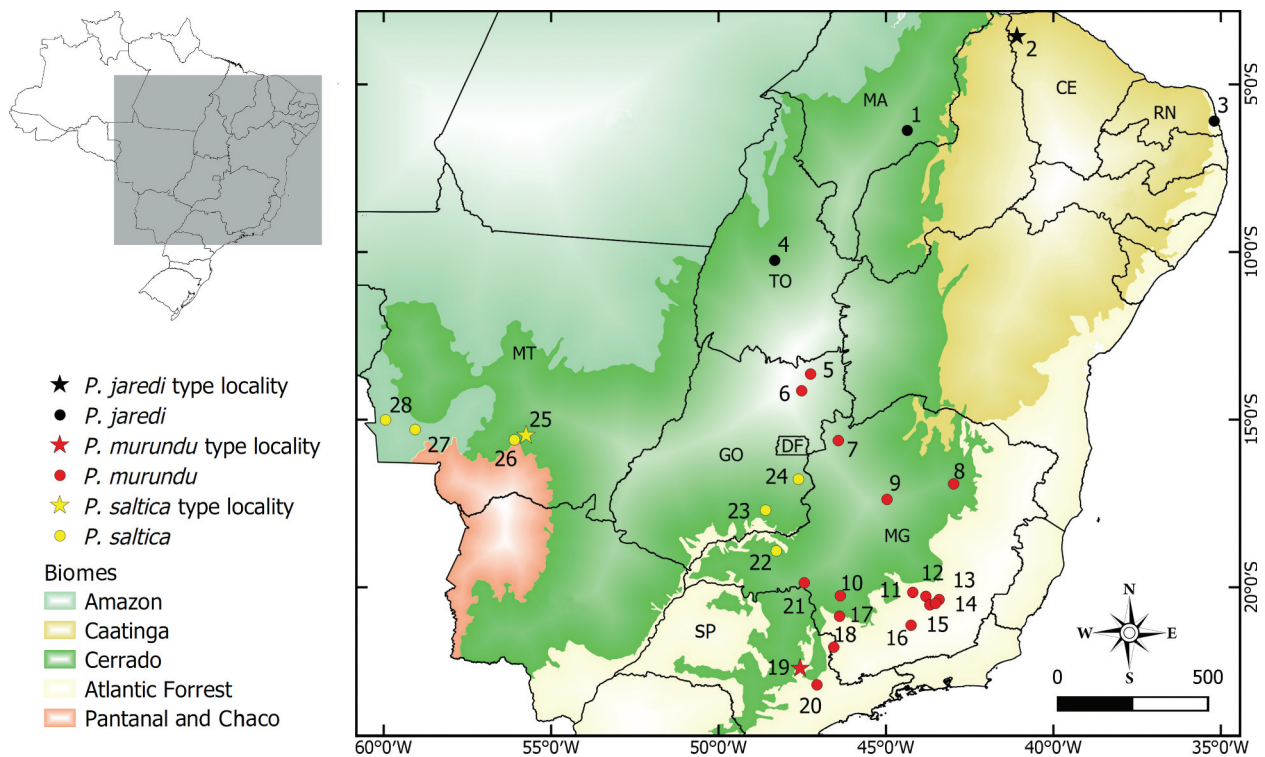


Figure 1. Brazilian biomes and updated geographic distributions of the three long-legged species: *P. saltica*, *P. murundu*, and *P. Jaredi*. Municipalities: (1) Mirador, MA; (2) Viçosa do Ceará, CE; (3) Nísia Floresta, RN; (4) Palmas, TO; (5) Teresina de Goiás, GO (Chapada dos Veadeiros); (6) Alto Paraíso de Goiás, GO (Chapada dos Veadeiros); (7) Buritis, MG; (8) Botumirim, MG; (9) Buritizeiro, MG; (10) São Roque de Minas, MG (Serra da Canastra National Park); (11) Brumadinho, MG; (12) Itabirito, MG; (13) Mariana, MG; (14) Lavras Novas, MG; (15) Ouro Branco, MG; (16) São João del-Rei, MG; (17) Alpinópolis, MG; (18) Poços de Caldas, MG; (19) Rio Claro, SP; (20) Campinas, SP; (21) Sacramento, MG; (22) Uberlândia, MG; (23) Caldas Novas, GO; (24) Cristalina, GO; (25) Chapada dos Guimarães, MT; (26) Cuiabá, MT; (27) Vale de São Domingos, MT; and (28) Vila Bela da Santíssima Trindade, MT. Brazilian states: CE: Ceará; GO: Goiás; MA: Maranhão; MG: Minas Gerais; MT: Mato Grosso; SP: São Paulo; RN: Rio Grande do Norte; and TO: Tocantins. DF: Distrito Federal [Federal District]. Data from the present study combined with previous studies: Toledo et al. (2010), Toledo (2010), Pansonato et al. (2013, 2014), Andrade et al. (2016a), and Andrade et al. (2016b).

Goiás using a Zeiss Stemi 2000 stereo microscope coupled to an ocular micrometer, except snout-vent length (SVL), which was taken with a Mitutoyo Absolute Digital Caliper (to the nearest 0.1 mm) using a stereo microscope. Morphometric traits were measured according to Watters et al. (2016): SVL, head length (HL), head width (HW), eye diameter (ED), snout length (SL), internarial distance (IND), hand length (HAL), thigh length (TL), tibia length (or shank length, TBL), and foot length (FL). Snout shape was measured according to Heyer et al. (1990).

For comparisons with the other long-legged species, we used the dataset provided in the description of *P. Jaredi* (Andrade et al. 2016a) with some additions: 20 topotypic adult males of *P. saltica* from Chapada dos Guimarães, State of Mato Grosso, Brazil; nine individuals from the type series (adult males) of *P. murundu*, 11 adult males from the Serra da Moeda, Brumadinho, State of Minas Gerais, Brazil; and 19 topotypic adult males of *P. Jaredi*, in addition to 14

adult males of *P. Jaredi* from Nísia Floresta, State of Rio Grande do Norte, Brazil (Appendix A).

Bioacoustics

All recordings (.WAV format) generated for this study were obtained with the following digital recorders: Marantz PMD 671 (Marantz, Japan) and Boss BR-864 (Roland Corporation, USA), both coupled to Sennheiser ME67/K6 directional microphone (Sennheiser electronic GmbH & Co. KG, Germany); and M-audio Microtrack II (M-audio, USA) and Marantz PMD 661 MKII (Marantz, Japan), both coupled to Sennheiser ME66/K6. Recorders were set at a sampling rate of 44.1 or 48 kHz and a resolution of 16 bits. Sound files are deposited in *Arquivo Sonoro da Coleção de Anuros da Universidade Federal de Uberlândia* [Sound Archive of anurans of Universidade Federal de Uberlândia] at UFU, and in *Fonoteca Neotropical Jacques Vielliard* [Sound Archive] (FNJV) at Unicamp (Table S1).

We analyzed calls using Raven Pro 1.5 software, version 64-bit (Bioacoustics Research Program 2014) with the following settings: window type = Hann, window size = 256 samples, 3 dB filter bandwidth = 248 Hz, brightness = 50%, contrast = 50%, overlap = 85% (locked), DFT size = 1024 samples (locked), and grid spacing (spectral resolution) = 43.1 Hz. Call duration is the time that males spend emitting the series of pulsed notes. Temporal traits were measured in oscillograms, and the spectral ones were measured in spectrograms. Raven obtained the peaks of dominant frequency through its 'Peak Frequency (Hz)' function. Call figures were generated using the Seewave v.1.6 package (Sueur et al. 2008) in the R version 3.6.0 64-bit (R Core Team 2019). Seewave settings were: Hanning window, 90% overlap, and 512 points resolution (FFT). Pulse terminology is in accordance with Magalhães et al. (2014), and overall acoustic terminology agrees with Köhler et al. (2017), in a note-centered approach. We calculated means and standard deviations considering mean values of individual males, whereas the range (variation) encompasses the minimum and maximum values for all call samples.

We recorded 19 long-legged males from Chapada dos Veadeiros highlands: 11 males from Alto Paraíso de Goiás and 8 from Teresina de Goiás. For acoustic comparisons of *Pseudopaludicola* long-legged species, we also used the dataset of Andrade et al. (2016a). Therefore, we analyzed six topotypic males of *P. murundu*, nine males of *P. saltica* from Uberlândia, State of Minas Gerais, and eight males of *P. jaredi* from its type locality and Nísia Floresta, State of Rio Grande do Norte, all located in Brazil. We also reanalyzed the original recordings from the species description of *P. murundu* (FNJV 12876; Toledo et al. 2010), one recording from the type locality (LH 676, Pansonato et al. 2014), and four recordings from São João del-Rei, State of Minas Gerais, Brazil (FNJV 12877–80; Toledo 2010). Regarding *P. saltica*, we reanalyzed three recordings (13A-01, 42A-06, LH-13) of topotypes described by Pansonato et al. (2013). Moreover, we analyzed novel data on *P. murundu* from Poços de Caldas (two males), São Roque de Minas (three males from Serra da Canastra National Park), Sacramento (one male), Buritis (one male), and Buritizeiro (two males), all these municipalities are in the State of Minas Gerais; *P. jaredi* from Palmas (three males), State of Tocantins; and *P. saltica* from Cristalina (four males) and Caldas Novas (three males), both in the State of Goiás (Figure 1), all located in Brazil. We also quantified the variability of the interpulse interval trait by its variance and the coefficient of variation [$CV = (SD/mean) \times 100$], and used the mean and variance in subsequent analyses (e.g., Discriminant Analysis) (see Andrade et al. 2016a). In Table S1 are presented further details on all new sound files analyzed.

We recorded calls of 37 adult males of *P. boliviana* from Brazil and Argentina, and analyzed 389 note groups and 1,600 notes. Further details on all recordings such as date, time, recorder, microphone, air temperature, and voucher specimen are presented in Table S1. Eight males were recorded in Argentina: seven from Corrientes, Capital Department, Corrientes Province (27°25'47"S, 58°42'36"W, 62 m a.s.l., datum = WGS84); and one from La Leonesa, Bermejo Department, Chaco Province (27°5'30"S, 58°45'10"W, 59 m a.s.l., datum = WGS84). Nineteen males were recorded around the Environmental Protection Area of Curiaú River, located in the municipality of Macapá, State of Amapá, Brazil (0°9'0.22"N, 51°2'18.33"W, 6 m a.s.l., datum = WGS84). Ten males were recorded in the Rancho do Papito, municipality of Cantá, State of Roraima, Brazil (2°45'31.75"N, 60°36'18.89"W, 75 m a.s.l., datum = WGS84) (Figure 2). Recordings of three males from Bolivia were provided by Ignácio de La Riva and Steffen Reichle by a request via *Fonoteca Zoológica* [Zoology Sound Archives] (FZ) of the *Museo Nacional de Ciencias Naturales de Madrid*. Two of these males were recorded in Santa Cruz de la Sierra, Santa Cruz; the other is from Reserva de la Biosfera Estación Biológica del Beni, San Borja.

For *P. boliviana* data, the variability of notes per minute trait was verified using between-male coefficient of variation (sensu Gerhardt 1991). We measured the within-male call variation by the coefficients of variation [$CV = (SD/mean) \times 100$] and calculated the average for each trait from individual male CV values. Acoustic traits with low within-male CV were classified as static; and traits with higher values, as dynamic (sensu Gerhardt 1991). Considering the call complexity of the species, some definitions are necessary. For our analysis, the advertisement call was considered as the total time males spent emitting a series of multi-pulsed note groups. These series can be spaced by intervals of silence, which were called interseries intervals. The multi-pulsed notes are consistently emitted in groups of 3–5 notes (= note groups) spaced by silence intervals, which were called intergroup intervals. Concatenated pulses are those in which no silence interval (100% amplitude modulation) can be recognized between them (Magalhães et al. 2014).

Statistical analysis

Considering the morphometric and acoustic datasets separately, we sought for discrimination among long-legged species by applying the DAPC function (Discriminant Analysis on Principal Components, version 2.1.1, *adegenet* package; Jombart 2008; Jombart &

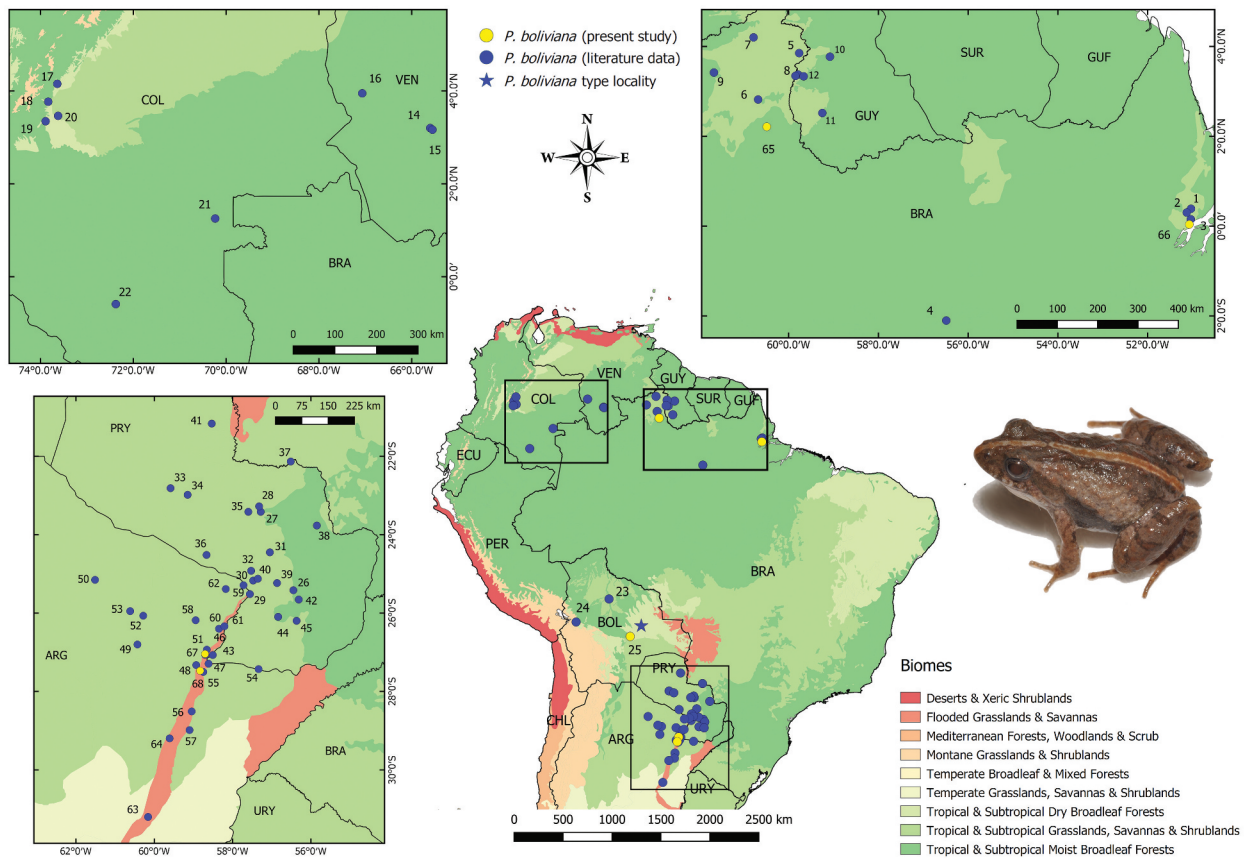


Figure 2. Parts of the South America map highlighting the biomes, known distribution, and our records of *Pseudopaludicola boliviana*. Inset: an adult male (AAG-UFU 5553) from Cantá, Roraima, Brazil. Localities: BRAZIL (BRA): State of Amapá: 1) *Campo Experimental de Cerrado* [Cerrado Experimental Field], Brazilian Agricultural Research Corporation (EMBRAPA), Macapá; 2) Distrito de Ariri, Macapá; 3) floodplains of Curiaú River, Macapá; State of Pará: 4) Terra Santa; State of Roraima: 5) Caracaranã; 6) Boa Vista; 7) Surumu; 8) Bonfim; 9) Ilha de Maracá. GUYANA (GUY): 10) Savanas de Rupununi; 11) Aishalton; 12) Moco-Moco Village; 13) Karanambu Lodge, Lethem. VENEZUELA (VEN): 14) Amazonas; 15) La Esmeralda, Amazonas; 16) Santa Bárbara, Amazonas. COLOMBIA (COL): Meta Department: 17) Villavicencio, 18) Cubarral, 19) San Juan de Arama, 20) Fuente de Oro; Vaúpes Department: 21) Mitú; 22) Araracuara, Amazonas. BOLIVIA (BOL): 23) El Beni; 24) La Paz; 25) Santa Cruz de la Sierra. PARAGUAY (PRY): Caaguazú Department: 26) Coronel Oviedo; Concepción Department: 27) Concepción, 28) Loreto; Central Department: 29) Villeta, 30) Estancia Surubi'i; San Pedro Department: 31) Villa del Rosario; Presidente Hayes Department: 32) Estancia Santa María de la Doce, 33) Estancia Fortín Salazar, Irala Fernandez district, 34) Estancia La Victoria, 35) Estancia Palo Santo, 36) Estancia la Golondrina; Amambay Department: 37) Bella Vista, 38) Estancia San Juan; Cordillera Department: 39) Estancia Saladillo, Caraguatay, 40) Cerro Vy, Emboscada district; Alto Paraguay Department: 41) Laguna General Díaz; Guairá Department: 42) Natalicio Talavera; Ñeembucú Department: 43) Estancia San José; Paraguari Department: 44) Parque Nacional Ybycuí; Caazapá Department: 45) Caazapá Town. ARGENTINA (ARG): Chaco Province: 46) Gral. Vedia, 47) Isla del Cerrito, 48) Reserva Natural Educativa Colonia Benítez, 49) Presidencia Roque Sáenz Peña, 50) General Güemes, 51) Las Palmas, 52) La Sardina, 53) Castelli; Corrientes Province: 54) Ita-Ibaté, 55) Corrientes, 56) Bella Vista (Playa Carrizal), 57) Santa Lucía; Formosa Province: 58) Reserva El Bagual, 59) Clorinda, 60) Tatané, 61) Trés Marias, 62) Frontera; Santa Fé Province: 63) Cayastá, 64) General Obligado (Reconquista). Our sampled localities: BRAZIL: State of Roraima: 65) Cantá; State of Amapá: 66) Macapá; ARGENTINA: Chaco Province: 67) La Leonesa; Corrientes Province: 68) Corrientes. The aforementioned points were obtained from the following literature: Parker (1935), Hoogmoed (1979), Lynch (1989), Lobo (1990, 1992, 1994), Reichle (1997), Reichle and Köhler (1998), De la Riva et al. (2000), Duré et al. (2004), Alcalde and Williams (2004), Brusquetti and Lavilla (2006), Cardozo and Lobo (2009), Cole et al. (2013), Pansonato et al. (2016), Cardozo et al. (2016), Lavilla et al. (2016), Costa-Campos et al. (2016).

Ahmed 2011). The DAPC performs analyses of the Principal Component scores (Jombart 2008). The application of a Discriminant Analysis (DA) to a few axes (preserving about 95% of the variance) of a Principal Component Analysis, as performed by DAPC, reduces the imbalance between objects and

traits (Jombart et al. 2010). The direct or indirect packages for discriminant analyses were run in R (R Core Team 2019). DAPC was the method applied in the last study on long-legged species (see Andrade et al. 2016a); therefore, we prefer to use the same analysis to make comparisons between both studies.

For the multivariate and statistical analyses of the long-legged species, we used all the morphometric features previously detailed and 23 adult males from Alto Paraíso de Goiás and Teresina de Goiás. For the acoustic analyses we used pulse duration, interpulse interval, interpulse interval variance, pulses per second, pulses per note, note duration, internote interval, notes per minute, and peak of dominant frequency. Acoustic and morphometric traits were tested for statistically significant differences between the population from Chapada dos Veadeiros and the long-legged species by the ‘Exact Wilcoxon Mann Whitney Rank Sum Test’, with the `wilcox_test` function implemented in the `Coin` package (Resampling Statistics model; Hothorn et al. 2008) in R. We tested the significance of differences among the variances of the interpulse interval of the three long-legged species with the Permutational Bartlett’s test of homogeneity of variances (‘`perm.bartlett.test`’ function) using the `RVAideMemoire` package (Hervé 2015). This function performs a permutational Bartlett’s test of homogeneity of k variances. Since these tests were done between species pairs, significance levels (P) were adjusted considering the number of pairs by the method of Holm (`p.adjust` function using `stats` package; R Core Team 2019). We considered significance when $P \leq 0.01$.

For *Pseudopaludicola boliviana*, we sought for call discrimination among populations by applying the randomForest (RF) model (`randomForest` v. 4.6–12 package; Liaw & Wiener 2002). Details on the randomForest algorithm and the package we used are in Breiman (2001) and Liaw and Wiener (2002). The RF results include an estimate of distances among the objects that are submitted to a Multidimensional Scaling (MDS) analysis by the ‘`rfPermute`’ package version 2.1.5 (Archer 2016), whose results can be plotted with the ‘`proximityPlot`’ function. All analyses were conducted in R. For the multivariate analysis and statistical tests, we used duration of note groups, intergroup intervals, number of notes per group, note duration, internote interval, number of pulses per note, pulse duration, pulse rate, note rate, peak of dominant frequency, minimum, and maximum of dominant frequency. We tested for significant differences in population medians through the Exact Wilcoxon Mann Whitney Rank Sum Test.

Phylogenetic inference and genetic distances for the *P. saltica* species group

We generated new sequences data for 17 long-legged specimens. They were from Alto Paraíso de Goiás ($n = 5$) and Teresina de Goiás ($n = 2$), Chapada dos

Veadeiros highlands, Cristalina ($n = 3$), and Caldas Novas ($n = 1$); all these localities in the state of Goiás. From Buritis ($n = 2$), Buritizeiro ($n = 1$), Parque Nacional Serra da Canastra, São Roque de Minas ($n = 1$), and Poços de Caldas ($n = 1$), these last four localities located in the State of Minas Gerais. And one from Palmas, state of Tocantins (Table S2).

We extracted total DNA and amplified a mitochondrial fragment including the partial sequences of 12S rRNA, tRNA-val and 16S rRNA genes (12S-tval-16S) or partial 16S following procedures described in Andrade et al. (2020). PCR products were purified by enzymatic reaction and sent to Macrogen Inc. Seoul, Republic of Korea, for sequencing. DNA sequences were submitted to quality trimming and assembled using Geneious R11 (Biomatter).

For the phylogenetic inference, we aligned the newly generated sequences with the sequences available from GenBank for *Pseudopaludicola* spp., including five outgroups (Table S2) in MAFFT v. 7.25 using E-INS-I strategy (Katoh & Standley 2013). We performed maximum likelihood analysis in RAXML software, v. 8.2.10 (Stamatakis 2014), using GTRCAT approximation and searching for the most likely tree 100 times. Then we estimated node support with 1,000 non-parametric bootstrap replicates. The consensus tree was edited in FigTree v. 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree>).

The average genetic distances within and between species were calculated for the 16S fragment flanked by the primers 16Sar-L and 16Sbr-H (Kessing et al. 1989), since this fragment was available for all samples included in the analyses. Estimates were conducted using the alignment generated in MAFFT v.7.25 imported in MEGA v.6 (Tamura et al. 2013), using uncorrected p -distance with pairwise deletion (ignoring any position with gaps).

Results

The specific status of the long-legged population of *Pseudopaludicola* from Chapada dos Veadeiros

We found the males calling in wet grassland (grasses < 20 cm tall) on soft and rocky substrates where water flows superficially during the rainy season. Specimens were found in natural and human-disturbed sites such as areas of cattle grassing. Males called from late afternoon to early evening and showed marked intraspecific variation in color patterns, size, and distribution of the enlarged dorsal tubercles (Figure 3). *Adenomera saci* Carvalho & Giaretta, 2013 was sharing the same habitat.

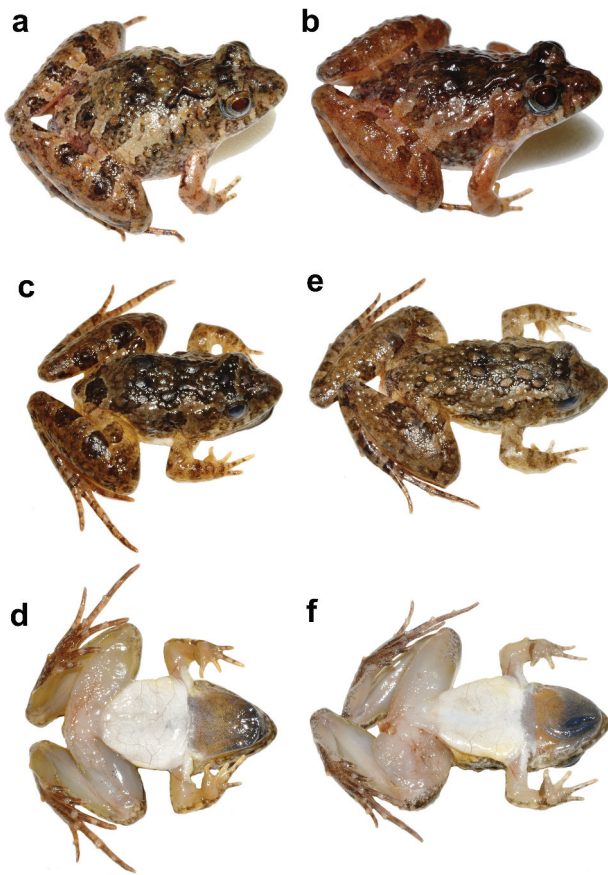


Figure 3. Adult males of *Pseudopaludicola murundu* from Chapada dos Veadeiros, State of Goiás, in life. AAG-UFU 3332, SVL = 16.6 mm (A); AAG-UFU 0785, SVL = 16.3 mm (B); dorsal and ventral views of the AAG-UFU 5098, SVL = 14.9 mm (C-D); dorsal and views of the AAG-UFU 5099, SVL = 15.2 mm (E-F). Note the marked intraspecific variation in color patterns and that of the enlarged tubercles on the back of the males.

We found morphometric differences between this population and the other three long-legged species (Exact Wilcoxon-Mann-Whitney Test: $P \leq 0.01$). The specimens of this population have shorter thigh, tibia, and foot lengths, and smaller eye diameter and head length compared to *P. saltica*. This population also differs from *P. murundu* for its larger SVL and head width, and from *P. jaredi* by having a shorter head and different snout and thigh lengths. The scatter plot of the DAPC showed a partial overlap among all clusters (Figure 4A).

The advertisement call of specimens from Chapada dos Veadeiros consists of a long series of pulsed notes (4–78 s). Notes vary from 47 to 146 ms in duration separated by intervals of 108–666 ms, and are emitted at a rate of 180–270 notes per minute; notes have a slightly increase in amplitude from the first to the second/third pulse (= midpoint of note), followed by a decrease in amplitude in the last pulses (Figure 5A).

Notes also have a slightly ascending frequency modulation over their duration (Figure 5A). Notes are composed of 2–6 non-concatenated pulses. Pulses last 3–27 ms, separated by intervals of 1–44 ms ($CV = 42.5 \pm 31.2$; 12.8–118.5) and are released at a rate of 34.7–50.4 pulses per second. Peaks of dominant frequency are between 5.0 and 6.4 kHz. Another weak frequency band can be recognized at a higher frequency, peaking between 9.8 and 12.6 kHz (11.1 ± 0.6 kHz; Figure 5A). In Table 1 we summarize the quantitative acoustic traits, and in Figure 5 we show the call figures of *P. murundu* from its type locality, *P. saltica*, and *P. jaredi*.

When comparing calls of this population with the three long-legged species, we found a difference in the internote intervals. We found differences with *P. saltica* in the variance of the interpulse interval, notes per minute, and the peak of the dominant frequency. With *P. jaredi*, the differences were in the notes per minute trait. We observed broad overlaps of the raw data of all analyzed variables of this population and those of *P. murundu*, *P. jaredi*, and *P. saltica* (Table 1). By the DAPC multivariate approach to acoustic dataset, no noticeable discrimination between the population of Chapada dos Veadeiros and *P. murundu* from other localities was yielded (Figure 4B). In contrast, we also found substantial differentiation among *P. murundu*, *P. saltica*, and *P. jaredi* (Figure 4B) by the DAPC, with separation along linear discriminant axis 1 (LD1) = 33%, whereas axis 2 (LD2) accounted for 27%. Peak of dominant frequency (49%), variance of the interpulse interval (15%), notes per minute (11%), and pulses per note (10%) mainly accounted for species separation along LD1 (Figure 4B). The variance of the interpulse interval (34%), notes per minute (29%), pulses per second (10%), and note duration (10%) mainly accounted for species separation along LD2 (Figure 4B).

The specimens from Chapada dos Veadeiros were assigned to *P. murundu* in the topology of the mitochondrial phylogeny (Figure 6), together with specimens of this same species from other sites. In addition, we found a low intraspecific genetic distance in *P. murundu* (0.9%), *P. saltica* (0.3%), and *P. jaredi* (0.1%) (Table 2). *Pseudopaludicola murundu* is the best taxonomic identity for the specimens from Chapada dos Veadeiros, based on their phylogenetic position, intraspecific genetic distances, and the absence of acoustic differentiation. We were unable to completely understand the relationships among the three long-legged species due to the low bootstrap supports of the nodes.

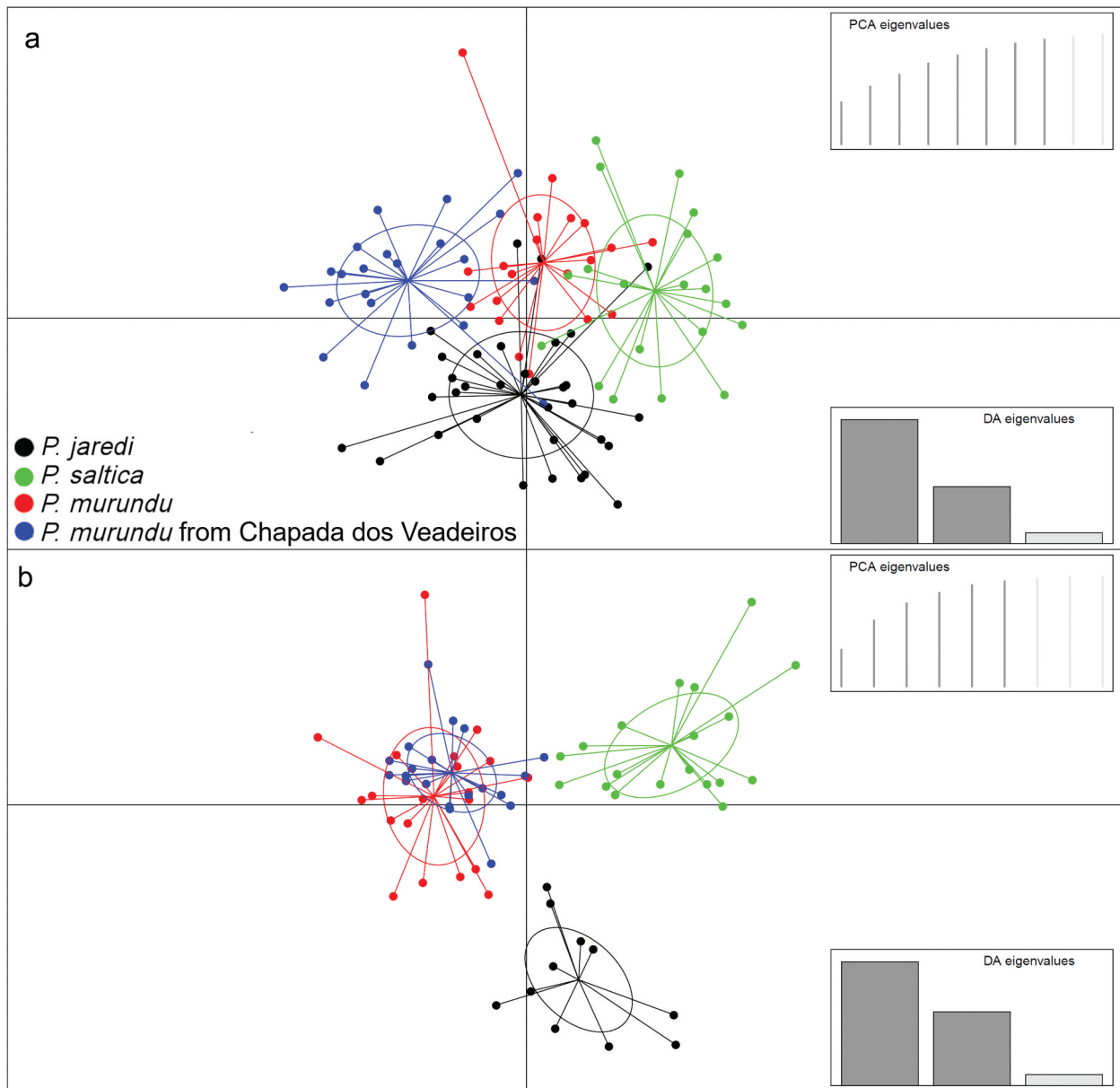


Figure 4. Scatterplot of the discriminant analysis of principal components (DAPC) in the morphometric (A) and acoustic (B) datasets of long-legged species of *Pseudopaludicola*: *P. murundu* from Chapada dos Veadeiros (blue), *P. murundu* (red), *P. jaredi* (black), and *P. saltica* (green). The two first axes in the morphometric data (8 first Principal Components [PCs], 95% retained variance); variance explained by the axes: LD1 = 38% (F-statistic = 49.0) and LD2 = 14% (F-statistic = 22.6) (A). The two first axes in the acoustic data (6 first PCs, 95% retained variance). LD1 = 33% (F-statistic = 75.4) and LD2 = 27% (F-statistic = 45.1) (B).

By adding more calls to former comparisons, and already considering the population from Chapada dos Veadeiros highlands as *P. murundu*, we were able to improve the characterizations of *P. murundu*, *P. jaredi*, and *P. saltica*. We found differences among all pair comparisons of these species in the peak of the dominant frequency and the variance of the interpulse interval traits. The internote interval and notes per minute differed between *P. saltica* vs. *P. murundu* and *P. jaredi* vs. *P. murundu*. In addition, we found a difference in the pulse duration between *P. saltica* and *P. murundu*.

New distributional data on three species from the *P. saltica* species group

Based on genetic information, we expanded the geographic distribution of *P. saltica*, *P. murundu*, and *P. jaredi*. The new records of *P. saltica* were the municipalities of Cristalina (about 880 km east of the type locality) and Caldas Novas (about 800 km southeast of the type locality), both in the State of Goiás. *Pseudopaludicola murundu* also occurs in the municipalities of Alto Paraiso de Goiás and Teresina de Goiás

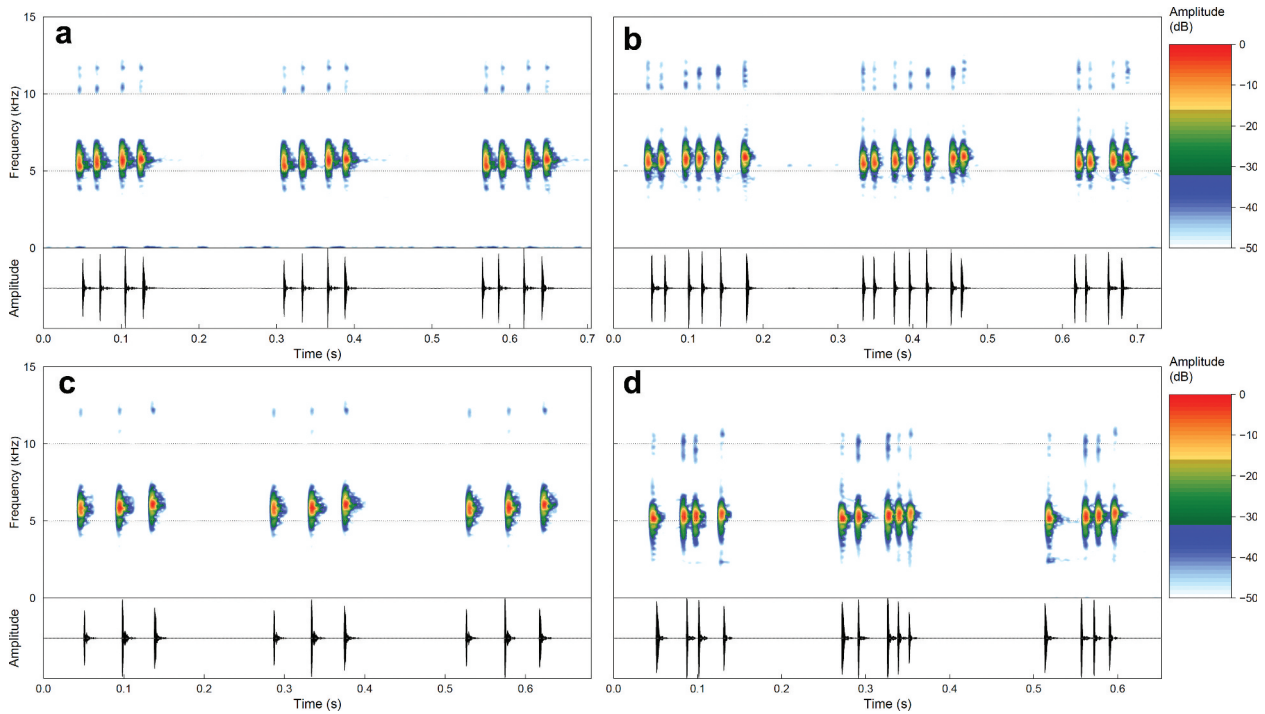


Figure 5. Audiospectrograms (above) and corresponding oscillograms (below) detailing notes (three notes with non-concatenated pulses) of the advertisement calls of the three species from the *Pseudopaludicola saltica* species group. (A) *P. murundu* from Chapada dos Veadeiros highlands, Goiás; (B) *P. jaredi* from FLONA de Nísia Floresta (National Forest), Nísia Floresta, Rio Grande do Norte; (C) *P. murundu* from its type locality, Rio Claro, São Paulo; and (D) *P. saltica* from Uberlândia, Minas Gerais. Sound files: A – Pseudop_murunduVeadeirosGO15dAAGm671; voucher AAG-UFU 3344 recorded on 29 November 2013 at 7:23 p.m.; air temperature of 23°C; B – ASUFRN236; voucher ZUEC 21860 recorded on 3 July 2013 around 8 p.m.; air temperature of 22°C; C – Pseudop_murunduRioClaroSP3aAAGm671; male recorded on 8 March 2015 at 10:34 p.m.; air temperature of 24°C; D – Pseudop_salticUberlMG4bAAGm; voucher AAG-UFU 2308 recorded on 19 March 2011 at 8:44 p.m.; air temperature of 23°C.

Table 1. Advertisement call traits based on the recordings of the *Pseudopaludicola saltica* species group. Mean \pm standard deviation (minimum–maximum); n = number of recorded specimens (number of analyzed notes); CV = Coefficient of variation. * = Rio Claro (State of São Paulo; type locality), São João del-Rei, Poços de Caldas, São Roque de Minas (Serra da Canastra National Park), Sacramento, Buritis, and Buritizeiro (all located in the State of Minas Gerais).

Trait	<i>P. murundu</i>		<i>P. jaredi</i>	<i>P. saltica</i>
	Chapada dos Veadeiros	Other localities*		
	$n = 19$ (280)	$n = 21$ (226)	$n = 11$ (304)	$n = 19$ (190)
Call duration (s)	37.1 ± 20.2 (4.1–77.9)	11.3 ± 5.3 (5.7–19.5)	33.6 ± 35.1 (7.0–108.5)	45.4 ± 18.0 (28.8–74.6)
Note duration (s)	0.102 ± 0.009 (0.047–0.146)	0.089 ± 0.021 (0.054–0.135)	0.105 ± 0.024 (0.037–0.178)	0.092 ± 0.025 (0.030–0.144)
Internote interval (s)	0.195 ± 0.052 (0.108–0.666)	0.141 ± 0.041 (0.070–0.427)	0.113 ± 0.024 (0.058–0.184)	0.130 ± 0.021 (0.080–0.198)
Notes per minute	226.2 ± 23.9 (180.0–270.0)	280.9 ± 63.3 (180.0–420.0)	350.3 ± 99.4 (240.0–600.0)	302.0 ± 74.3 (180.0–480.0)
Pulse duration (s)	0.011 ± 0.004 (0.003–0.027)	0.011 ± 0.002 (0.006–0.021)	0.009 ± 0.002 (0.004–0.010)	0.009 ± 0.002 (0.003–0.015)
Interpulse interval (s)	0.016 ± 0.004 (0.001–0.044)	0.016 ± 0.006 (0.000–0.043)	0.021 ± 0.007 (0.001–0.047)	0.019 ± 0.009 (0.001–0.077)
CV interpulse interval	42.5 ± 31.2 (12.8–118.5)	17.3 ± 6.2 (6.0–28.9)	60.6 ± 34.9 (15.0–115.2)	19.6 ± 9.8 (9.0–41.6)
Pulses per second	42.9 ± 3.7 (34.7–50.4)	46.5 ± 11.8 (28.0–58.8)	42.7 ± 7.9 (27.5–81.1)	47.6 ± 11.8 (24.0–74.6)
Pulses per note	4.3 ± 0.4 (2.0–6.0)	3.9 ± 0.5 (3.0–6.0)	4.4 ± 1.0 (2.0–7.0)	4.2 ± 1.0 (2.0–6.0)
Peak of dominant frequency (kHz)	5.6 ± 0.3 (5.0–6.4)	5.8 ± 0.2 (5.3–6.1)	5.4 ± 0.2 (5.0–6.0)	5.0 ± 0.2 (4.5–5.5)
Air temperature (°C)	19–24	17–26	22–25	20–26



Figure 6. Phylogenetic relationships of the *Pseudopaludicola* species based on the mitochondrial fragment 12S-tVal-16S, highlighting the long-legged species: *P. jaredi* (purple), *P. saltica* (green), and *P. murundu* (red). Numbers near the nodes are the support values from a maximum likelihood bootstrap analysis. Black dots represent bootstrap = 100; support below species levels were not shown.

Table 2. Estimates of average uncorrected p-distances between and within (in bold) species of the *Pseudopaludicola saltica* group. Within species distances include standard error.

	1	2	3	4	5	6	7	8	9
1 <i>P. jaredi</i>	0.001 (0.001)								
2 <i>P. saltica</i>	0.025	0.003 (0.002)							
3 <i>P. murundu</i>	0.026	0.030	0.009 (0.003)						
4 <i>P. matuta</i>	0.072	0.071	0.065	0.001 (0.001)					
5 <i>P. mineira</i>	0.058	0.057	0.055	0.037	0 (0)				
6 <i>P. florencei</i>	0.070	0.067	0.061	0.077	0.050	0.001 (0.001)			
7 <i>P. restinga</i>	0.067	0.065	0.062	0.065	0.051	0.035	0.001 (0.001)		
8 <i>P. pocoto</i>	0.066	0.064	0.060	0.066	0.051	0.038	0.035	0.012 (0.003)	
9 <i>P. falcipes</i>	0.088	0.089	0.071	0.084	0.074	0.080	0.078	0.076	0.004 (0.002)

(Chapada dos Veadeiros; about 950 km north of the type locality), both in the State of Goiás; and in the municipalities of Buritis (about 760 km north of the type locality), Buritizeiro (about 610 km northeast of

the type locality), São Roque de Minas (Serra da Canastra National Park, about 270 km northeast of the type locality), and Poços de Caldas (about 120 km northeast of the type locality), all in the State of Minas

Geráis. Merely based on the dark coloration of vocal sacs with longitudinal folds and acoustic evidence, we identified specimens from a population from the municipality of Sacramento (about 290 km north of the type locality), State of Minas Gerais as *P. murundu* (for details on these analyzed specimens, see Appendix A). We did not have access to the genetic data on this population. Lastly, *P. jaredi* also occurs in the municipality of Palmas (about 1,000 km southwest of the type locality), State of Tocantins (Figure 1).

Calling sites and call of *P. boliviana*

We heard vocalizations and collected male specimens of *Pseudopaludicola boliviana* on grassland in disturbed areas in Brazil. Males started calling in the late afternoon and continued until around 9:30 p.m. In Cantá, State of Roraima, males called from sandy soil covered by low grasses (< 30 cm height), on the edge of a waterlogged area at an abandoned fishpond (no fish present) that was partially empty. They were also vocalizing amidst grasses under a water tank, which periodically overflowed, forming a constant shallow pool on the ground. In Macapá, State of Amapá, males were in an artificial open area around a shallow seasonal pond (dimensions: 10 × 10 m; depth of the pond: < 15 cm), with low and sparse grasses.

We recorded all the specimens from Argentina in partially urbanized areas. In La Leonesa (Chaco Province), the recorded individual was calling in an agricultural area, on the edge of a paddy field. In Corrientes (Corrientes Province), several individuals were recorded on the edges of flooded areas, among low grasses (< 5 cm height) and calling in syntopy with *P. falcipes*. Some individuals were calling at the shore of a wetland with flooded soil and amidst low vegetation, whereas other males were calling from sites more distant from this shore (distance of 100 m), on a drier soil and higher grasses (50–100 cm height). We found these last individuals associated with a narrow artificial water channel, calling at its edges or on its irregular walls.

The advertisement call of *P. boliviana* consists of a series of 6–255 groups of multi-pulsed notes (= note groups). These series last 0.8–73.6 s and are separated by intervals of 0.6–19.9 s (Figure 7A). The note groups last 53–118 ms, separated by intervals of 71–528 ms (the red line in Figure 7B represents a group of four multi-pulsed notes). These groups have 3–5 notes each (Figure 7C). The notes have a regular structure, duration, interval, and number of pulses. Notes last 7–25 ms, separated by intervals of 1–14 ms, and are released at a rate of 687–1289 notes per minute. Notes have 2–8 concatenated pulses each, these pulses last 1–10 ms; they are released at a rate

of 167–389 pulses per second (Figure 7B). Peaks of the dominant (= fundamental harmonic) frequency are between 4091 and 5512 Hz, the minimum frequency of the dominant band ranges between 3187 and 4996 Hz, and the maximum frequency of the dominant band ranges between 4350 and 5943 Hz. Notes have up to three harmonics; the second ranges between 7795 and 10,723 Hz. The air temperature of recorded calls varied from 20 to 30°C. In Table 3 we summarize call quantitative traits from the four studied populations.

The following traits were classified as static (within-male CV < 5%): duration of the note groups (mean = 3.3%, SD = 3.6 [0.6–13.3]), number of notes per group (mean = 2.2 %, SD = 4.1 [0.0–13.1]), dominant frequency (mean = 1.6%, SD = 0.4 [1.0–2.7]), minimum dominant frequency (mean = 1.5%, SD = 0.7 [0.6–4.4]), and maximum dominant frequency (mean = 1.7%, SD = 0.6 [0.9–3.9]). The other traits were classified as dynamic (CV < 9.0%). Additionally, the between-male coefficient of notes per minute was 22%, and such variability between males is sufficient to classify this trait as dynamic.

According to the randomForest analysis, there was an overlap among all four partitions, which was stronger among the Northern populations (Figure 8), with different degrees of classification error (Table 4). In addition, the raw values of all traits overlapped (Table 3). The only noticeable difference between Northern and Southern populations was in the intergroup intervals ($P < 0.01$), but the ranges of this variable also overlapped (Table 3).

Discussion

The new distribution records shown in this study help elucidate the distribution of the long-legged species of the genus *Pseudopaludicola*. To date, Teresina de Goiás (GO), in the Chapada dos Veadeiros highlands, is the northernmost location of the distribution of *P. murundu*; Cristalina, State of Goiás, is the easternmost location of the occurrence of *P. saltica*; and Uberlândia, State of Minas Gerais, its southernmost location. The recorded presence of *P. jaredi* in Palmas, State of Tocantins, expanded its distribution about 560 km southwest of Mirador, State of Maranhão, its westernmost previously known location (Andrade et al. 2016b). Therefore, these long-legged species have wide distributions throughout Brazilian open fields of the Atlantic Forest, Caatinga, Cerrado, and Pantanal biomes. There is no geographical distribution overlap among these long-legged species, and there are no syntopic reports of two or more of them so far. Future studies that provide novel genetic and acoustics data would

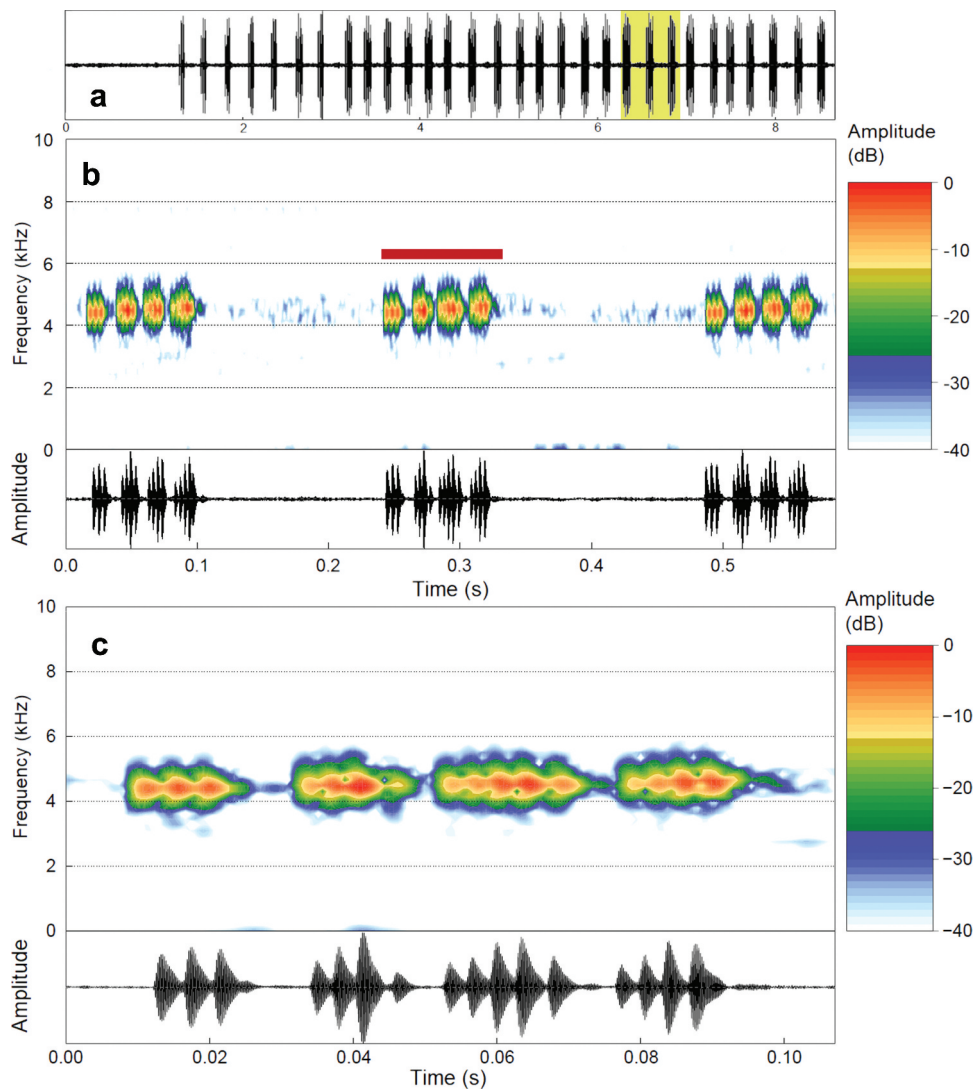


Figure 7. (A) Oscillogram of an entire advertisement call with a series of 30 groups of multi-pulsed notes of *Pseudopaludicola boliviana* from the municipality of Cantá, State of Roraima, Brazil. (B) Spectrogram (top) and corresponding oscillogram (below) of the stretch highlighted in yellow in (A), detailing three groups of multi-pulsed notes (= note groups); the red line represents a single group. (C) Spectrogram (top) and corresponding oscillogram of the note group highlighted in (B), detailing four multi-pulsed notes; notice the concatenated nature of the pulses (no intervals). The male was recorded on 24 July 2016 at 7:31 p.m.; air temperature of 27°C (label: Pseudop_bolivianaCantaRR6aAAGm671).

be valuable to further clarify the distributional limits of these three long-legged *Pseudopaludicola* species.

Toledo et al. (2010) suggested, as a morphological diagnostic character between *P. murundu* and *P. saltica*, the aspect of the nuptial pads on the outer part of the finger I of adult males, which are larger and brighter in *P. murundu*, and smaller and darker in *P. saltica*. Haddad and Cardoso (1987) pointed out that nuptial pads were more developed in specimens of *P. saltica* from the type locality than in long-legged specimens collected and recorded in localities of the states of Minas Gerais and São Paulo. These specimens from Minas Gerais and São Paulo mentioned by Haddad and Cardoso (1987) are currently assigned to

P. murundu (Toledo et al. 2010; Pansonato et al. 2014; Andrade et al. 2016a). We did not find differences in the nuptial pads of adult males of *P. saltica*, *P. murundu*, and *P. jaredi*. Therefore, nuptial pads are not a useful diagnostic morphological character for these species. Moreover, the observation of this character in preserved specimens might be influenced by fixation and preservation methods.

After pair comparisons between *P. jaredi* vs. *P. murundu* and *P. jaredi* vs. *P. saltica*, we found differences in the peak of dominant frequency, note duration, and variance of the interpulse interval (Andrade et al. 2016a). In addition, we reported differences between *P. jaredi* and *P. saltica* in the number of pulses per note

Table 3. Advertisement call traits from four populations of *Pseudopaludicola boliviana*. Mean \pm SD (minimum–maximum); n = number of recorded males (number of analyzed notes).

Traits	Bolivia $n = 3$ (112)	Argentina $n = 8$ (356)	Amapá, Brazil $n = 19$ (754)	Roraima, Brazil $n = 10$ (378)
Duration of note groups (ms)	87 \pm 13 (71–106)	85 \pm 17 (59–118)	84 \pm 4 (58–93)	76 \pm 6 (53–89)
Intergroup intervals (ms)	181 \pm 69 (103–347)	261 \pm 48 (163–528)	181 \pm 26 (71–312)	164 \pm 38 (90–369)
Number of note groups per call	25 \pm 20 (11–39)	47 \pm 29 (11–98)	130 \pm 72 (17–255)	48 \pm 31 (6–116)
Number of notes per group	4.0 \pm 0.0 (4–4)	4.8 \pm 0.4 (4–5)	4.0 \pm 0.1 (3–4)	3.9 \pm 0.1 (3–4)
Note duration (ms)	17 \pm 2 (9–23)	15 \pm 2 (8–24)	15 \pm 2 (8–23)	15 \pm 2 (7–25)
Internote interval (ms)	7 \pm 2 (2–12)	4 \pm 1 (1–8)	8 \pm 2 (3–14)	5 \pm 1 (1–12)
Notes per minute	1074 \pm 304 (723–1253)	845 \pm 107 (687–985)	843 \pm 209 (775–1137)	983 \pm 146 (863–1289)
Number of pulses per note	4.4 \pm 0.2 (3–6)	3.9 \pm 0.4 (2–7)	4.3 \pm 0.7 (2–8)	4.0 \pm 0.4 (2–7)
Pulse duration (ms)	4 \pm 1 (2–9)	4 \pm 1 (1–10)	4 \pm 0 (1–10)	4 \pm 0 (1–9)
Pulse rate	274 \pm 42 (190–364)	271 \pm 40 (167–389)	281 \pm 25 (187–389)	263 \pm 28 (176–389)
Dominant frequency (Hz)	4502 \pm 317 (4091–4996)	4913 \pm 249 (4436–5512)	4721 \pm 132 (4350–4996)	4587 \pm 256 (4134–5254)
Minimum dominant frequency (Hz)	3938 \pm 408 (3187–4565)	4504 \pm 216 (4134–4996)	4275 \pm 152 (3919–4608)	4247 \pm 243 (3790–4780)
Maximum dominant frequency (Hz)	4873 \pm 352 (4436–5814)	5295 \pm 286 (4823–5943)	5031 \pm 122 (4651–5340)	4886 \pm 266 (4350–5599)
Air temperature (°C)	-	20.1–30.0	26	27.0–30.0

(Andrade et al. 2016a). Our results corroborate the differences previously reported in the peak of the dominant frequency and in the variance of the interpulse interval among the calls. However, we were unable to find differences in the note duration between *P. jaredi* vs. *P. murundu* and *P. jaredi* vs. *P. saltica*. Furthermore, we did not find a difference in the number of pulses per note when comparing *P. jaredi* with *P. murundu*.

Acoustic data was helpful to delimit species in the *P. saltica* species group, but not in the case of the populations of *P. boliviana*. Although a slight difference was observed between the Argentine population and those from the northern distribution, our data were unable to definitely corroborate the one or two species hypotheses. We did not notice any quantitative difference between acoustic data presented by us and those from previous studies (Márquez et al. 1995; De la Riva et al. 2000; Duré et al. 2004; Pansonato et al. 2016; Costa-Campos et al. 2016); all acoustic variables compared overlapped in their amplitudes. However, there are conflicts in terminologies between our call characterization and those of previous studies (Márquez et al. 1995; Duré et al. 2004; Pansonato et al. 2016; Costa-Campos et al. 2016). In short, the authors of all these previous studies characterized the advertisement call of *P. boliviana* as consisting of a stereotyped structure of 2–5 multi-pulsed notes

spaced by roughly equal intervals. We called here these well-stereotyped structures of notes as ‘groups of multi-pulsed notes,’ actually the advertisement call of *P. boliviana* is composed of long series of these well-stereotyped note groups. Contrary to this disagreement of what would be considered as the advertisement call of *P. boliviana*, there is a consensus on what notes are within the call. In fact, the call structure of *P. boliviana* is unique to the genus. We believe that the terminology used here better describes its call, allowing interspecific comparisons, a homology-based nomenclature (Hepp & Pombal 2019), and the use of the note-centered approach of Köhler et al. (2017). In addition, it is worth pointing out that we were unable to notice any relevant quantitative difference between the populations sampled in this study, regardless of the broad geographic range under consideration.

De la Riva et al. (2000) suggested that the Northern (concerning the Amazon River) populations of *P. boliviana* might be considered a different species yet to be described. On the other hand, we found a lack of differentiation among the calls of all populations in our study. Myers and Donnelly (2001) suspected that there is another species under the name of *P. boliviana* in Southern Venezuela, considering that the possible unnamed species lacks the heel tubercle and seems to be a larger species than *P. llanera* or

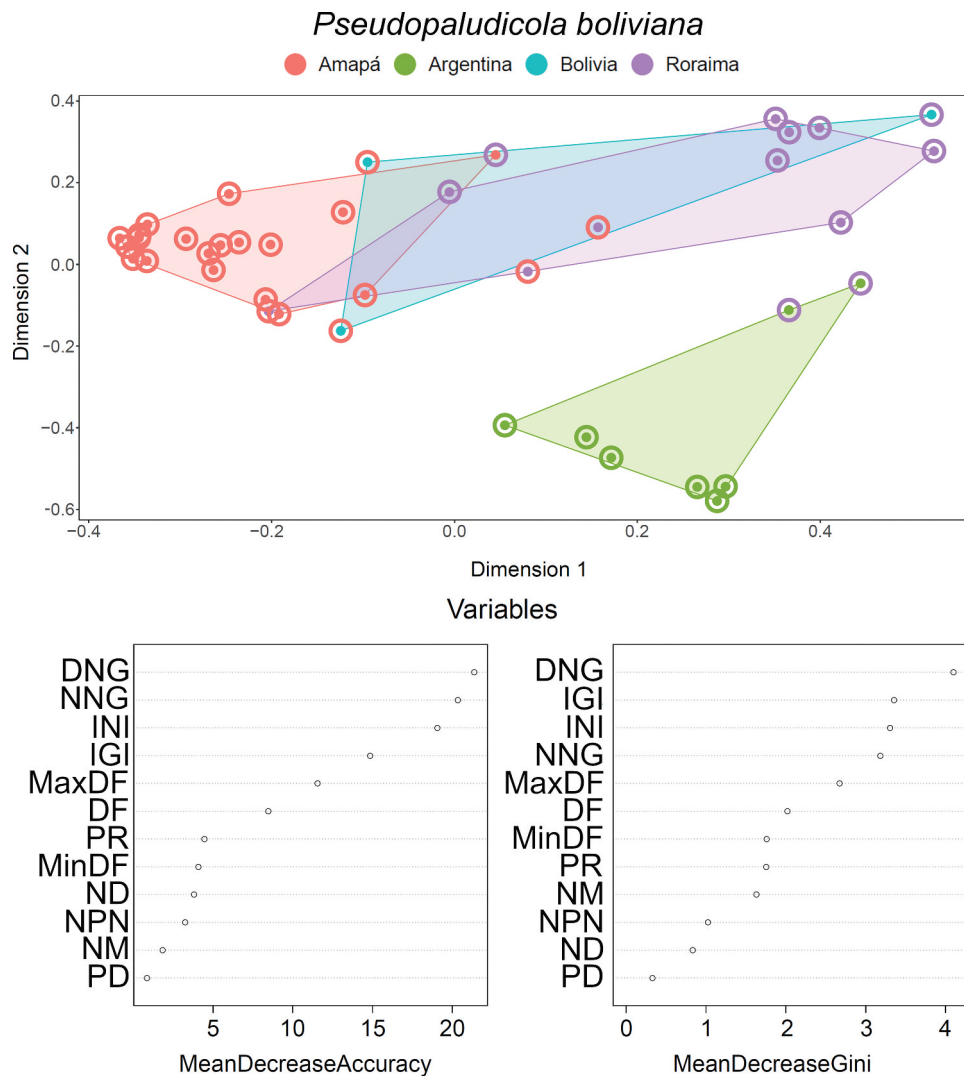


Figure 8. First and second dimensions of the multidimensional scaling on the proximity scores from the randomForest analysis considering acoustic traits from males of the four studied populations (top). Each dot represents an analyzed male. Dotcharts of variable importance score as indicated by the randomForest analysis (below). Abbreviations: DNG = duration of note groups; IGI = intergroup intervals; NNG = number of notes per group; ND = note duration; INI = internote intervals; NM = notes per minute; NPN = number of pulses per note; PD = pulse duration; PR = pulse rate; DF = dominant frequency; MinDF = Minimum dominant frequency; MaxDF = Maximum dominant frequency.

Table 4. Confusion matrix for all adult males analyzed from the four populations of *Pseudopaludicola boliviana*, based on acoustic data, by a randomForest model. Settings: number of tree permutations = 1,000; number of variables tried at each split = 3.0; error rate = 22.5%.

	Amapá, Brazil	Argentina	Bolivia	Roraima, Brazil	Class. error
Amapá, Brazil	18	0	0	1	0.05
Argentina	0	6	0	2	0.25
Bolivia	2	0	0	1	1.00
Roraima, Brazil	3	0	0	7	0.30

P. boliviana. Despite being aware of this morphological distinction, we suggest the need for integrative approaches in future taxonomic studies on these

Venezuelan populations. Integrative approaches have proved to be a good way to elucidate the richness of the genus *Pseudopaludicola* (e.g., Pansonato et al. 2016; Andrade et al. 2018a, 2018b, 2019, 2020; Cardozo et al. 2018). These approaches are important for the description of new species, but also for a better characterization of closely related ones (e.g., *P. ternetzi* and *P. ameghini*, Andrade et al. 2017; *P. boliviana* and *P. motorzinho*; Pansonato et al. 2016), as well as species with a putative wide geographic distribution that lack in-depth studies, which is the case of those within the *P. pusilla* species group.

Genetic information on *P. boliviana* is scarce; there are only sequences of mitochondrial DNA (mtDNA)

from two Argentine specimens (Veiga-Menoncello et al. 2014). We were unable to make clear taxonomic decisions due to the acoustic undifferentiation among the populations of *P. boliviana* sampled in our study and the lack of genetic and morphological data available, especially on specimens from the Bolivian populations. In addition, it is worth emphasizing the need to sample the entire accepted distribution of this species to avoid large geographical sampling gaps. Therefore, we recommend carrying out further genetic, morphological, and phylogeographic studies on *P. boliviana*, in agreement with previous suggestions (De la Riva et al. 2000; Myers & Donnelly 2001).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix A. Examined specimens

Pseudopaludicola jaredi: BRAZIL: CEARÁ: Viçosa do Ceará: CFBH 32609, 32614, 32,617–25, ZUEC 20477–84; Nisia Floresta: ZUEC 21858–72; TOCANTINS: Palmas: AAG-UFU 2743.

Pseudopaludicola murundu: BRAZIL: SÃO PAULO: Águas de Santa Bárbara: ZUEC 20507–8; Rio Claro: AAG-UFU 5125–6, CFBH 8235–42, ZUEC 14284–90; MINAS GERAIS: Brumadinho: ZUEC 16396–8, 16442–3, 19549, 19551, 19555, 19557–78, 19560; Buritis: AAG-UFU 1769–71; Buritizeiros: AAG-UFU 3361–4; Poços de Caldas: AAG-UFU 4134, 4382–3; Sacramento: AAG-UFU 4401–4405; Santana do Riacho: ZUEC 2323; São João del-Rei: ZUEC 16447–52, 16455–6; São Roque de Minas: AAG-UFU 0608, 0662; GOIÁS: Alto Paraíso de Goiás: AAG-UFU 0111, AAG-UFU 3323–4, AAG-UFU 3344, AAG-UFU 5098–101, ZUEC 24182–91; Teresina de Goiás: 24696–700.

Pseudopaludicola saltica: BRAZIL: MATO GROSSO: Chapada dos Guimarães: ZUEC 14228, 14230–3, 14235, 14239–40, 14244, 14247, 14272, 5134–46, 5854–5; MINAS GERAIS: Buritis: AAG-UFU 1769–71; Buritizeiro: AAG-UFU 3361–64, Uberlândia: AAG-UFU 2308, 2630, 4598, 4631, 4735, 4707–11; GOIÁS: Cristalina ZUEC 24664.