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A new species of long-legged *Pseudopaludicola* from northeastern Brazil (Anura, Leptodactylidae, Leiuperinae)

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Abstract. A recently published phylogeny corroborated the monophyly of the genus *Pseudopaludicola* and revealed several potential undescribed taxa. In this first integrative taxonomic study within the genus *Pseudopaludicola*, we describe the sister clade to the remaining long-legged species (*Pseudopaludicola saltica* + *Pseudopaludicola murundu*), the third recognized species of the monophyletic *P. saltica* clade, as a new species from northeastern Brazil. The new species is included in the *P. saltica* species group based on morphological (the presence of long hind limbs) and molecular evidence (mitochondrial genes). It is diagnosed by single, dark, subgular vocal sac with dark longitudinal folds in males, the presence of eleven pairs of chromosomes, and by an advertisement call composed of notes with up to seven non-concatenated pulses separated by regular interpulse intervals. We also describe the karyotype and tadpoles of the new species and compare them with the other long-legged species. Our populations are supported as an undescribed and independently evolving species within the *P. saltica* clade based on the generalized mixed Yule-coalescent (GMYC) species delimitation method. Although almost morphologically cryptic to *P. saltica* and *P. murundu*, this new species is distinguishable by means of acoustical and genetic traits.

Key words. Advertisement call, GMYC, integrative taxonomy, karyotype, *Pseudopaludicola saltica* clade, species delimitation.

Introduction

At present, the genus of dwarf swamp frogs, *Pseudopaludicola* Miranda-Ribeiro, 1926, includes 18 species (Frost 2015) that occur throughout South America, east of the Andes (Lynch 1989). *Pseudopaludicola* is recognized as monophyletic based on osteological (Lobo 1995) and external morphological features, such as the presence of a hypertrophied antebrachial tubercle (Lynch 1989, Lobo 1995), as well as by molecular evidence based on mitochondrial DNA (Veiga-Menoncello et al. 2014). Lynch (1989) proposed the *Pseudo-*

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paludicola pusilla (Ruthven, 1916) species group, including *P. boliviana* Parker, 1927, *P. ceratophyes* Rivero & Serna, 1985, *P. llanera* Lynch, 1989, and *P. pusilla*, all of which share the presence of T-shaped terminal phalanges. In subsequent analyses, Lobo (1995) and Veiga-Menoncello et al. (2014) corroborated the monophyly of the *P. pusilla* group. Cardozo & Suárez (2012) withdrew *P. canga* Giaretta & Kokubum, 2003 from the *P. pusilla* group. Cardozo & Toledo (2013), based on the lack of differentiation in both advertisement call and morphology, placed *P. riopiedadensis* Mercadal de Barrio & Barrio, 1994 as a junior synonym of *P. ternetzi* Miranda-Ribeiro, 1937. Pansonato et al. (2014a) considered *P. serrana* Toledo, 2010 a junior synonym of *P. murundu* Toledo, Siqueira, Duarte, Veiga-Menoncello, Recco-Pimentel & Haddad, 2010. Carvalho et al. (2015) revisited the diagnoses of the species with trilled advertisement calls (*P. canga*; *P. hyleaustralis* Pansonato, Morais, Ávila, Kawashita-Ribeiro, Strussmann & Martins, 2012; *P. facureae* Andrade & Carvalho, 2013; and *P. parnaiba* Roberto, Cardozo & Ávila, 2013) and found that *P. canga* and *P. parnaiba* could not be distinguished from each other by morphology/morphometric, colour pattern, and bioacoustics traits.

Although advertisement call features have provided information relevant to the intrageneric taxonomy of *Pseudopaludicola* (as mentioned above), integrative taxonomic studies including molecular evidence are still scarce for the genus, even though these are equally important for describing biological diversity (Glaw et al. 2010, Padial et al. 2010). Despite the considerable rise in number of species described in the past five years (6 species; Andrade & Carvalho 2013, Magalhães et al. 2014, Pansonato et al. 2014b, and references therein), a recent molecular phylogenetic analysis of *Pseudopaludicola* revealed several populations that may constitute undescribed species (Veiga-Menoncello et al. 2014), suggesting that the diversity within the genus is still underestimated. Such complex taxonomy and recent findings emphasize the need for a more thorough taxonomic study of the genus, using multiple sources of evidence.

Within Clade I (2n = 22 chromosomes), a subgroup of long-legged species can be recognized, the *P. saltica* (Cope, 1887) group (see Toledo 2010, Veiga-Menoncello et al. 2014), which includes *P. saltica*, *P. murundu*, and an additional species, *Pseudopaludicola* sp. (aff. *saltica*), described herein. This species (sister to *P. saltica* + *P. murundu*) was considered undescribed by Veiga-Menoncello et al. (2014), and is restricted to northeastern Brazil, while *P. saltica* and *P. murundu* are distributed in central and southeastern Brazil (Toledo 2010, Toledo et al. 2010, Pansonato et al. 2014a). Based on adult and larval morphology, advertisement call, molecular data, and chromosome morphology, we herein describe the third species of the *P. saltica* group from northeastern Brazil.

Material and methods

Reference specimens

Specimens (adults and tadpoles) of the type series of the new species were collected and recorded in the Serra das Flores, municipality of Viçosa do Ceará, state of Ceará (CE), Brazil (03°23'07" S, 41°09'29" W; 700 m above sea level [a.s.l.]; datum = WGS84) by D. Loebmann. Additional specimens (ZUEC 21858–72) were collected and recorded in the Floresta Nacional (FLONA) de Nísia Floresta, municipality of Nísia Floresta, state of Rio Grande do Norte (RN), Brazil (06°04'47.92" S, 35°10'57.22" W; 51 m a.s.l.; datum = WGS84) by F. M. de Magalhães, D. J. Santana & A. A. Garda (see Fig. 1). Reference specimens and tadpoles have been deposited in the Célio F. B. Haddad amphibian collection (CFBH) at the Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Rio Claro, São Paulo, Brazil, and the amphibian collection of the Museu de Zoologia "prof. Adão José Cardoso" (ZUEC), Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil. Specimens analysed from the Universidade Federal de Uberlândia frog collection, Uberlândia, Minas Gerais, Brazil, are referred to as AAG-UFU.

Morphometry

We measured morphometric characters of 19 adult males and two adult females from the type locality and 15 adult males and four adult females from FLONA de Nísia Floresta under a stereomicroscope Zeiss Stemi 2000 coupled to an ocular micrometer. Eight measurements follow Duellman (2001): snout–vent length (SVL), head length (HL), head width (HW), internarial distance (IND), snout–eye distance (SED) (snout length), eye diameter (ED), tibia length (SL) (shank length), and foot length (FL); three measurements follow Heyer et al. (1990): forearm length (FAL), hand length (HAL), and thigh length (THL). We measured the SVL of the adults with a Mitutoyo Absolute digital calliper (to the nearest 0.1 mm) under a stereomicroscope. For morphologic/morphometric comparisons, we also measured 20 adult males of *P. saltica* from the municipality of Chapada dos Guimarães (type locality), state of Mato Grosso and nine adult males of *P. murundu* from the district of Itapé (type locality), municipality of Rio Claro, state of São Paulo and 11 adult males from the Serra da Moeda, municipality of Brumadinho, state of Minas Gerais (see Appendix).

Bioacoustics

We recorded eight males and analysed a total of 24 advertisement calls and 224 notes for the new species. We recorded calls (one male) from Viçosa do Ceará (CE) with a Sony cassette tape recorder (TCM-150) coupled to a directional microphone Yoga® (HT 81 Boom) positioned

about 2 m from the calling male. Recordings were made between 19:00–21:00 h and digitised at 44.1 kHz and 16-bit resolution. We recorded vocalizations (seven males) from FLONA with a Tascam DR-40 digital recorder coupled to a Sennheiser ME66/K6 directional microphone. We analysed all calls with Raven Pro 1.5, 64-bit version (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 = 1,024 samples (locked); grid spacing (spectral resolution) = 43.1 Hz. Temporal traits were measured in the oscillogram, and spectral traits were measured in the spectrogram. Raven Pro 1.5 obtained the peaks of dominant frequency and other frequency bands automatically through its "Peak Frequency (Hz)" function. We generated call figures using the Seewave v.1.6 package (Sueur et al. 2008) in R version 3.0.2 (R Core Team 2014). Seewave settings were: Hanning window, 85% overlap, and 516 points resolution (FFT). Call duration is the time that males spend emitting a single series of pulsed notes. Note and pulse terminologies follow Magalhães et al. (2014), and overall acoustic terminology follows Duellman & Trueb (1994) and Toledo et al. (2015). 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. For each advertisement call, we analysed ten notes and all pulse/interpulse intervals that comprise these notes. Pulse rate was calculated as pulses per second. We noticed that within each note, the variability of the interpulse interval was higher in *P. saltica* and more constant in the other two species, therefore quantified this variability through the Coefficient of Variation [CV = (SD/mean) × 100], and used the mean and variance in subsequent analyses (e.g., Discriminant Analysis).

Additionally, for acoustic comparisons, we recorded six topotypic males of *P. murundu* on 08 March 2015 and nine males of *P. saltica* from Uberlândia, state of Minas Gerais, Brazil. We also reanalysed 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. 2014a), and four recordings from São João Del Rei, state of Minas Gerais (FNJV 12877–80, Toledo 2010); as far as *P. saltica* is concerned, we reanalysed three recordings (13A-01, 42A-06, LH-13) of topotypes described by Pansonato et al. (2013).

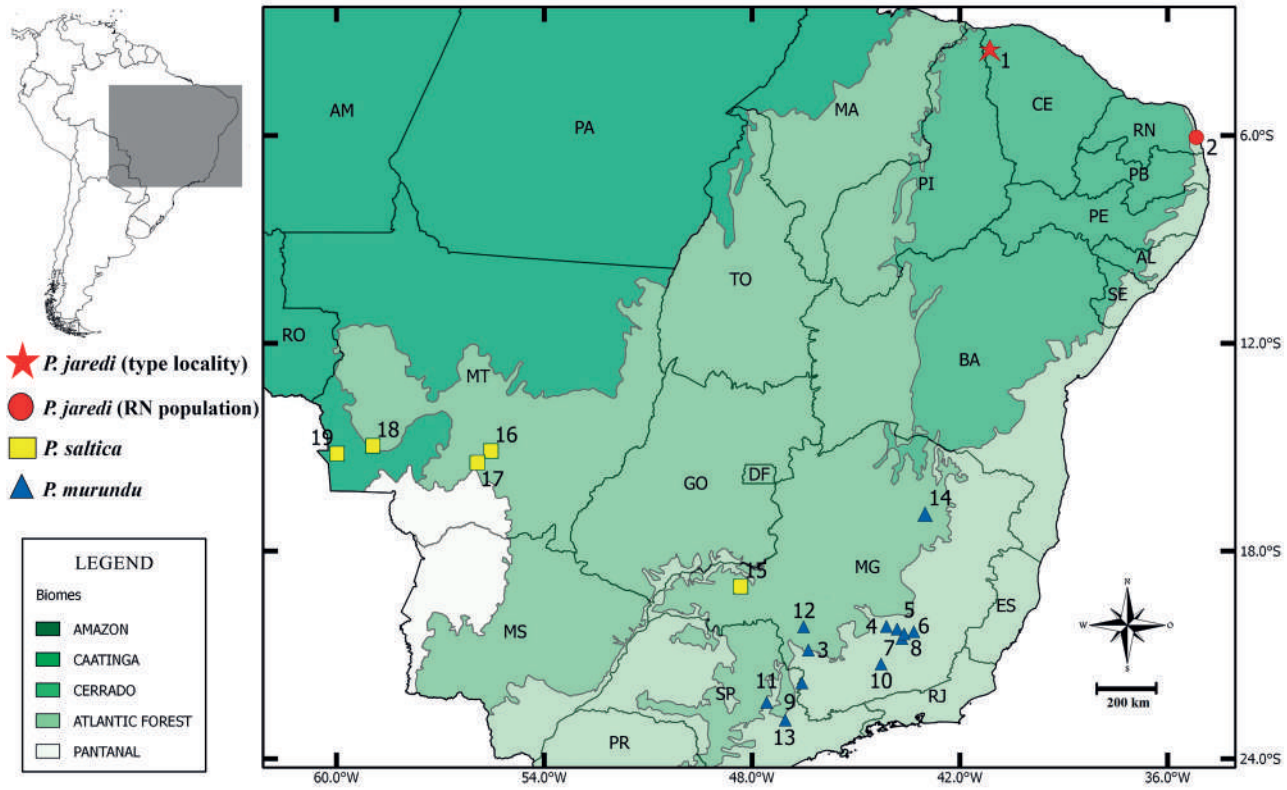

Figure 1. Geographic distribution of *Pseudopaludicola saltica* clade members (sensu Toledo et al. 2010, Toledo 2010, Pansonato et al. 2013, Pansonato et al. 2014a, and this work). Municipalities: 1 – Viçosa do Ceará (CE; type locality of new species); 2 – Nísia Floresta (RN); 3 – Alpinópolis (MG); 4 – Brumadinho (MG); 5 – Itabirito (MG); 6 – Mariana (MG); 7 – Ouro Branco (MG); 8 – Lavras Novas (MG); 9 – Poços de Caldas (MG); 10 – São João Del Rei (MG); 12 – São Roque de Minas (MG); 14 – Botumirim (MG); 11 – Rio Claro (SP; type locality of *P. murundu*); 13 – Campinas (SP); 15 – Uberlândia (MG); 16 – Chapada dos Guimarães (MT; type locality of *P. saltica*); 17 – Cuiabá (MT); 18 – Vale de São Domingos (MT); 19 – Vila Bela da Santíssima Trindade (MT). Brazilian States: RN – Rio Grande do Norte; CE – Ceará; MG – Minas Gerais; SP – São Paulo; and MT – Mato Grosso.

Voucher specimens for call recordings: *Pseudopaludicola* new species: ZUEC 21858–61; *P. murundu*: AAG-UFU 5126; and *P. saltica*: AAG-UFU 2308, 2612.

Tadpole morphology

We assigned the tadpoles to the new species of *Pseudopaludicola*, because the only other anuran species that reproduces in the pond they were found in was *Pleurodema diplolister* (Peters, 1870), which has very distinct larvae (e.g., oral disc with lateral folds and only one anterior gap around the entire oral disc in *P. diplolister* larvae; see Peixoto 1982). We measured eight specimens at Gosner's (1960) stages 27 to 29, and nine specimens at stages 35 and 37, respectively. The following measurements of tadpoles were taken according to Altig (2007): body length, tail length, maximum tail height, tail muscle height, tail muscle width, total length, oral disc width, internarial distance, and interorbital distance. The following additional measurements were taken according to Eterovick & Brandão (2001): eye diameter, nostril diameter, eye–nostril distance, and nostril–snout distance. The labial tooth row formula was composed as suggested by Altig & McDiarmid (1999). The terminology for the gap configuration in the oral disc marginal papillae follows Vera Candioti et al. (2011). Measurements of tadpoles were taken (to the nearest 0.01 mm) with a micrometric ocular coupled to an Olympus SZ40 stereomicroscope.

Statistical analysis

Considering the (multivariate) morphological and acoustic datasets, we discriminated between populations/species by applying two functions: (1) "randomforest" (RF) (randomForest package, Liaw & Wiener 2002), and (2) "dapc" (adegenet package, see Jombart 2008, Jombart et al. 2010). The RF algorithm constructs many (e.g., 500) classification trees using bootstrap samples from the original dataset and then generates classifiers and aggregate results by voting to classes (Breiman 2001). About one-third of the instances were left out of the sample because the training set for the current tree was drawn by sampling with replacements. This oob (out-of-bag) data was used to obtain a running unbiased estimate of the classification error as trees were added to the forest. After each tree was built, all of the data were run down the tree, and proximities were computed for each pair of instances. If two instances occupied the same terminal node, their proximity was increased by one. At the end of the run, proximities were normalized by dividing them by the total number of trees. Proximities were used to replacing missing data, locate outliers, and produce illuminating low-dimensional views of the data (Breiman 2001).

Classic Discriminant Analysis (DA) depends on multivariate normality (Pohar et al. 2004) and a larger number of objects than variables. The multivariate normality assumption was tested with the "mardiaTest" (MVN package, Korkmaz et al. 2014) and applied only to the acoustic data. Applying DA on a few axes (preserving about 95% of the variance) of a Principal Component Analysis, as performed by the "dapc", reduces the imbalance between objects and variables (Jombart et al. 2010). For the call analyses, points plotted in the scatterplot figure correspond to the mean values obtained from each individual recorded. DA ellipses are centred around means, their widths and heights are determined by variances, and covariance sets their slopes (Dray & Dufour 2007). Despite the lack of normality in our morphometric datasets, the results of "dapc" were evaluated for both datasets within an exploratory context and to assess their congruence in relation to "randomForest" results. The direct or indirect packages related to the application of both Discriminant Functions were computed in R (R Core Team 2014).

We used the following morphological variables for both Discriminant Analyses and statistical tests*:* SVL, HL, HW, ED, SED, IND, FAL, HAL, THL, SL, and FL; variables for acoustic analyses were pulse duration, interpulse interval, interpulse interval variance, pulses/second, note duration, internote interval, notes/second, pulses/note, minimum of dominant frequency, maximum of dominant frequency, and peak of dominant frequency. Considering that both analyses were highly concordant in species discrimination (see results), we present the RF results in tables and DAPC in scatterplots.

We tested all morphometric and acoustic variables for the statistical significance of their differences between population/species with the Exact Wilcoxon Mann Whitney Rank Sum Test using the Coin package (Resampling Statistics, function "wilcox_test", Hothorn et al. 2008) in R. We tested the significance of the differences found between the variances of the interpulse interval of the three species with the Permutational Bartlett's test of homogeneity of variances (function "perm.bartlett.test") using RVAideMemoire (Hervé 2015). This function performs a permutational Bartlett's test of homogeneity of k variances. As these tests were done between species pairs, we adjusted the significance levels ("P") considering the number of pairings with the Holm method (p.adust function in R). We assumed significance when $P \leq 0.05$.

Chromosomal morphology

We transported live specimens from the type locality to the laboratory for chromosome studies at UNICAMP, state of São Paulo, Brazil: one female (ZUEC 21004) and four males (ZUEC 20999; 21001; 21003; 21006). We obtained mitotic metaphases from the epithelium of intestinal cell suspensions according to King & Rofe (1976) following treatment in vivo with 2% colchicine solution for at least four hours. We stained the slides with Giemsa 10% and examined them with a photomicroscope Olympus BX60. We based our morphometric analyses on at least three metaphases of each individual, and the chromosomal classi-

fication relative to centromeric position was conducted according to the criteria proposed by Green & Sessions (1991).

Phylogenetic tree estimation and species delimitation

We wanted to assess the evolutionary independence of lineages between populations from the states of CE and RN in comparison to other *Pseudopaludicola* species and obtain confirmation whether these two populations represented the same species by means of molecular evidence. We sequenced fragments of 12S and 16S ribosomal RNA mitochondrial genes from four individuals from the RN population (deposited in GenBank, accession numbers: KT455497–455504), while sequences from two specimens from the CE population were obtained from GenBank (accession numbers KJ147033; KJ147034). We used six specimens from each of the three species of the *P. saltica* clade, including topotypes of all species (see Veiga-Menoncello et al. 2014). Genomic DNA was extracted from liver tissue using the Phenol-Chloroform protocol (Sambrook & Russell 2006) (see Supplementary table 1). We used the primers MVZ59/MVZ50 of Graybeal (1997) and 16Sa/16Sb of Palumbi (1996) under the following PCR conditions: 1× buffer, dNTP at 0.2 mM, each primer at 0.2 µM, $MgCl_2$ at 2mM, 1U Taq polymerase and 2 µl of template DNA, in a total reaction volume of 25 µl. We used the following cycling program: 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 50°C for 30 s, and 72°C for 1 min, and concluding with a 5-min extension at 72°C. PCR products were purified with Ethanol/Sodium Acetate and sent for sequencing to Macrogen Inc. (Seoul, South Korea). We also included all 12S and 16S sequences from *Pseudopaludicola* specimens available in GenBank, plus three additional outgroups [*Leptodactylus pentadactylus* (Laurenti, 1768), *Pleurodema diplolister*, and *Physalaemus nattereri* (Steindachner, 1863); see Supplementary table 1 for all specimens accession numbers]. Sequence alignment was performed using the default settings of the MUSCLE algorithm (Edgar 2004) as incorporated in the software MEGA v. 6.0.6 (Tamura et al. 2013). Hypervariable regions within the alignment were removed with GBLOCK v. 0.91b (Castresana 2000) and excluded from the matrix. The final alignment contains partial sequences of 12S and 16S mitochondrial genes (totalling a 975-bp concatenated dataset) from 93 specimens, representing 13 of the 18 currently recognized *Pseudopaludicola* species, three candidate species (referred as *Pseudopaludicola* sp.), and the three outgroups previously mentioned.

We then estimated a Bayesian ultrametric mitochondrial gene tree with BEAST v. 1.8 software (Drummond et al. 2012) creating a GTR+I+G model as suggested by the Akaike Information Criterion (Akaike 1974) in jModeltest version 2.1.6 (Darriba et al. 2012). We performed a run with 20 million generations, sampling every 2,000 steps using a Birth–death tree prior. We checked for stationary posterior distributions, effective sample sizes (ESS) above 200, and convergence between runs by examining parameter traces with the software Tracer v. 1.6 (Rambaut et al. 2014). We annotated tree files and computed the maximum clade credibility (MCC) tree with TreeAnnotator v. 1.8 (Drummond et al. 2012).

To objectively delimit species based on the mitochondrial dataset, we used both the maximum likelihood (ML) and the Bayesian implementation of the generalized mixed Yule-coalescent (GMYC) model, which delimit independently evolving species using single-locus data (Pons et al. 2006). The ML method implements a model-based analysis to locate threshold points (or nodes) in the genealogy where transitions in branching rates reflect either inter- or intraspecific evolutionary processes, using an ultrametric gene tree as a guide (Pons et al. 2006). We used the single-threshold version of the ML method incorporated in the R v. 3.0.2 package 'splits'. We then applied the Bayesian implementation of the GMYC model to account for uncertainties in genealogy estimation (Reid & Carstens 2012) with the R v. 3.0.2 package 'bGMYC', which calculates the marginal posterior probabilities of species limits from the posterior distribution of ultrametric trees reconstructed with BEAST. For the bGMYC analysis, a post-burn-in sample of 100 trees was used to calculate the posterior distribution of the GMYC model. The vector of starting parameters for the model was set to c(1,1,45), while scaling parameters were set to c(15,20,0.5). Priors of parameters t1 and t2 were set to 18 and 90, respectively. Remaining priors were set as default. We ran the bGMYC analysis for 100,000 generations, with a burn-in of 90,000 generations, and a thinning interval of 100 samples.

Additionally, between-groups mean distances between the 13 analysed species (including the new species) and three candidate species were computed with MEGA v. 6.0.6 using uncorrected and Tamura-Nei-corrected (Tamura & Nei 1993) distances. One specimen from the municipality of Andaraí (GenBank accession KJ147016), state of Bahia, was grouped with *P. pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 (from the municipality of Novas Russas, state of Ceará) because of its closer phylogenetic relatedness in comparison to two specimens from the municipality of Andaraí (see Results; Veiga-Menoncello et al. 2014).

Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The LSID (Life Science Identifier) for this publication is: urn:lsid:zoobank.org:pub:87A6C747-4227-4328-908E-C7DF7F4F3873. The electronic edition of this work was published in a journal with an ISSN, has been archived, and is available from the digital repository www.salamandra-journal.com.

Species description

Pseudopaludicola jaredi sp. n.

(Figs 2–4, Tables 1, 2)

ZooBank LSID: urn:lsid:zoobank.org:pub:87A6C747-4227-4328-908E-C7DF7F4F3873
Pseudopaludicola sp. (aff. *saltica*): Loebmann & Haddad (2010)
Pseudopaludicola sp. (aff. *saltica*): Veiga-Menoncello et al. (2014)

Holotype: Adult male (CFBH 32609; Figs 2, 3) collected in Serra das Flores, municipality of Viçosa do Ceará, state of Ceará (03°23'07" S, 41°09'29" W, 700 m a.s.l.) by D. Loebmann on 3 February 2009.

Paratopotypes: Eighteen males: ZUEC 20475, ZUEC 20477–84, CFBH 32617–25; and two females: ZUEC 20476 and CFBH 32614. All adult specimens were collected on 3 February 2009 by D. Loebmann.

List of additional specimens: Brazil: state of Rio Grande do Norte, municipality of Nísia Floresta, Floresta Nacional de Nísia Floresta (FLONA): ZUEC 21858–21872; state of Ceará, municipality of Viçosa do Ceará, Serra das Flores: nine tadpoles (CFBH 32626) were collected on 14 May 2008 and twenty tadpoles (ZUEC 20485) on 20 April 2009.

Diagnosis: *Pseudopaludicola jaredi* sp. n. is assigned to the genus *Pseudopaludicola* by having a hypertrophied antebrachial tubercle (see Lynch 1989, Lobo 1995), and to the *P. saltica* species group by its tibio-tarsal articulation reaching beyond the tip of the snout when the legs are adpressed to the body (Lobo 1995). The new species is characterized by the following combination of characters: (1) long hind limbs; (2) single, dark, subgular vocal sac with dark longitudinal folds; (3) white to light brown nuptial pads present in males, covering the external part of the thumb; (4) eleven pairs of chromosomes; and (5) advertisement call composed of notes with up to seven non-concatenated pulses separated by regular interpulse intervals.

Comparison with other species: *Pseudopaludicola jaredi* sp. n. is promptly distinguished from species of the *P. pusilla* group by the absence of either T-shaped terminal phalanges or expanded toe tips (Lynch 1989, Lobo 1995, Cardozo & Suárez 2012). The phalanges of the new species are similar in shape to those of *P. falcipes* (Hensel, 1867) (see Fig. 2B in Cardozo & Suárez 2012). The new species can also be distinguished from *P. ceratophyes* by the absence of enlarged palpebral tubercle (Lynch 1989) and from *P. boliviana* by the absence of an enlarged, conical tubercle on the heel. *Pseudopaludicola jaredi* sp. n. is distinguished from all species of the genus, except *P. saltica* and *P. murundu*, by having long hind limbs, with the tibio-tarsal articulation reaching beyond the tip of snout when the leg is adpressed to the body. The new species is distinct from *P. saltica* by its males having a dark vocal sac, shorter thigh ($P = 0.007$), shank ($P < 0.001$), and foot ($P = 0.007$) lengths, and a smaller eye diameter ($P = 0.015$); from *P. murundu* by its greater shank length ($P = 0.023$) and head width ($P < 0.001$), and wider internarial ($P = 0.002$) and eye–snout distances ($P < 0.001$).

The tadpoles within the *P. saltica* species group are so similar in external morphology that they are indistinguishable based on most of the characters examined (see Giaretta & Facure 2009, Toledo et al. 2010, Toledo 2010).

Pseudopaludicola jaredi sp. n. is differentiated from *P. canga*, *P. giarettai* Carvalho, 2012, *P. hyleaustralis*, *P. facureae*, and *P. parnaiba* by its advertisement call composed of pulsed notes, whereas all the five abovementioned species emit non-pulsed notes (Giaretta & Kokubum 2003, Carvalho 2012, Pansonato et al. 2012, Andrade & Carvalho 2013, Roberto et al. 2013). Its note structure of 2–7 non-concatenated pulses distinguishes the call of *P. jaredi* sp. n. from those of *P. mystacalis* (Cope, 1887) (12–14 concatenated pulses [= lack of interpulse interval], Pansonato et al. 2014a) and *P. boliviana* (calls with five notes and 3–6 concatenated pulses each, Duré et al. 2004).

The new species differs from other congeners [values within square brackets] with which it shares notes with

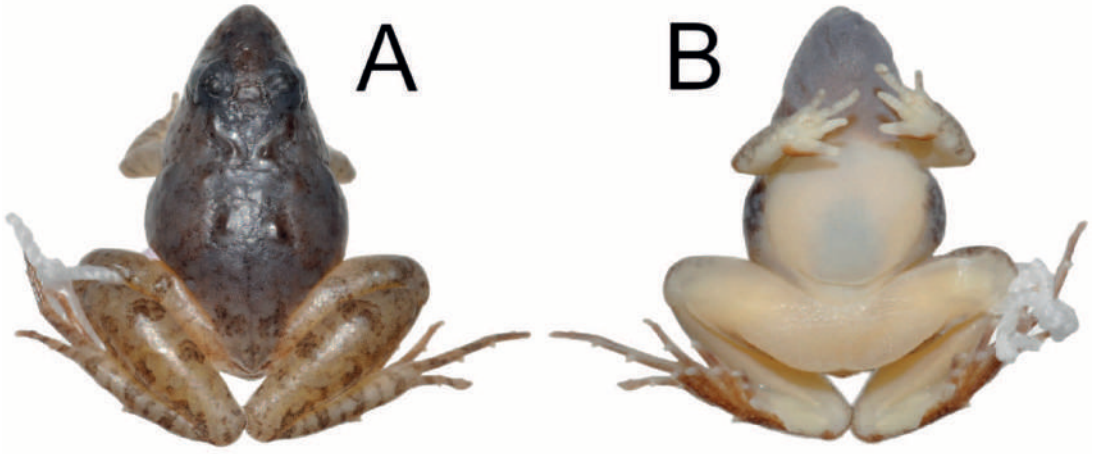

Figure 2. *Pseudopaludicola jaredi* sp. n., adult male, holotype (CFBH 32609), dorsal (A) and ventral (B) views. SVL = 15.3 mm.

non-concatenated pulses by the following acoustic traits: *P. ternetzi* has shorter internote intervals (84–184 [18–61] ms) and lower peaks of dominant frequency (5.0–5.9 [3.6–3.8] kHz, Cardozo & Toledo 2013); *P. ameghini* (Cope, 1887) has a lower peak of dominant frequency [3.2–4.4] kHz (Pansonato et al. 2013); *P. mineira* Lobo, 1994 has a shorter note duration (56–178 [mean 40 ± 4] ms), fewer pulses per note (2–7 [2–3]), and lower peaks of dominant frequency [4.3–4.8 kHz] (Pereira & Nascimento 2004); *P. falcipes* has a shorter note duration [mean 40 ms] and internote interval [mean 70 ms] (Haddad & Cardoso 1987); *P. pocoto* has a longer note duration [126–290 ms], longer interpulse interval [49–166 ms], and lower pulse rate (17–26 [10–18] pulses/second, Magalhães et al. 2014); and *P. atragula* Pansonato, Mudrek, Veiga-Menoncello, Rossa-Feres, Martins & Strüssmann, 2014 has a longer note duration [300–700 ms], higher number of pulses per note [9–36], and lower peak of dominant frequency [3.6–4.2 kHz] (Pansonato et al. 2014b).

As far as its most closely related species are concerned, *Pseudopaludicola jaredi* sp. n. is distinguished from *P. murundu* by its lower peak of dominant frequency (P = 0.003), longer note duration (P = 0.006), lower variance in interpulse intervals (P = 0.008), and higher number of pulses per note (P = 0.002); and from *P. saltica* by having regular (low variance) interpulse intervals (CV = 43.3 ± 7.8 [33.5–56.2] in the new species and CV = 90.6 ± 17.2 [53.0–111.8] in *P. saltica*, P = 0.006), a higher dominant frequency (P = 0.003), and shorter note duration (P < 0.001). In addition, the new species can be distinguished from *P. murundu* and *P. saltica* by emitting notes with up to seven pulses [up to six pulses/note in these species].

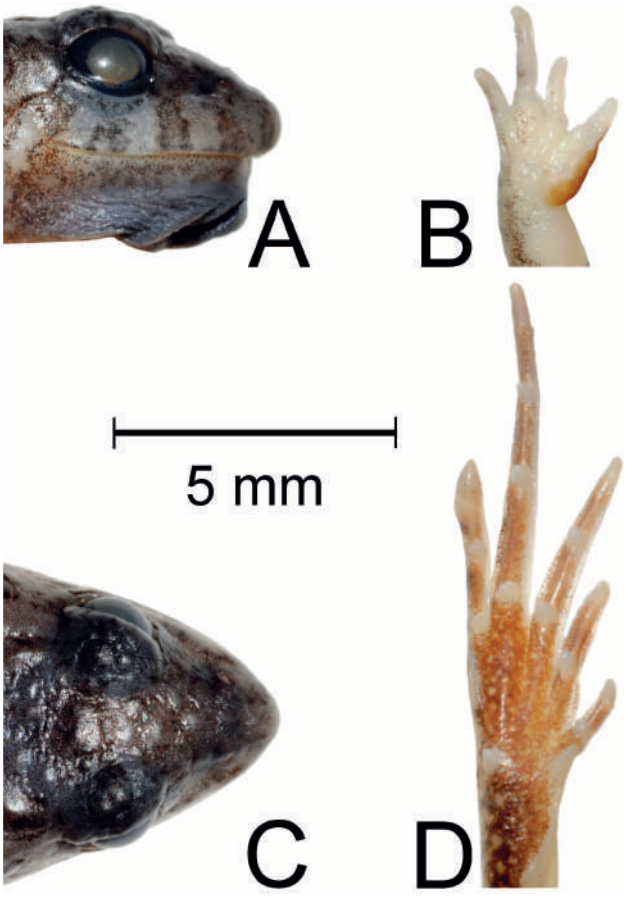

Figure 3. *Pseudopaludicola jaredi* sp. n., adult male, holotype (CFBH 32609), lateral view of head (A); ventral view of right hand (B); dorsal view of head (C); and ventral view of foot (D). Scale bar = 5 mm.

Table 1. Morphometric characters of the *Pseudopaludicola jaredi* sp. n. type series (including holotype) from the municipality of Viçosa do Ceará, state of Ceará, Brazil; and adult males from the Floresta Nacional (FLONA) de Nísia Floresta, state of Rio Grande do Norte, Brazil. Values presented in millimetres as mean ± standard deviation (minimum–maximum); n = number of measured specimens.

Characters	Type series			FLONA
	Holotype (male)	Males (n=19)	Females (n=2)	Males (n=14)
Snout–vent length (SVL)	15.1	15.4±0.9 (13.9–16.6)	16.1–17.0	15.0±0.6 (14.3–16.2)
Head length (HL)	4.6	4.7±0.4 (3.9–5.6)	5.0–5.1	4.6±0.3 (4.2–5.0)
Head width (HW)	5.2	5.3±0.2 (5.0–5.9)	6.1–6.2	5.5±0.2 (5.2–5.9)
Internarial distance (IND)	1.4	1.5±0.1 (1.3–1.7)	1.6–1.7	1.5±0.1 (1.3–1.6)
Eye diameter (ED)	1.8	1.9±0.2 (1.6–2.4)	2.1	2.0±0.1 (1.8–2.1)
Snout–eye distance (SED)	2.9	2.7±0.2 (2.4–3.0)	2.8–3.0	2.7±0.1 (2.5–2.9)
Hand length (HAL)	4.5	4.2±0.1 (4.0–4.5)	4.3–4.4	4.1±0.2 (3.8–4.6)
Thigh length (THL)	8.5	8.6±0.3 (8.1–9.3)	9.8–10.2	9.1±0.4 (8.5–9.8)
Shank length (TBL)	10.6	10.1±0.6 (9.1–11.0)	10.4–11.2	10.1±0.3 (9.5–10.5)
Foot length (FL)	10.1	9.7±0.3 (9.1–10.3)	10.5–10.6	9.7±0.4 (9.0–10.4)

Both multivariate approaches (randomForest and dapc) to morphology yielded no noticeable discrimination between the three species (see Table 4 and Fig. 5). Multivariate analyses of acoustic traits revealed a considerable discrimination of the new species from *P. saltica* and *P. murundu* (Table 5), with only one male of the new species being classified as *P. murundu* due to its higher dominant frequency. Accordingly, the dapc (Fig. 5) resulted in perfect discrimination of the three species. Interpulse interval variance, peak of dominant frequency, and note duration were the main sources of variation (about 80%), facilitating the distinction of *P. jaredi* sp. n. from *P. saltica* and *P. murundu*.

Pseudopaludicola jaredi sp. n. [2n = 22 chromosomes] also differs in chromosome number from *P. mystacalis* [2n = 16], *P. canga*, *P. facureae*, *P. atragula* [2n = 18],

P. ameghini, and *P. ternetzi* [2n = 20] (Duarte et al. 2010, Fávero et al. 2011, Veiga-Menoncello et al. 2014).

Description of the holotype: Body elliptic and broad (Fig. 2, Table 1). Head triangular, slightly longer than wide. Snout sub-elliptical in dorsal view and rounded in profile (sensu Heyer et al. 1990; Fig. 3). Eye protuberant, its diameter larger than the interorbital distance; interorbital area flat; pupil rounded; upper eyelid with 3–4 discrete tubercles. Nostril not protuberant and closer to the snout tip than to the eye. Canthus rostralis rounded, smooth; loreal region slightly concave. Single subgular vocal sac, externally expanded, large, and with longitudinal folds; choanae well separated from each other; vocal slits present. Tympanum indistinct; a dermal fold extending from of the posterior margin of the eye to the insertion of the arm. Mouth opening ventral. Vomerine teeth absent (unnoticeable also to the touch). Tongue elliptical,

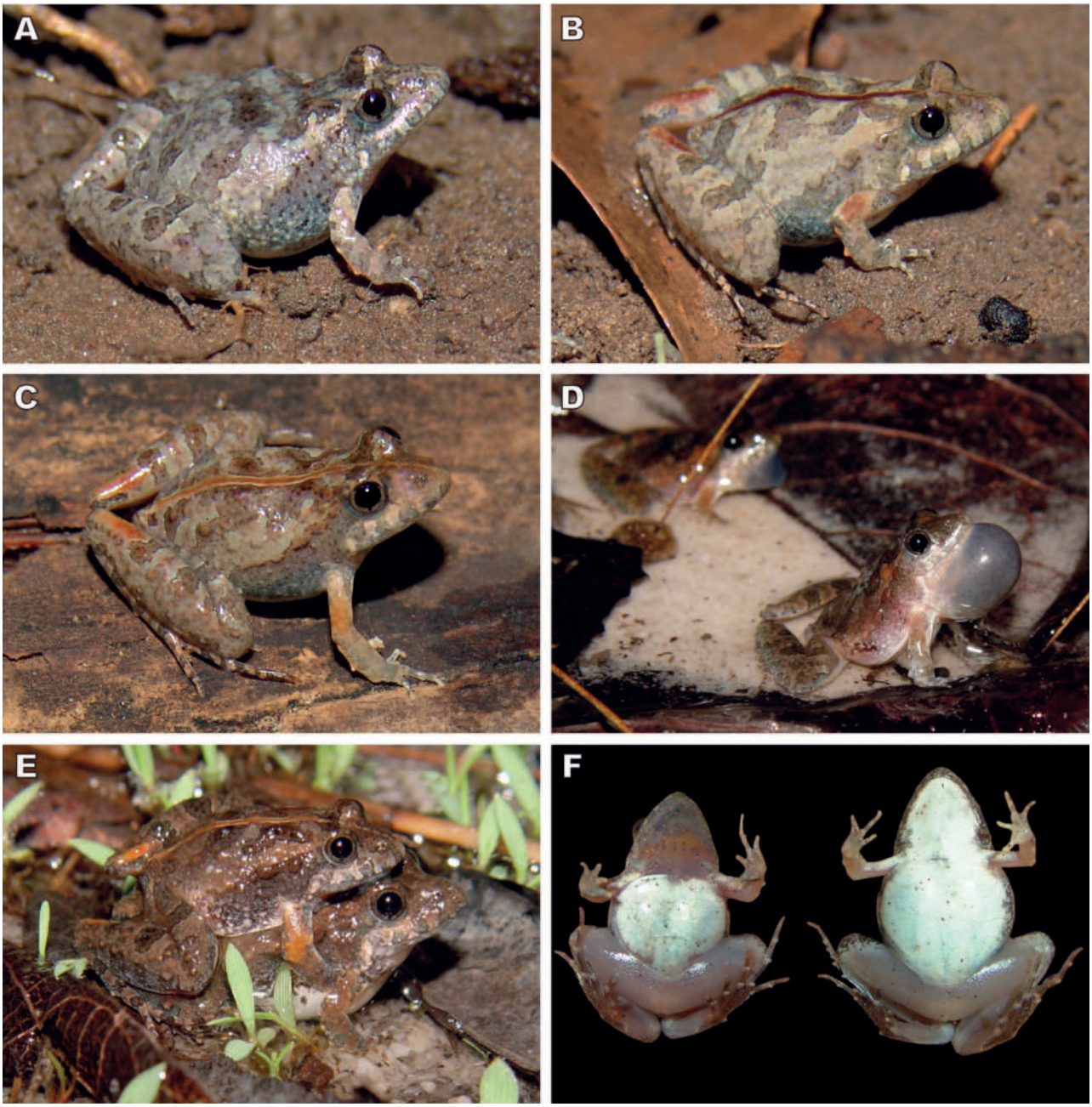

Figure 4. Specimens of *Pseudopaludicola jaredi* sp. n. in life from the municipality of Viçosa do Ceará, state of Ceará, Brazil. A) Male vertebral line absent; B) male with red vertebral line; C) male with red vertebral line; D) male vocalizing in the presence of a satellite male; E) couple in axillar amplexus; F) male and female highlighting the sexual dimorphism by the presence of the dark vocal sac in males.

Table 2. Advertisement call traits of the *Pseudopaludicola saltica* species group: *P. jaredi* sp. n. from the municipalities of Viçosa do Ceará, state of Ceará (type locality), and Nísia Floresta, state of Rio Grande do Norte; *P. saltica* from the municipalities of Chapada dos Guimarães, state of Mato Grosso (type locality) and Uberlândia, state of Minas Gerais; and *P. murundu* from the municipalities of Rio Claro (type locality), state of São Paulo, and São João Del Rei, state of Minas Gerais. Mean ± SD (minimum–maximum). n = number of specimens recorded (number of analysed notes). CV = Coefficient of variation.

	P. jaredi sp. n.	*P. murundu*	*P. saltica*
Variables	n=8 (224)	n=12 (120)	n=12 (120)
Call duration (s)	33.6±35.1 (7.0–108.5)	11.3±5.3 (5.7–19.5)	45.4±18.0 (28.8–74.6)
Note duration (s)	0.115±0.019 (0.056–0.178)	0.080±0.020 (0.027–0.126)	0.076±0.015 (0.030–0.108)
Internote interval (s)	0.125±0.013 (0.084–0.184)	0.117±0.016 (0.079–0.184)	0.122±0.016 (0.080–0.198)
Notes/second	5.3±1.0 (4.0–6.9)	5.2±0.9 (4.0–7.0)	5.6±1.2 (4.0–8.0)
Pulse duration (s)	0.010±0.002 (0.006–0.018)	0.011±0.002 (0.002–0.018)	0.009±0.002 (0.003–0.015)
Interpulse interval (s)	0.018±0.003 (0.001–0.046)	0.015±0.006 (0.001–0.039)	0.017±0.009 (0.001–0.048)
CV interpulse interval	43.3±7.8 (33.5–56.2)	46.9±18.7 (25.9–78.3)	90.6±17.2 (53.0–111.8)
Pulses/second	21.1±2.6 (17.1–25.7)	18.3±4.0 (10.8–25.0)	20.9±5.1 (13.0–28.0)
Pulses/note	4.9±0.7 (2.0–7.0)	3.7±0.5 (2.0–6.0)	4.0±1.2 (2.0–6.0)
Peak of dominant frequency (Hz)	5429.7±222.3 (5081.8–5986.2)	5827.9±230.7 (5081.8–6375.0)	5032.5±272.4 (4478.9–5531.2)
Min. dominant frequency (Hz)	4541.0±525.4 (4317.7–5057.1)	4730.5±354.8 (3852.2–5398.5)	3531.7±766.3 (2553.4–4718.0)
Max. dominant frequency (Hz)	6447.9±436.8 (6000.2–6676.1)	7091.2±294.0 (5952.2–8015.1)	6686.8±384.4 (5916.1–7830.0)

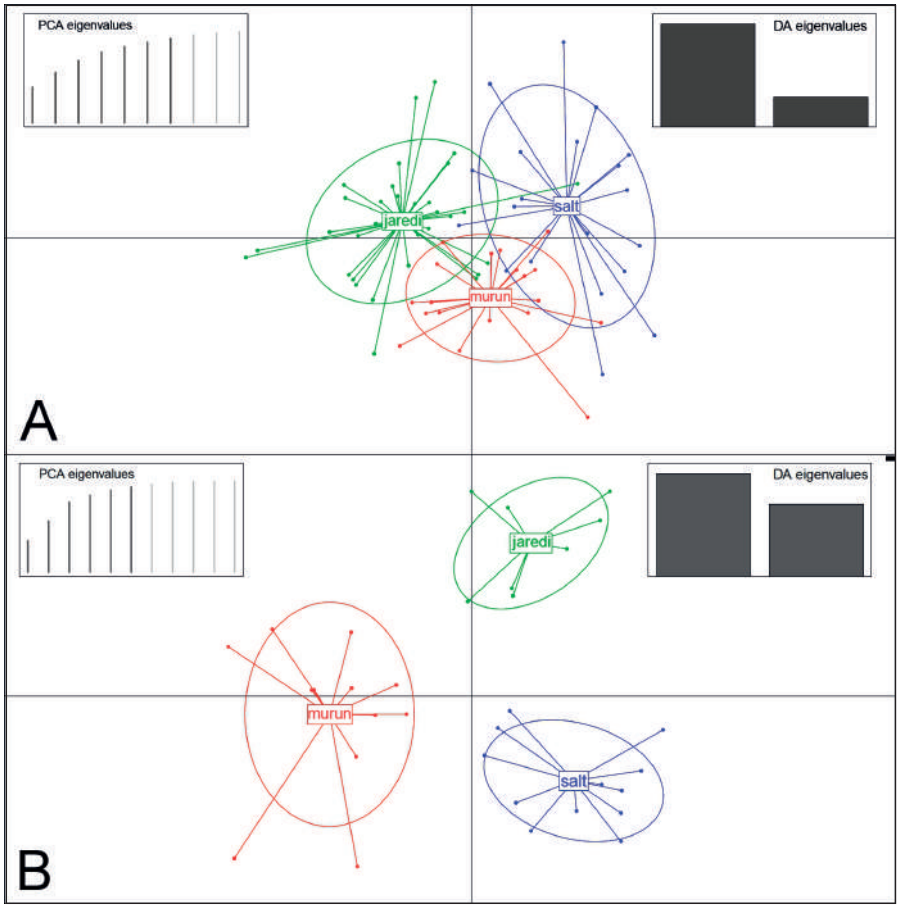

Figure 5. A) Scatterplot with the two first axes of the Discriminant Analysis of the first seven Principal Components of the morphometric dataset of the *Pseudopaludicola saltica* species group (inset top left: 45 and 13% of the explained variance proportion between DAPC axes retained, respectively); and B) two first axes of the DAPC scatterplot of the first six Principal Components of call traits (inset top left: 56 and 39% of the explained variance proportion between DAPC axes retained, respectively). Abbreviations: jaredi – *P. jaredi* sp. n.; murun – *P. murundu*; and salt – *P. saltica*.

Table 3. Measurements of 17 tadpoles of *Pseudopaludicola jaredi* sp. n. at Gosner's (1960) stages 35 and 37 (n = 9) and stages 27–29 (n = 8). Values presented in millimetres as mean ± SD (minimum–maximum).

Character	Stages 27 to 29	Stages 35 and 37
Body length (BL)	6.00±0.87 (4.68–7.23)	6.66±0.30 (6.34–7.28)
Tail length (TAL)	12.78±1.90 (10.19–15.21)	15.03±0.87 (13.82–16.03)
Maximum tail height (MTH)	2.45±0.45 (1.90–3.05)	2.47±0.30 (2.10–3.14)
Tail muscle height	1.15±0.16 (0.96–1.43)	1.56±0.23 (1.26–1.89)
Tail muscle width (TMH)	1.09±0.10 (0.93–1.25)	1.37±0.14 (1.13–1.58)
Total length (TL)	18.78±2.58 (15.40–21.53)	21.69±0.97 (20.30–22.86)
Oral disc width (ODW)	1.21±0.18 (1.02–1.45)	1.22±0.13 (0.98–1.44)
Internarial distance (IND)	0.89±0.06 (0.81–1.00)	1.04±0.09 (0.95–1.19)
Interorbital distance (IOD)	0.83±0.26 (0.58–1.35)	0.88±0.13 (0.72–1.07)
Eye diameter (ED)	0.71±0.12 (0.56–0.94)	0.86±0.09 (0.74–1.01)
Nostril diameter (ND)	0.16±0.05 (0.07–0.24)	0.13±0.03 (0.09–0.19)
Eye–nostril distance (END)	0.47±0.14 (0.29–0.70)	0.46±0.11 (0.32–0.62)
Nostril–snout distance (NSD)	1.12±0.22 (0.96–1.64)	1.44±0.20 (1.05–1.74)

Table 4. Confusion matrix for species of the *Pseudopaludicola saltica* group based on morphometric data by means of a Random Forests model. Settings: number of tree permutations = 500; number of variables tried at each split = 3.0; error rate = 36.99%.

	P. jaredi sp. n.	*P. murundu*	*P. saltica*	Classification error
P. jaredi sp. n.	26	4	3	0.21
P. murundu	5	11	4	0.45
P. saltica	5	6	9	0.55

Table 5. Confusion matrix for species of the *Pseudopaludicola saltica* group based on acoustic data by means of a Random Forests model. Settings: number of tree permutations = 500; number of variables tried at each split = 3.0; error rate = 6.25%.

	P. jaredi sp. n.	*P. murundu*	*P. saltica*	Classification error
P. jaredi sp. n.	7	1	0	0.12
P. murundu	0	12	0	0.00
P. saltica	1	0	11	0.08

free posteriorly; without pigmentation at its base. Flanks with discrete granules. One ovoid antebrachial tubercle present in the first quarter of the forearm. Finger and toe tips not expanded. Outer and inner metacarpal tubercles well defined, rounded. About three rounded supernumerary tubercles on the hand. Only one conical subarticular tubercle at the base of each finger; finger III with an extra subarticular tubercle between the first and second phalanges. Thumb with a keratinised, light brown nuptial pad, extending from the base of the hand to the proximal limit of the terminal phalanx, covering almost the entire external portion of the finger. No finger webbing. Fingers slightly fringed along their sides. Relative finger lengths (when adpressed to one another) I < IV < II < III (Fig. 3). Outer and inner metatarsal tubercles well defined, conical; the internal one larger than the external one; the external one more protuberant than the internal one. Toes with well-defined, single, enlarged, and conical subarticular tubercles (Figs 2, 3). Supernumerary tubercles absent on the foot. Toes webbed basally and extensively fringed along their sides to almost their tips. A well-developed fold from the internal metatarsal tubercle to the mid-ventral tarsus, continuing as fringes towards the tip of toe V. Relative toe lengths (when adpressed to one another) I < II < III = V < IV (Fig. 3). Hind limb robust and long with its tibio-tarsal articulation reaching beyond the tip of the snout. Thigh shorter than tibia; foot longer than thigh and slightly shorter than tibia. Transverse stripes on thighs (2–3), shanks (3–4), feet (3–4), and forearms (2–3). Calcaneus appendices absent. Belly skin smooth. Skin of ventral thighs (sit pad) granulated. Dorsal surfaces of head, body, and limbs smooth, interspersed with some tubercles; the skin covering the scapular region has two arc-shaped, granular folds. One gland on either side of the cloaca, cloacal region smooth (Fig. 2). For measurements of the holotype, see Table 1.

Colour pattern of the holotype in preservative: Back brown with grey spots; light beige belly. Back darker than the dorsal faces of limbs; coloration of the plantar face similar to that of dorsal legs; palmar face almost without pigmentation. Region between mouth and eyes with alternating vertical dark brown and light beige stripes. Ventral faces of arms and legs light beige. Dorsal faces of arms light beige with brown spots; dorsal faces of legs beige with brown transversal discontinuous stripes. Thin dark vertebral line from the snout tip to cloacal region. Light beige nuptial pads with brown margins (Fig. 2).

Variation in type series: Back colour varies from grey to brown, with dark grey or dark brown irregular spots. In adult males, the vocal sac can be dark as well, as can be the ventral faces of arms and legs. The belly is consistently beige. Females differ by having a white throat and being slightly larger than males (Table 1). When present (5 out of 18 paratopotypes), the vertebral line can be red, orange, or yellow. When a vertebral line is present, a spot with the same colour is visible on the upper arms (Fig. 4). Conversely, the specimens collected at the municipality of Nísia Floresta do not exhibit a vertebral line, and such fea-

ture was not observed in other specimens in the field either (F. M. Magalhães pers. obs.).

Etymology: The specific name is a noun, honouring Dr. Carlos Alberto Gonçalves Silva Jared, a Brazilian biologist and enthusiastic herpetologist who has dedicated his career to the study of amphibian and reptile morphology and behaviour. Besides his great scientific contributions, Carlos Jared makes everybody at his side enthusiastic about herpetology. Jared has also spent several years conducting field surveys in northeastern Brazil, where *Pseudopaludicola jaredi* sp. n. occurs.

Distribution: *Pseudopaludicola jaredi* sp. n. is known from the type locality, Serra das Flores, municipality of Viçosa do Ceará, state of Ceará, 03°23'07" S, 41°09'29" W, 700 m a.s.l., and Floresta Nacional (FLONA) de Nísia Floresta, municipality of Nísia Floresta, state of Rio Grande do Norte, Brazil. The FLONA de Nísia Floresta is situated about 700 km southeast of the type locality (Fig. 1).

Natural history notes: Loebmann & Haddad (2010) conducted a 24-month period of field work in Planalto of Ibiapaba and only one population of *P. jaredi* sp. n. was found there. Besides *P. jaredi* sp. n., two congeneric species were recorded on the Planalto of Ibiapaba: *P. mystacalis* and *P. pocoto*. These species do not occur syntopically with *P. jaredi* sp. n., which in turn co-occurs with at least five other frog species: *Leptodactylus vastus* Lutz, 1930, *Physalaemus cuvieri* Fitzinger, 1826, *Pleurodema diplolister*, *Proceratophrys caramaschii* Cruz, Nunes & Juncá, 2012, and *Scinax* sp. (gr. *ruber*). At the municipality of Nísia Floresta, *Elachistocleis cesarii* (Miranda-Ribeiro, 1920), *Leptodactylus troglodytes* Lutz, 1926, *P. diplolister*, *Scinax fuscomarginatus* (Lutz, 1925), and the congeneric *P. mystacalis* occur in sympatry with *P. jaredi* sp. n. *Pseudopaludicola jaredi* sp. n. is nocturnal and breeds during the rainy season (February to May in the state of Ceará and from June to August in the state of Rio Grande do Norte), mainly during the first rains. Males call in choruses during the breeding season in thinly flooded grasslands (up to 1 cm in depth). Satellite behaviour was observed in populations in the state of Ceará (Fig. 4). Amplectant pairs deposit their eggs in the water where exotrophic tadpoles develop (i.e., reproductive mode 1 of Haddad & Prado 2005).

Advertisement call: Quantitative variables are summarized in Table 2. The advertisement call of the new species consists of a long (7.0–108.5 s) series of pulsed notes (18–574 notes/call). Notes vary from 56–178 ms in duration separated by intervals of 84–184 ms; notes have a slightly ascending frequency modulation throughout their duration and are emitted at a rate of 4.0–6.9 notes/second. Notes are composed of 2–7 non-concatenated pulses. Pulses last from 6 to 18 ms, separated by intervals of 1–46 ms (variance = 5.9 ± 1.3; 0.2–39.2), and released at a rate of 17 to 26 pulses/second. Dominant frequency peaks between 5,082 and 5,986 Hz; minimum frequency ranges from 4,318 to 5,057 Hz, and the maximum from 6,000 to 6,676 Hz. Another emphasized frequency band may be accessed at a higher frequency (Fig. 6), peaking from 9,862 to 12,102 Hz (10,922 ± 369). Air temperature of recorded calls varied from 22.2 to 24.0°C.

Tadpole description (Table 3, Fig. 7): Body flattened ventrally (body height/body width = 0.78; 0.69–0.96), elliptical in dorsal and ventral views. Body length about 30% (27–36%) of total length. Snout oval in dorsal view and sloped in lateral view. Nostrils rounded, dorsolaterally directed, closer to the eyes (0.48 ± 0.12; 0.29–0.7) than to the tip of the snout (1.37 ± 0.22; 0.96–1.74). Small dorsolaterally orientated eyes (eye diameter/body width = 0.22; 0.19–0.28). Spiracle single and sinistral at mid-body, with a short free tube (see Fig. 7). Tail muscle about 56% of the total tail height. Dorsal and ventral fins rise near the tail/body junction. Oral disc anteroventral, without emarginations. Marginal papillae in the lateral region and on the median lower labium with two ventrolateral gaps. One wider gap in the dorsal marginal papillae. The g1-gap is present in 13 specimens analysed. Labial tooth row formula 2(2)/2–3[1]. Narrow jaw sheaths with triangular serration; lower jaw U-shaped and upper jaw sheath arc-shaped with long lateral processes. In preservative, dorsum dark brown, tail beige, and fins transparent with few scattered brown spots; internal organs visible in ventral view through the transparent venter.

Karyotype description: The karyotype of *Pseudopaludicola jaredi* sp. n. consists of 2n = 22 chromosomes organized in seven metacentric pairs (1, 2, 5, 7, 9, 10, and 11), three submetacentric pairs (3, 4, and 6), and one telocentric pair (8). A remarkable size heteromorphism was observed on the long arm of pair 8 (ZUEC 20999; 21001; 21003; 21006), due to a secondary constriction being present in one of the homologues, which resulted in morphs 8 and 8' (Fig. 8; Table 6).

Phylogenetic tree estimation and species delimitation: The mtDNA gene tree recovered with BEAST is generally congruent with the Bayesian tree topology inferred by Veiga-Menoncello et al. (2014). The main difference is that *P. mineira* was recovered as a sister taxa to *P. pocoto* + *Pseudopaludicola* sp. (Andaraí/BA) with high posterior probability (previously recovered as sister taxa to the *P. saltica* clade with a Bayesian approach; Veiga-Menoncello et al. 2014). Also, the mitochondrial gene tree confirmed (with high posterior probability) that *P. jaredi* sp. n. populations from the states of CE and RN are reciprocally monophyletic relative to the sister clade formed by *P. saltica* + *P. murundu* (aggroupment recovered with lower posterior probability). The GMYC ML analysis identified 19 evolutionary entities (confidence interval 16–29), including three evolutionary entities within the *P. saltica* clade, and 18 genetic clusters (confidence interval 16–24) with a significant model of species delimitation (χ^2 = 13.21, P = 0.001). The mean number of evolutionary entities delim-

ited by the bGMYC analysis was 19 (conspecificity probability threshold = 0.5; confidence interval 16–32), and 25 genetic clusters with a 95% HPD probability interval, including 3 singletons. Most of the ML entities match those coalescent units with the highest marginal probabilities (which corresponds to the currently recognized or candi-

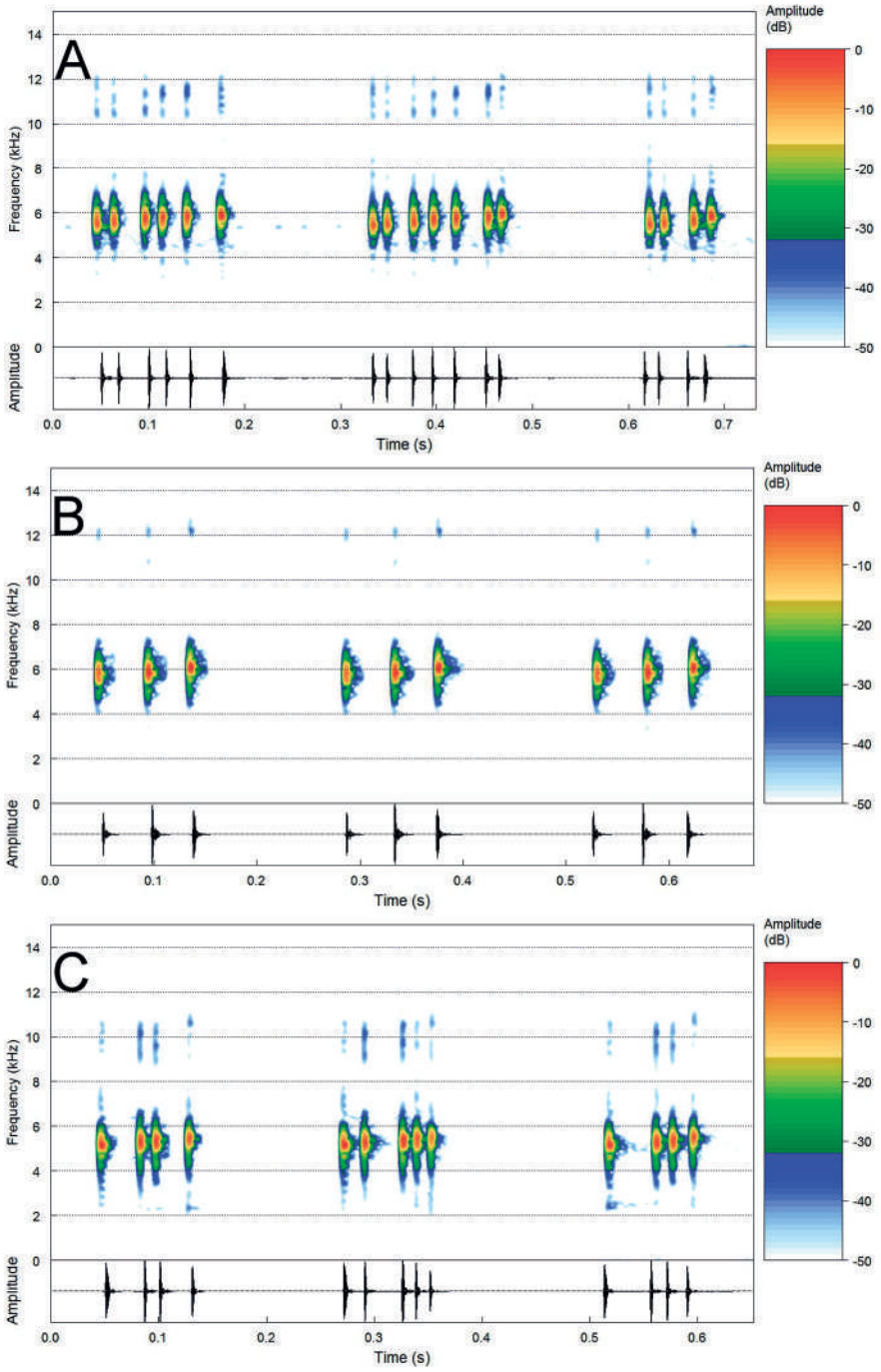

Figure 6. Audiospectrograms (above) and corresponding oscillograms (below) detailing three pulsed notes of the advertisement calls of species of the *Pseudopaludicola saltica* group (note that pulses are non-concatenated). A) *Pseudopaludicola jaredi* sp. n. from the Floresta Nacional de Nísia Floresta, municipality of Nísia Floresta, state of Rio Grande do Norte. Sound file: ASUFRN236; approximately 20:00 h, 03 July 2013; air 22°C. Vouchered recording (ZUEC 21860). B) *Pseudopaludicola murundu* from the municipality of Rio Claro, state of São Paulo. Sound file: Pseudop_murunduRioClaroSP3aAAGm671; 22:34 h, 08 March 2015; air 24°C, water 25°C. Unvouchered recording. C) *Pseudopaludicola saltica* from the Clube de Caça e Pesca Itororó de Uberlândia, municipality of Uberlândia, state of Minas Gerais. Sound file: Pseudop_salticUberlMG4bAAGm; 20:44 h, 19 March 2011; air 23°C, water 25.8°C. Vouchered recording (AAG-UFU 2308).

Table 6. Morphometry and classification of chromosomes of *Pseudopaludicola jaredi* sp. n.. The chromosomal classification relative to centromeric positions follows Green & Session (1991): M – metacentric; SM – submetacentric; T – telocentric.

	Chromosome number											
	1	2	3	4	5	6	7	8	8'	9	10	11
Relative size	15.72	13.25	12.41	11.12	9.79	8.39	8.05	6.74	3.83	6.09	4.64	4.43
Arm ratio	1.03	1.23	2.03	1.99	1.13	1.70	1.26	16.5	8.4	1.14	1.08	1.01
Classification	M	M	SM	SM	M	SM	M	T	T	M	M	M

date species in this genus). Specifically, the delimitation of the three species within the *P. saltica* clade is supported by a high marginal probability (> 95%), providing additional evidence that *P. jaredi* sp. n. is an independently evolving species within the *P. saltica* clade, which also includes *P. saltica* and *P. murundu* (see Fig. 9).The divergences between *P. jaredi* sp. n. and other species of the *P. saltica* species group varied from 1.4 to 2% with both uncorrected and corrected p-distance methods (Table 7). The overall genetic distance (GD) between all *Pseudopaludicola* species ranged from 1.4 to 15%, and the average distance is 8% with the uncorrected and 9% with a corrected p-distance.

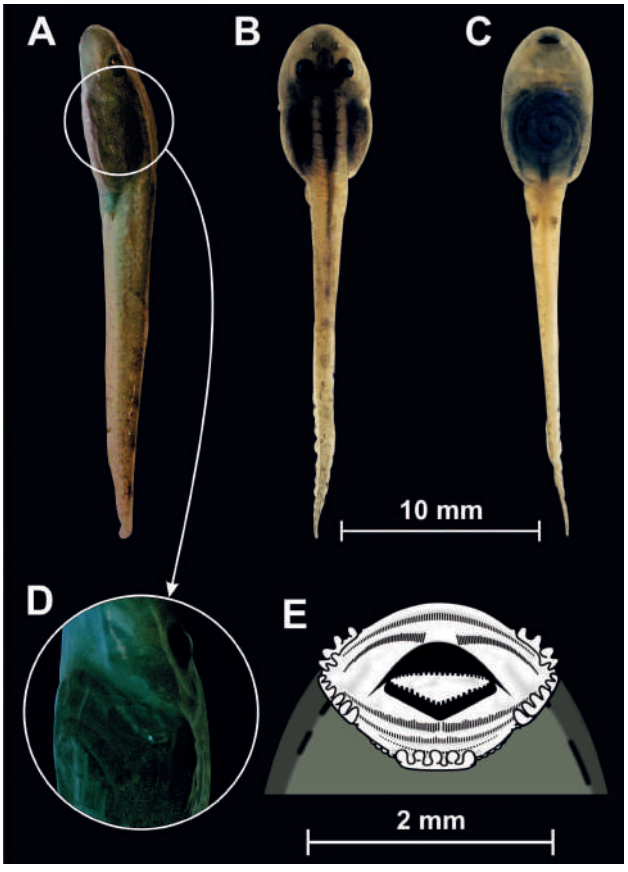

Figure 7. Tadpole (stage 37) of *Pseudopaludicola jaredi* sp. n. from the municipality of Viçosa do Ceará, state of Ceará, Brazil. A) Sinistrolateral, B) dorsal, and C) ventral views; D) Spiracle zoom view and E) ventral view of oral disc.

Discussion

By adding more calls to former comparisons, we were able to improve the differential diagnosis between *Pseudopaludicola murundu* and *P. saltica* (Fig. 5; Table 5). *Pseudopaludicola murundu* has pulsed notes with pulses separated by regular interpulse intervals (low variance) and a higher dominant frequency (Fig. 6), whereas *P. saltica* calls have pulses separated by irregular interpulse intervals (P = 0.05) and a lower dominant frequency (P < 0.001, Fig. 6; further details in Table 2). Moreover, our statistical morphometric analysis also demonstrated that *P. saltica* has larger head (P = 0.005), thigh (P = 0.004), and shank (P = 0.002) lengths than *P. murundu*.

The diploid number of 2n = 22 described herein for *P. jaredi* sp. n. has also been reported for the other species of the *P. saltica* group (Duarte et al. 2010, Toledo et al. 2010). A comparison between the karyotypes of *P. saltica* (Duarte et al. 2010), *P. murundu* (Toledo et al. 2010), and *P. jaredi* sp. n. revealed strong similarities. Nevertheless, interspecific differences were observed with regard to the morphology of pair 8. In *P. jaredi* sp. n. and *P. saltica* females, both pairs of chromosome 8 are telocentric (Duarte et al. 2010). Conversely, in males of *P. jaredi* sp. n. and *P. murundu* (Toledo et al. 2010), this same pair is also composed of telocentric chromosomes, but with a re-

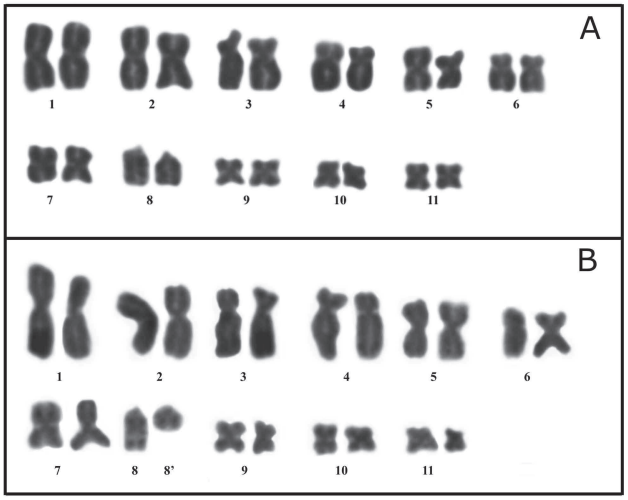

Figure 8. Karyotypes of *Pseudopaludicola jaredi* sp. n. after conventional Giemsa staining. A) One female (ZUEC 21004) and B) one male (ZUEC 21006).

Table 7. Average genetic distances between *Pseudopaludicola* species as per the mtDNA concatenated dataset. Values below the diagonal are uncorrected *p*-distances and values above the diagonal are corrected p-distances using the Tamura-Nei model. Pfal – *P. falcipes*; Pame – *P. ameghini*; Pfac – *P. facureae*; Pmys – *P. mystacalis*; Pter – *P. ternetzi*; Pcan – *P. canga*; Ppcn – *Pseudopaludicola* sp. (Poconé/MT); Psal – *P. saltica*; Patr – *P. atragula*; Pmur – *P. murundu*; Pbar – *Pseudopaludicola* sp. (Barreirinhas/MA); Ppoc – *P. pocoto*; Pand – *Pseudopaludicola* sp. (Andaraí/BA); Pmin – *P. mineira*; Pjar – *P. jaredi* sp. n.; Pbol – *P. boliviana*. Values below 5% are highlighted in bold.

	Pfal	Pame	Pfac	Pmys	Pter	Pcan	Ppcn	Psal	Patr	Pmur	Pbar	Ppoc	Pand	Pmin	Pjar	Pbol
Pfal		0.101	0.141	0.138	0.105	0.123	0.090	0.061	0.148	0.051	0.122	0.056	0.053	0.054	0.058	0.077
Pame	0.092		0.094	0.082	**0.017**	0.070	0.084	0.094	0.093	0.083	0.069	0.098	0.096	0.097	0.088	0.086
Pfac	0.123	0.085		0.083	0.098	0.053	0.132	0.130	**0.029**	0.125	0.052	0.142	0.139	0.142	0.131	0.126
Pmys	0.119	0.075	0.075		0.087	0.069	0.126	0.142	0.081	0.132	0.066	0.145	0.140	0.132	0.131	0.118
Pter	0.096	**0.017**	0.088	0.079		0.072	0.089	0.100	0.095	0.089	0.072	0.104	0.104	0.105	0.095	0.089
Pcan	0.109	0.066	**0.049**	0.063	0.067		0.111	0.124	0.062	0.114	**0.018**	0.124	0.119	0.125	0.120	0.109
Ppcn	0.083	0.078	0.116	0.111	0.082	0.100		0.091	0.136	0.082	0.119	0.095	0.092	0.094	0.086	**0.043**
Psal	0.057	0.086	0.114	0.123	0.092	0.110	0.084		0.128	**0.014**	0.122	**0.044**	**0.043**	**0.044**	**0.018**	0.088
Patr	0.127	0.084	**0.028**	0.074	0.086	0.058	0.119	0.113		0.125	0.058	0.138	0.138	0.139	0.128	0.130
Pmur	**0.048**	0.077	0.111	0.116	0.083	0.102	0.076	**0.014**	0.111		0.113	**0.038**	**0.040**	**0.039**	**0.015**	0.075
Pbar	0.108	0.065	**0.049**	0.061	0.067	**0.018**	0.107	0.109	0.054	0.101		0.120	0.121	0.125	0.119	0.111
Ppoc	0.052	0.090	0.123	0.124	0.095	0.110	0.086	**0.042**	0.119	**0.036**	0.106		**0.027**	**0.035**	**0.041**	0.083
Pand	0.050	0.088	0.120	0.121	0.094	0.106	0.084	**0.041**	0.119	**0.038**	0.107	**0.026**		**0.033**	**0.041**	0.079
Pmin	0.051	0.088	0.123	0.116	0.095	0.111	0.086	**0.042**	0.121	**0.037**	0.111	**0.034**	**0.031**		**0.040**	0.077
Pjar	0.054	0.082	0.115	0.115	0.087	0.107	0.079	**0.018**	0.113	**0.014**	0.106	**0.039**	**0.039**	**0.038**		0.078
Pbol	0.072	0.079	0.111	0.105	0.082	0.099	**0.041**	0.081	0.114	0.070	0.100	0.077	0.074	0.072	0.072	

markable size heteromorphism (see Fig. 8). In *P. saltica* males, the NOR-bearing pair 8 is heteromorphic with telocentric and submetacentric homologues, characterizing an XX/XY sex-determination system for this species with telocentric X and submetacentric Y (Duarte et al. 2010). Sexual dimorphism in chromosomal morphology was not mentioned by Toledo et al. (2010) in the description of *P. murundu*. Unfortunately, only males were analysed by these authors (a juvenile male was erroneously identified as a female). Therefore, further studies are necessary to provide evidence and confirm that the size heteromorphism observed between the homologues of pair 8, as seen here in *P. jaredi* sp. n., is a male-specific chromosome, indicating a morphological differentiation of XY/XX sex chromosomes, and whether *P. murundu* also shares this putative sex-specific difference, so that the existence of heteromorphic sex chromosomes could be considered a synapomorphy of the *P. saltica* species group.

The overall mitochondrial genetic distance (GD) between members of the *P. saltica* clade is low (1.4–2%) compared to the average GD of *Pseudopaludicola* species evaluated herein (8–9%). Nevertheless, the currently recognized species also have equally low genetic distances, as seen in *P. ameghini* and *P. ternetzi* (around 2%), and *P. atragula* and *P. facureae* (around 3%). Pansonato et al. (2014b) highlighted this low divergence between conspecific lineages of *Pseudopaludicola* species and also arrived at a similar result using a partial fragment of 16S. This pattern of GD is commonly observed in other cryptic species complexes of frogs such as *Engystomops* and *Hypsiboas* (less than 3% of GD between some species; Funk et al. 2012), *Physalaemus* (l.6–4.5% of GD between species; Ron et al. 2005, Funk et al. 2007), and *Ameerega* (1.8–5% of GD between species; Lötters et al. 2009). Conversely to mitochondrial DNA, morphological and especially acoustic traits might evolve more rapidly in response to natural or sexual selection, allowing for rapid trait differentiation even in geographically close populations (see Boul et al. 2007, Funk et al. 2012), which may also be the case in these mentioned *Pseudopaludicola* species. Because of the subjectivity involved in establishing a threshold in distancing methods, more objective methods that account for variation in tree topology and identify threshold points in the genealogy representing speciation processes (such as bGMYC) are highly desirable when dealing with mitochondrial data (Reid & Carstens 2012).

This is the first integrative taxonomic study including morphological (adult and larval), acoustic, chromosomal, and molecular evidence in a description of a species of the genus *Pseudopaludicola*. Given that almost all distinct lines of evidence have yielded congruent results, we have unequivocally confirmed that *P. jaredi* sp. n. is an independently evolving species within the *P. saltica* clade. Moreover, our integrative results reinforce that *P. serrana* is a junior synonym of *P. murundu* (Pansonato et al. 2014a) because all sequenced individuals (including three sequences from topotypical *P. serrana*) were recovered as a single species in the delimitation analysis. Besides recovering almost all currently recognized species as independently evolving units (corroborating the current taxonomy of the genus), both ML and Bayesian GMYC analysis indicated that some widely distributed species could just as well comprise more than one taxon. For instance, the BEAST gene tree recov-

ered three main lineages within *P. mystacalis* with high posterior probability, and such lineages have less than 0.05% of marginal posterior probability of being conspecific according to the bGMYC analysis (see Fig. 9). Additionally, these analyses also recovered as independently evolving entities three candidate species (*Pseudopaludicola* sp. sensu Veiga-Menoncello et al. 2014). Such results reinforce the notion that the species richness within *Pseudopaludicola* is likely underestimated, as was highlighted by the first molecular phylogenetic assessment of this genus conducted by Veiga-Menoncello et al. (2014). Finally, integrative taxonomic studies (such as ours) can significantly improve species descriptions and delimitation, especially when dealing with a rich and morphologically cryptic group, as is the case with the species of the genus *Pseudopaludicola*.

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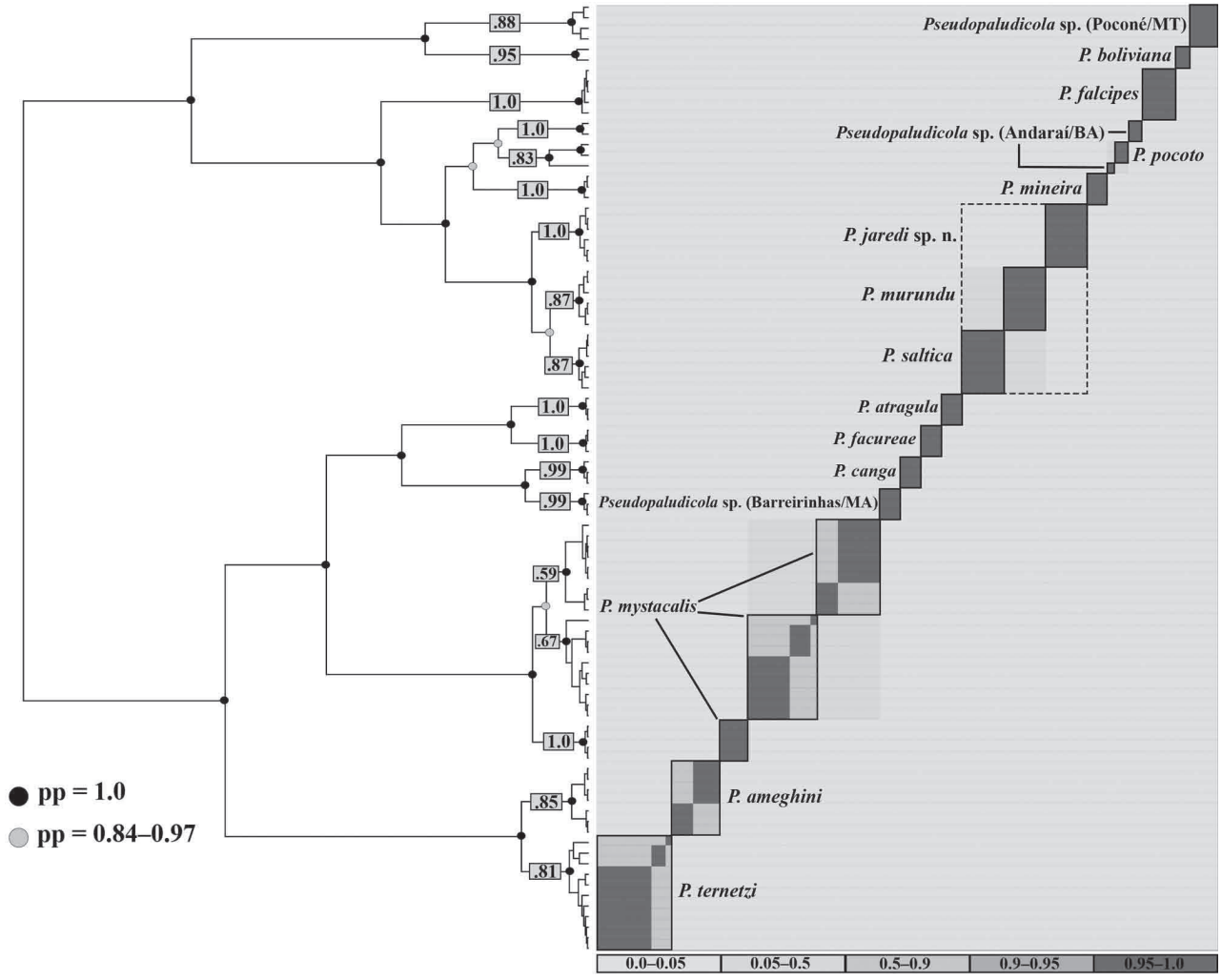

Figure 9. Summary of species delimitation analyses using maximum likelihood (ML) and Bayesian implementations of the Generalized Mixed Yule Coalescent model for the genus *Pseudopaludicola*, with a focus on the *P. saltica* clade (depicted with dashed lines). The topology represents the maximum clade credibility tree from BEAST with the respective node posterior probabilities (values indicated by the circles). The ML entities identified by the GMYC method are outlined with continuous contours. Numbers are the posterior probabilities of species identities sampled from a posterior distribution of 100 trees generated in BEAST. The greyscale plot is a sequence-by-sequence matrix coloured by pairwise posterior probabilities of conspecifity, where off-diagonal patterns indicate uncertainty of species limits owing to topological variation of the phylogenetic tree.

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Appendix
Examined specimens

Pseudopaludicola ameghini: Brazil: Mato Grosso: Chapada dos Guimarães (ZUEC 14138–14139; 14141–14145).

Pseudopaludicola canga: Brazil: Pará: Marabá: Serra dos Carajás (ZUEC 9990; 10034; 14370; 14372–14374; 14378).

Pseudopaludicola facureae: Brazil: Minas Gerais: Uberlândia (AAG-UFU 0853–0855; ZUEC 13651–13652; 14215; 14218–14219; 14221; 14224).

Pseudopaludicola falcipes: Brazil: Rio Grande Do Sul: Porto Alegre (ZUEC 14008; 14022; 14162–14166; 14168).

Pseudopaludicola mineira: Brazil: Minas Gerais: Jaboticatubas (ZUEC 1574; 1582–1583; 1586–1587; 1590; 1593–1595; 1600; 1604; 1617).

Pseudopaludicola murundu: Brazil: São Paulo: Águas de Santa Bárbara (ZUEC 20507–20508), Rio Claro (AAG-UFU 5125–5126; CFBH 8235–8242; ZUEC 14284–14290). Brazil: Minas Gerais: Brumadinho (ZUEC 16396–16398; 16442–16443; 19549; 19551; 19555; 19557–19578; 19560), Santana do Riacho (ZUEC 2323), São João del Rei (ZUEC 16447–16452; 16455–16456).

Pseudopaludicola mystacalis: Brazil: Goiás: Itapirapuã (ZUEC 10222). Brazil: Mato Grosso: Cáceres (ZUEC 10286), Chapada dos Guimarães (ZUEC 5115; 5117; 5119; 5121; 10685). Brazil: Mato Grosso Do Sul: Três Lagoas (16720; 16949). Brazil: Tocantins: Formoso do Araguaia (ZUEC 10154).

Pseudopaludicola saltica: Brazil: Mato Grosso: Chapada dos Guimarães (ZUEC 14228; 14230–14233; 14235; 14239–14240; 14244; 14247; 14272; 5134–5146; 5854–5855). Brazil: Minas Gerais: Uberlândia (AAG-UFU 2308; 2630; 4598; 4631; 4735; 4707–4711).

Pseudopaludicola ternetzi: Brazil: Minas Gerais: Uberlândia (ZUEC 14036–14039; 14170–14171). Brazil: Tocantins: Formoso do Araguaia (ZUEC 10140–10143; 10145; 10147; 10150; 10153).

Supplementary material
Additional information is available in the online version of this article at http://www.salamandra-journal.com

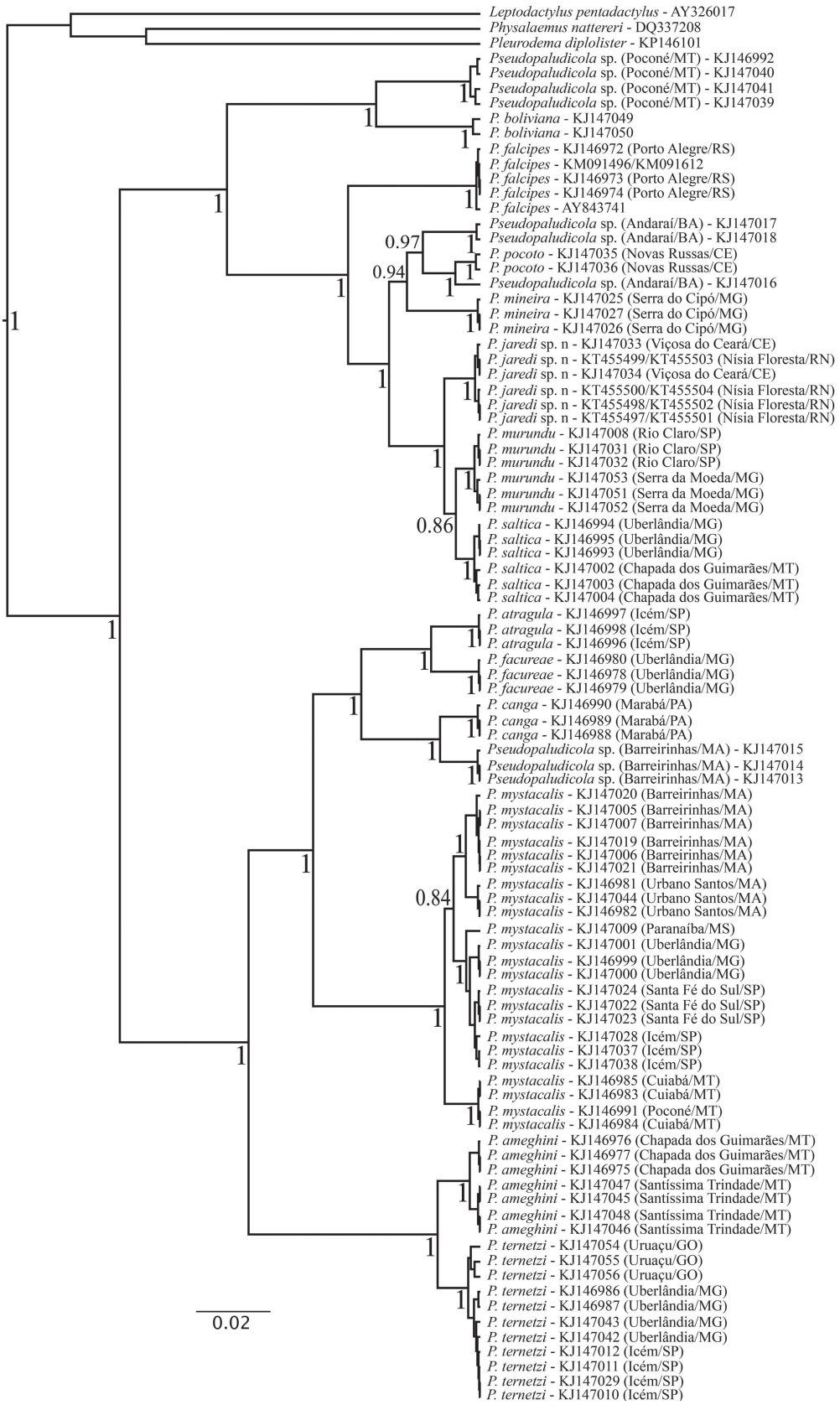
Supplementary table 1. GenBank accession numbers.

Supplementary figure 1. Maximum clade credibility mitochondrial gene tree of *Pseudopaludicola*.

Supplementary table 1. GenBank accession numbers.

	Taxa	GenBank accession number 12S	16S
1	*Pseudopaludicola ameghini*	KJ146975	KJ146975
2	*Pseudopaludicola ameghini*	KJ146976	KJ146976
3	*Pseudopaludicola ameghini*	KJ146977	KJ146977
4	*Pseudopaludicola ameghini*	KJ147045	KJ147045
5	*Pseudopaludicola ameghini*	KJ147046	KJ147046
6	*Pseudopaludicola ameghini*	KJ147047	KJ147047
7	*Pseudopaludicola ameghini*	KJ147048	KJ147048
8	*Pseudopaludicola atragula*	KJ146996	KJ146996
9	*Pseudopaludicola atragula*	KJ146997	KJ146997
10	*Pseudopaludicola atragula*	KJ146998	KJ146998
11	*Pseudopaludicola boliviana*	KJ147049	KJ147049
12	*Pseudopaludicola boliviana*	KJ147050	KJ147050
13	*Pseudopaludicola canga*	KJ146988	KJ146988
14	*Pseudopaludicola canga*	KJ146989	KJ146989
15	*Pseudopaludicola canga*	KJ146990	KJ146990
16	*Pseudopaludicola facureae*	KJ146978	KJ146978
17	*Pseudopaludicola facureae*	KJ146979	KJ146979
18	*Pseudopaludicola facureae*	KJ146980	KJ146980
19	*Pseudopaludicola falcipes*	AY843741	AY843741
20	*Pseudopaludicola falcipes*	KJ146972	KJ146972
21	*Pseudopaludicola falcipes*	KJ146973	KJ146973
22	*Pseudopaludicola falcipes*	KJ146974	KJ146974
23	*Pseudopaludicola falcipes*	KM091496	KM091612
24	*Pseudopaludicola jaredi* sp. n.	KJ147033	KJ147033
25	*Pseudopaludicola jaredi* sp. n.	KJ147034	KJ147034
26	*Pseudopaludicola jaredi* sp. n.	KT455497	KT455501
27	*Pseudopaludicola jaredi* sp. n.	KT455498	KT455502
28	*Pseudopaludicola jaredi* sp. n.	KT455499	KT455503
29	*Pseudopaludicola jaredi* sp. n.	KT455500	KT455504
30	*Pseudopaludicola mineira*	KJ147025	KJ147025
31	*Pseudopaludicola mineira*	KJ147026	KJ147026
32	*Pseudopaludicola mineira*	KJ147027	KJ147027
33	*Pseudopaludicola murundu*	KJ147008	KJ147008
34	*Pseudopaludicola murundu*	KJ147031	KJ147031
35	*Pseudopaludicola murundu*	KJ147032	KJ147032
67	*Pseudopaludicola murundu*	KJ147051	KJ147051
68	*Pseudopaludicola murundu*	KJ147052	KJ147052
69	*Pseudopaludicola murundu*	KJ147053	KJ147053
39	*Pseudopaludicola mystacalis*	KJ146984	KJ146984
40	*Pseudopaludicola mystacalis*	KJ146985	KJ146985
41	*Pseudopaludicola mystacalis*	KJ146991	KJ146991
42	*Pseudopaludicola mystacalis*	KJ146999	KJ146999
43	*Pseudopaludicola mystacalis*	KJ147000	KJ147000
44	*Pseudopaludicola mystacalis*	KJ147001	KJ147001
45	*Pseudopaludicola mystacalis*	KJ147005	KJ147005
46	*Pseudopaludicola mystacalis*	KJ147006	KJ147006
47	*Pseudopaludicola mystacalis*	KJ147007	KJ147007
48	*Pseudopaludicola mystacalis*	KJ147009	KJ147009

	Taxa	GenBank accession number 12S	16S
49	*Pseudopaludicola mystacalis*	KJ147019	KJ147019
50	*Pseudopaludicola mystacalis*	KJ147020	KJ147020
51	*Pseudopaludicola mystacalis*	KJ147021	KJ147021
52	*Pseudopaludicola mystacalis*	KJ147022	KJ147022
53	*Pseudopaludicola mystacalis*	KJ147023	KJ147023
54	*Pseudopaludicola mystacalis*	KJ147024	KJ147024
55	*Pseudopaludicola mystacalis*	KJ147028	KJ147028
56	*Pseudopaludicola mystacalis*	KJ147037	KJ147037
57	*Pseudopaludicola mystacalis*	KJ147038	KJ147038
58	*Pseudopaludicola mystacalis*	KJ147044	KJ147044
59	*Pseudopaludicola pocoto*	KJ147035	KJ147035
60	*Pseudopaludicola pocoto*	KJ147036	KJ147036
61	*Pseudopaludicola saltica*	KJ146993	KJ146993
62	*Pseudopaludicola saltica*	KJ146994	KJ146994
63	*Pseudopaludicola saltica*	KJ146995	KJ146995
64	*Pseudopaludicola saltica*	KJ147002	KJ147002
65	*Pseudopaludicola saltica*	KJ147003	KJ147003
66	*Pseudopaludicola saltica*	KJ147004	KJ147004
70	*Pseudopaludicola* sp. (Andaraí/BA)	KJ147016	KJ147016
71	*Pseudopaludicola* sp. (Andaraí/BA)	KJ147017	KJ147017
72	*Pseudopaludicola* sp. (Andaraí/BA)	KJ147018	KJ147018
73	*Pseudopaludicola* sp. (Barreirinhas/MA)	KJ147013	KJ147013
74	*Pseudopaludicola* sp. (Barreirinhas/MA)	KJ147014	KJ147014
75	*Pseudopaludicola* sp. (Barreirinhas/MA)	KJ147015	KJ147015
76	*Pseudopaludicola* sp. (Poconé/MT)	KJ146992	KJ146992
77	*Pseudopaludicola* sp. (Poconé/MT)	KJ147039	KJ147039
78	*Pseudopaludicola* sp. (Poconé/MT)	KJ147040	KJ147040
79	*Pseudopaludicola* sp. (Poconé/MT)	KJ147041	KJ147041
80	*Pseudopaludicola ternetzi*	KJ146986	KJ146986
81	*Pseudopaludicola ternetzi*	KJ146987	KJ146987
82	*Pseudopaludicola ternetzi*	KJ147010	KJ147010
83	*Pseudopaludicola ternetzi*	KJ147011	KJ147011
84	*Pseudopaludicola ternetzi*	KJ147012	KJ147012
85	*Pseudopaludicola ternetzi*	KJ147029	KJ147029
86	*Pseudopaludicola ternetzi*	KJ147042	KJ147042
87	*Pseudopaludicola ternetzi*	KJ147043	KJ147043
88	*Pseudopaludicola ternetzi*	KJ147054	KJ147054
89	*Pseudopaludicola ternetzi*	KJ147055	KJ147055
90	*Pseudopaludicola ternetzi*	KJ147056	KJ147056
91	*Pleurodema diplolister*	KP146101	KP146101
92	*Physalaemus nattereri*	DQ337208	DQ337208
93	*Leptodactylus pentadactylus*	AY326017	AY326017



Supplementary figure 1. Maximum clade credibility mitochondrial gene (12S and 16S) tree of *Pseudopaludicola* recovered in BEAST.