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A new species of *Ischnocnema* Reinhardt and Lütken, 1862 (Anura: Brachycephalidae) of the *I. lactea* species series from southeastern Brazil

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Abstract

We describe a new species of *Ischnocnema* from the Serra da Bocaina mountain range, state of São Paulo, southeastern Brazil, based on morphological, bioacoustic, and mtDNA data. The new species is retrieved with high support values within the *I. lactea* species series as the sister species of *I. spanios*. *Ischnocnema bocaina* **sp. nov.** is characterized by its medium size (18.6–19.0 mm), a smooth venter, a rounded snout in dorsal view and acuminate in lateral view, a slightly expanded subgular, single vocal sac, a round and whitish, poorly-developed glandular-appearing nuptial pad on the dorsal surface of the thumb, and a nonpulsed advertisement call with 9 to 18 notes. We raise to 38 the number of *Ischnocnema* species, the 12th described in the past 10 years.

Key words: Atlantic Rainforest**,** Bioacoustics, Brachycephaloidea, Integrative taxonomy, Molecular phylogeny

Introduction

The Neotropical genus *Ischnocnema* Reinhardt & Lütken, 1862 is a taxonomically challenging group of ground-dwelling direct-developing frogs that encompasses some morphologically cryptic species complexes (Gehara *et al.* 2013, 2017; Taucce *et al.* 2018a). Much of its diversity (about 30%) has been described during the past ten years, and presently it comprises 37 species (Frost 2018) , from which 34 are allocated in five species series (Taucce *et al.* 2018b). Among these, the *I. lactea* series currently contains ten described species (Canedo & Haddad 2012): *I. concolor* Targino, Costa & Carvalho-e-Silva 2009, *I. gehrti* (Miranda-Ribeiro 1926), *I. holti* (Cochran 1948), *I. lactea* (Miranda-Ribeiro 1923), *I. melanopygia* Targino, Costa & Carvalho-e-Silva 2009, *I. nigriventris* (Lutz 1925), *I. paranaensis* (Langone & Segalla 1996), *I. randorum* (Heyer 1985), *I. spanios* (Heyer 1985), and *I. vizottoi* Martins & Haddad, 2010. The series is endemic to the Brazilian Atlantic rainforest, occurring in the Brazilian states of Paraná, São Paulo, Minas Gerais, and Rio de Janeiro.

Hedges *et al.* (2008) were the first to define the *I. lactea* species series, based on the former *Eleutherodactylus lacteus* group of Lynch & Duellman (1997) and considering several morphological characters. However, some of the species they included in the series have been posteriorly reallocated in other groups, like "*Eleutherodactylus*" *bilineatus* Bokermann "1975" 1974*,* currently considered *incertae sedis* within Holoadeninae (Canedo & Haddad 2012); *I. bolbodactyla* (Lutz 1925)*;* currently allocated in the *I. verrucosa* series (Canedo & Haddad 2012); *I. venancioi* (Lutz 1958)*,* currently allocated in the *I. venancioi* series (Taucce *et al.* 2018b); and *I. manezinho* (Garcia 1996) and *I. sambaqui* (Castanho & Haddad, 2000), both currently unassigned to any series (Canedo & Haddad 2012). Despite consisting a phylogenetically solid group currently (Canedo & Haddad 2012; Taucce *et al.* 2018a; b), these recent rearrangements, along with new information on the morphology of some members of the *I. lactea* series (Targino & Carvalho-e-Silva 2008; Berneck *et al.* 2013; Silva-Soares *et al.* 2018), suggest that the morphological

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characters proposed by Hedges *et al.* (2008) are now outdated and not applicable anymore. All these aspects make classic morphology-based taxonomy approaches difficult to be applied to the group as the only line of evidence. Because of the taxonomic difficulties faced with the genus and its species series, we based our description herein on an integrative approach that combines three lines of evidence: mtDNA sequences, morphological, and bioacoustic characters.

Material and methods

Molecular analyses. In order to allocate the new species in one of the five currently recognized species series, we used a matrix that includes 28 of the 37 described species of *Ischnocnema* and two species of *Brachycephalus* Günther, 1858 as outgroups (Appendix I). The sister-group relationship between these two genera has been extensively tested (Canedo & Haddad 2012; Padial *et al.* 2014; Taucce *et al.* 2018a; b). We chose a fragment of the 16S ribosomal RNA gene (16S), which is commonly used in anuran taxonomy (Fouquet *et al.* 2007; Lyra *et al.* 2017; Vences *et al.* 2005), to perform our analyses. We used the primers 16S-AR (CGCCTGTTTATCAAAAACAT) and 16S-BR (GACCTGGATTACTCCGGTCTGA) to amplify our fragment (Palumbi *et al.* 1991).

We extracted whole cellular DNA from 99.5% ethanol liver tissue using a standard ammonium precipitation method (Lyra *et al.* 2017), and performed PCR amplification using Taq DNA Polymerase Master Mix (Ampliqon S/A, Denmark) and Axygene Maxygene thermocyclers. The PCR program consisted of a 3-min initial denaturing step at 95°C, followed by 35 cycles of 20 s at 95°C, 20 s at 50°C, and 45 s at 72°C, followed by a final extension step of 5 min at 72°C. We purified PCR product following Lyra *et al.* (2017) and sequenced it in both directions with a BigDye Terminator Cycle Sequencing Kit (version 3.0, Applied Biosystems) in an ABI 3730 automated DNA sequencer (Applied Biosystems) at Macrogen Inc. (Seoul, South Korea).

We performed the alignment with MAFFT v7.273 (Katoh & Standley 2013) using the E-INS-i algorithm (--*genafpair* command), which is adapted for sequences with conserved domains flanked by regions rich in gaps. We conducted a search for the best-fitting nuclear model with PartitionFinder v2.1.1 (Lanfear *et al.* 2017) using the corrected Akaike information criterion (AICc; Hurvich & Tsai 1989) and considering the whole 16S fragment as a single partition. PartitionFinder starts its analysis constructing a maximum likelihood tree, and we used PhyML 3.0 (Guindon *et al.* 2010) implemented on it for this purpose. The resulting best-fitting nucleotide substitution model was the general time-reversible (GTR) with proportion of invariant sites estimates and a gamma distribution of rates across sites.

We reconstructed phylogenetic trees using both Bayesian inference and maximum likelihood optimality criteria. For the first we used two independent runs of 1.0 x 10^7 generations, starting with random trees and four Markov chains (one cold), sampled every 1,000 generations in MrBayes 3.2.6 (Ronquist *et al.* 2012). We discarded 25% of generations and trees as burnin. We used the standard deviation of split frequencies (should be less than 0.01) and Estimated Sample Size (should be larger than 200) to assess run convergence. We performed maximum likelihood analysis using RAxML 8.2.10 (Stamatakis 2014), searching the most likely tree 100 times. To assess support, we conducted 1,000 nonparametric bootstrap replicates. We computed uncorrected pairwise distances using R v3.3.3 (R Core Team 2018) with the packages APE v3.4 (Paradis *et al.* 2004) and SPIDER v1.3-0 (Brown *et al.* 2012). In order to reduce the effect of the alignment on the genetic distances, we deleted the sites with gaps in a pairwise way (using *pairwise.deletion=T* in the *dist.dna* command).

Morphological analyses. The specimens used in the morphological analyses are housed in the following institutions: CFBH (Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil), MZUSP (Museu de Zoologia da Universidade de São Paulo, São Paulo, São Paulo, Brazil), and UFMG (Coleção de Anfíbios da Universidade Federal de Minas Gerais, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil). A complete list of specimens examined is given in Appendix II.

We took following measurements to the nearest 0.05 mm with a Mitutoyo® digital caliper under a stereomicroscope: snout-vent length (SVL), head length (HL), head width (HW), forearm length (FAL), hand length (HAL), thigh length (THL), tibia length (TL), tarsal length (TAL), foot length (FL), eye diameter (ED), tympanum diameter (TD), eye to nostril distance (END), internarial distance (IND), distance between the anterior margins of the eyes (AMD), maximum width of disk on third Finger (3FD), and maximum width of disk on fourth Toe (4TD). SVL, HL, HW, FAL, TL, FL, ED, TD, END, and IND follow Duellman (1970); 3FD and 4TD follow Heyer (1984); HAL, THL, and TAL follow Heyer *et al.* (1990); and AMD follows Garcia *et al.* (2003). Sex was determined by the observation of secondary sexual characters of male specimens (presence of nuptial pads and vocal slits) and by direct observation of eggs through the belly wall in female specimens. Morphological nomenclature follows previous literature on Brachycephaloidea (Duellman & Lehr 2009; Hedges *et al.* 2008; Heyer 1984; Heyer *et al.* 1990).

Call analyses. We recorded advertisement calls from the new species using a Marantz PMD 222 tape recorded coupled to an AudioTechnica AT835b unidirectional microphone. We carried out recordings at 44.1 kHz sampling frequency and we analyzed them with the software Raven pro 1.5 (Bioacoustics Research Program 2011). We produced spectrograms using window size of 512 samples, 99% overlap, DFT of 512 samples, and window type Hann. We obtained spectrogram and oscillogram figures using tuneR version 1.0 (Ligges *et al.* 2013) and seewave version 2.0.2 (Sueur *et al.* 2008) packages of R platform version 3.3.3 (R Core Team 2018). We produced spectrogram figures with window length of 512 samples, 99% overlap, and window name Hanning. Voucher specimen (MZUSP 138663) is housed at MZUSP and call recordings (CBUFMG 1015–1018) are housed Coleção Bioacústica da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais (CBUFMG).

We took the following acoustic parameters: call duration (Köhler *et al.* 2017), call rise time (Hepp & Canedo 2013), dominant frequency (Köhler *et al.* 2017), notes per call, note (repetition) rate (Taucce *et al.* 2018b), and note repetition rate acceleration (Gehara *et al.* 2013). Call and note concepts follow Köhler *et al.* (2017).

Results

Ischnocnema bocaina sp. nov.

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Holotype. MZUSP 138663, adult male, collected at Estação Ecológica do Bananal, municipality of Bananal, state of São Paulo, southeastern Brazil, by H. Zaher and P.C.A. Garcia on 18 January 2008.

Paratopotype. MZUSP 138664, adult male, collected with the holotype.

Diagnosis. In the *I. lactea* species series by phylogenetic placement (Fig. 1). *Ischnocnema bocaina* sp. nov. is distinguished from all other species of the *I. lactea* series by the following combination of characters: (1) medium size (SVL in males 18.6–19.0 mm); (2) snout rounded in dorsal view (Fig. 2); (3) tip of the snout acuminate in lateral view (Fig. 3); (4) dorsum smooth with granular flanks; (5) venter smooth; (6) head longer than wide; (7) eyelid tubercles absent; (8) dentigerous process of the vomer present; (9) vocal sac single, subgular, slightly expanded; (10) tips of Fingers II–IV expanded, truncated; (11) glandular-appearing nuptial pad on dorsal surface of the thumb, rounded, poorly-developed, whitish, extending to the posterior half of the thenar tubercle; (12) toe lengths formula I < II < V < III < IV; (13) calcar tubercle present, well-developed; (14) 9–18 notes per call; (15) advertisement call duration 1000–2010 ms; (16) note rate 8.1–9.0 notes/s; (17) dominant frequency 2325–2746 Hz; (18) nonpulsed advertisement call.

Comparison with other species. We based the comparisons both on collection specimens and literature data (references follow in parenthesis at the end of each species comparison). The new species differs from *I. concolor* by its larger size (*I. bocaina* sp. nov. males 18.6–19.0 mm SVL; *I. concolor* males 15.0–18.4 mm SVL); smooth venter (slightly areolate in *I. concolor*); rounded snout in dorsal view (subacuminate in *I. concolor*); acuminate snout in lateral view (rounded in *I. concolor*); slightly expanded vocal sac (moderately expanded in *I. concolor*); tips of Fingers II–IV well-developed and truncate (moderately developed and rounded in *I. concolor*); toe lengths formula I < II < V < III < IV (I < II < III < V < IV in *I. concolor*); and well-developed calcar tubercle (calcar tubercle absent in *I. concolor;* Targino *et al.* 2009). *Ischnocnema bocaina* sp. nov. differs from *I. gehrti* by its rounded snout in dorsal view (truncate in *I. gehrti*) and its acuminate snout in lateral view (subacuminate in *I. gehrti*; Pombal & Cruz 1999). From *I. holti*, the new species differs by its smooth venter (slightly areolate in *I. holti*); head longer than wide (wider than long in *I. holti*); acuminate snout in lateral view (rounded in *I. holti*); slightly expanded vocal sac (not expanded in *I. holti*); whitish nuptial pad (translucent in *I. holti*); and toe lengths formula I < II < V < III < IV (I < II < III < V < IV in *I. holti;* Targino & Carvalho-e-Silva 2008). *Ischnocnema bocaina* sp. nov. differs from *I. lactea* by its smaller size (*I. bocaina* sp. nov. males 18.6–19.0 mm SVL; *I. lactea* males 19.6–26.7 mm SVL); its smooth dorsum and venter (rugose and moderately granular in *I. lactea*); acuminate snout in lateral view (obtuse in *I. lactea*); the absence of upper eyelid tubercles (present in *I. lactea*); its single nuptial pad (double in *I. lactea*); its toe lengths formula I < II < V < III < IV (I < II < III < V < IV in *I. lactea*); and the advertisement call with 9–18 non-pulsed notes (one pulsed note in *I. lactea*; Silva-Soares *et al.* 2018). The new species differs from *I. melanopygia* by its smooth venter (venter slightly areolate in *I. melanopygia*); snout rounded in dorsal and acuminate in lateral views (respectively subacuminate and rounded in *I. melanopygia*); and its slightly expanded vocal sac (not expanded in *I. melanopygia*; Targino *et al.* 2009). *Ischnocnema bocaina* sp. nov. differs from *I. nigriventris* by its smooth dorsum and venter (respectively shagreen with tubercles or warts and weakly areolate in *I. nigriventris*); head longer than wide (wider than long in *I. nigriventris*); snout rounded in dorsal and acuminate in lateral views (respectively nearly-rounded and rounded in *I. nigriventris*); by the absence of upper eyelid tubercles (present in *I. nigriventris*); by the slightly expanded vocal sac (not expanded in *I. nigriventris*); its toe lengths formula I < II < V < III < IV (I < II < III = V < IV in *I. nigriventris*); and the advertisement call with 9–18 notes and call duration of 1000–2010 ms (2–4 notes and 194–565 ms in *I. nigriventris*; Berneck *et al.* 2013). The head longer than wide (as long as wide in *I. paranaensis*); snout acuminate in lateral view (rounded in *I. paranaensis*); vomerine teeth present (absent in *I. paranaensis*); toe lengths formula I < II < V < III < IV (I < II < III < V < IV in *I. paranaensis*); and well-developed calcar tubercle differ *I. bocaina* sp. nov. from *I. paranaensis* (calcar absent in *I.* paranaensis; Langone & Segalla 1996). *Ischnocnema bocaina* sp. nov. differs from *I. randorum* by its larger size (*I. bocaina* sp. nov. males 18.6–19.0 mm SVL; *I. randorum* males 11.8–15.0 mm SVL); its smooth venter (weakly granular in *I. randorum*); rounded snout in dorsal and acuminate in lateral views (respectively subovoid and rounded in *I. randorum*); the presence of dentigerous processes of the vomer (absent in *I. randorum*); its slightly expanded vocal sac (greatly expanded in *I. randorum*); its toe lengths formula I < II < V < III < IV (I < II < III < V < IV in *I. randorum*); and the nonpulsed advertisement call with 9–18 notes, note rate 8.1–9.0 notes/s, and dominant frequency 2325–2756 Hz (pulsed single note advertisement call with note rate 1.3–2.6 and dominant frequency 3800–5200 Hz in *I. randorum*; Heyer 1985; Heyer *et al.* 1990). *Ischnocnema bocaina* sp. nov differs from *I. spanios,* its sister species, by its larger size (*I. bocaina* sp. nov. males 18.6–19.0 mm SVL; *I. spanios* males 14.7–15.3 mm SVL); its rounded snout in dorsal and acuminate in lateral views (respectively subovoid and rounded in *I. spanios*); its slightly expanded vocal sac (not expanded in *I. spanios*); the presence of a nuptial pad (absent in *I. spanios*); and its toe lengths formula I < II < V < III < IV (I < II < III = V < IV in *I. spanios*; Heyer 1985; Heyer *et al.* 1990). Finally, *I. bocaina* sp. nov. differs from *I. vizottoi* by its larger size (*I. bocaina* sp. nov. males 18.6–19.0 mm SVL; *I. vizottoi* males 13.3–16.6 mm SVL); its smooth dorsum and venter (respectively slightly rugose and granular in *I. vizottoi*); its rounded snout in dorsal and acuminate in lateral views (respectively sub-elliptical and acuminate-rounded in *I. vizottoi*); its slightly expanded vocal sac (moderately expanded in *I. vizottoi*); its toe lengths formula I < II < V < III < IV (I < II < III = V < IV in *I. vizottoi*); its well-developed calcar tubercle (small or absent in *I. vizottoi*); and its advertisement call with 9–18 notes, call duration 1000–2010 ms and dominant frequency 2325–2756 Hz (advertisement call with a single note, call duration 38–72 ms, and dominant frequency 3417–3763 Hz in *I. vizottoi*; Martins & Haddad 2010).

Genetic distances of mitochondrial 16S rRNA fragment within and between members of the *Ischnocnema lactea* species series are given in Table 1.

Description of holotype. Adult male with vocal slits, subgular vocal sac, and nuptial pads; medium-size (SVL = 18.6 mm). Head slightly longer than wide; head length 36% of the SVL, head width 34% of the SVL; snout rounded in dorsal view, acuminate in lateral view; nostril elliptical, slightly protuberant, oriented laterally, located near the tip of snout; *canthus rostralis* slightly distinct, curved; loreal region slightly concave; two poorly-developed, rounded, postrictal tubercles on each side about the same size; eye protuberant, oriented laterally; eye diameter 42% of head length; palpebral tubercles absent; tympanum barely distinct, rounded; tympanic membrane undifferentiated; annulus present, barely visible externally, its dorsal portion hidden; tympanum diameter 45% of eye diameter; supratympanic fold absent; vocal slits present; vocal sac subgular, distinct, one visible fold on each side of the jaw; tongue large, elliptical, without posterior notch; choanae elliptical, larger than the dentigerous processes of the vomer; dentigerous processes of the vomer round, located posteromedially to choanae, medially separated by a gap approximately the same size as one dentigerous process; vomerine teeth present.

Forelimb slender; palmar tubercle barely distinct, cordiform, its diameter approximately equal that of the thenar tubercle; thenar tubercle barely distinct, elliptical; glandular-appearing nuptial pad on dorsal surface of the thumb, round, poorly-developed, whitish, extending to the posterior half of the thenar tubercle; palm smooth; supernumerary tubercles absent; single subarticular tubercles flat, rounded, large; fingers slender, without fringes; tip of Finger I slightly expanded, rounded; tip of Finger II moderately expanded, truncated; tips of Fingers III and IV fairly expanded, truncated, with ungual flaps indented; Finger I half the size of Finger II; finger lengths formula I < II < IV < III.

TABLE 1. Uncorrected pairwise genetic distances (given in percentage) of mitochondrial 16S rRNA fragment (ca. 600bp) within (in bold) and between members of the *Ischnocnema lactea* species series. Within species distances are in bold. Data are shown as min–max where appropriate.

	I. bocaina sp. nov.	*I. spanios*	*I. nigriventris*	*I. randorum*	*I. concolor*	*I. vizottoi*	*I. melanopygia*	*I. holti*	*I. lactea*
I. bocaina sp. nov.	**NA**								
I. spanios	6.3–6.9	**0.0–1.9** **(*n* = 6)**							
I. nigriventris	13.6–13.7	12.0–12.5	**0.0–0.2** **(*n* = 3)**						
I. randorum	12.1	12.0–12.2	10.0–10.1	**0.0** **(*n* = 2)**					
I. concolor	13.6	12.2–12.7	7.0	8.6	**0.0** **(*n* = 2)**				
I. vizottoi	14.1	12.5–13.1	6.7–7.0	8.9	1.4	**0.0** **(*n* = 2)**			
I. melanopygia	10.7	10.9–11.1	10.5–10.7	9.8	10.0	10.2	**0.0** **(*n* = 2)**		
I. holti	13.6	15.1–15.5	13.9–14.5	12.6	13.7	12.9	12.0	**0.0** **(*n* = 2)**	
I. lactea	13.9–14.8	14.9–16.2	15.5–16.4	12.6–13.2	13.1–14.2	12.9–14.0	12.7–13.2	9.2–10.2	**0.0–2.7** **(*n* = 7)**

Hindlimb slightly robust; shank slightly longer than thigh; tibia length 49% of SVL; thigh length 48% of SVL; moderately-developed, conical calcar tubercle present; tarsal folds absent; foot length 47 % of SVL; inner metatarsal tubercle elliptical, twice as large as outer metatarsal tubercle; outer metatarsal tubercle rounded; sole of foot smooth; supernumerary tubercles absent; single subarticular tubercles present, flat, rounded; toes long, slender, without fringes; tips of Toes I and V slightly expanded, rounded; tips of Toes II–IV moderately expanded, truncated, ungual flaps indented on Fingers III and IV; toe lengths formula I < II < V < III < IV.

Dorsal skin smooth; venter smooth; flanks granulated; posterior portion of ventral surface of thighs coarsely shagreen; discoidal and thoracic folds present.

Coloration of holotype. Dorsum diffused marbled rust, brown, and dark-brown, with two medial white spots at the shoulder girdle (Fig. 4); head with clear brown stripe between eyes; lips with sparse white spots; flanks diffused marbled rust, brown, and dark-brown; posterior half with sparse white spots; dorsal surfaces of the limbs banded brown and dark-brown with sparse rust blotches. Venter, venter surfaces of the limbs, and gular region marbled light-brown, brown, and rust; gular region darker than rest of the ventral region. Posterior surfaces of thighs brown. In preservative, rust becomes nut-brown and all other colors become lighter.

Measurements of holotype (in millimeters). SVL 18.6; HL 6.6; HW 6.3; ED 2.8; TD 1.3; END 1.5; IND 1.8; AMD 3.8; FAL 3.7; HAL 5.3; 3FD 1.1; THL 8.9; TL 9.1; TAL 4.7; FL 8.8; 4TD 0.8.

Variation. The paratopotype is somewhat faded, but it is overall similar to the holotype, except that its dentigerous processes of the vomer are barely distinct (distinct in the holotype). It also lacks the two medial white spots at the shoulder girdle. Measurements of the paratopotype (in millimeters): SVL 19.0; HL 7.3; HW 7.0; ED 2.7; TD 1.1; END 2.1; IND 2.0; AMD 3.4; FAL 3.6; HAL 5.6; 3FD 1.1; THL 9.4; TL 9.4; TAL 5.1; FL 8.8; 4TD 1.0.

Phylogenetic relationships and genetic distances. The Bayesian inference and the maximum likelihood analyses yielded similar topologies (Fig. 1). We recovered the *I. lactea* species series with high support (1.0 of posterior probability and 98% of bootstrap), with *I. bocaina* sp. nov. as the sister species of *I. spanios*, also with high support (1.0 of posterior probability and 98% of bootstrap). The uncorrected pairwise distance of partial 16S rRNA between these two species was among the lowest of all species pairs (6.3–6.9%, Table 1).

Etymology. The specific epithet refers to the Bocaina Mountain Range (*Serra da Bocaina*, in Portuguese), where the type locality of the species is located, in recognition of the great biodiversity importance of this mountain range. The name is used here as a noun in apposition.

Vocalization. We recorded two types of calls, call 1 (*n* = 11; Fig. 5A; Table2) and call 2 (*n* = 10; Fig. 5B; Table 2). The first one was composed of 9 to 18 non-pulsed notes ($\overline{X}$ = 15.6 ± 2.3), with the energy gradually increasing in each note through the call, until reaching a peak typically on the penultimate or last note (Fig. 5A). Call 1 duration ranged from 1000 to 2010 ms ($\overline{X}$ = 1760 ± 260) and call rise time ranged from 91.6–99.4% ($\overline{X}$ = 97.8 ± 2.2) of the call. Note repetition rate was 8.1–9.0 notes/s ($\overline{X}$ = 8.3± 0.3) and note repetition rate acceleration ranged from –13.2 to 0.2 ($\overline{X}$ = –6.1 ± 4.4). Dominant Frequency was 2325–2756 Hz ($\overline{X}$ = 2576 ± 146). Call 2 was composed of one non-pulsed note. with duration ranging from 13 to 14 ms ($\overline{X}$ = 13.7 ± 0.0) and Dominant Frequency ranging from 2756–2842 Hz ($\overline{X}$ = 2834 ± 27). We considered call 1 the advertisement call and call 2 the territorial call because of the similarities of each call with the advertisement and territorial calls of *I. nigriventris* (Berneck *et al.* 2013).

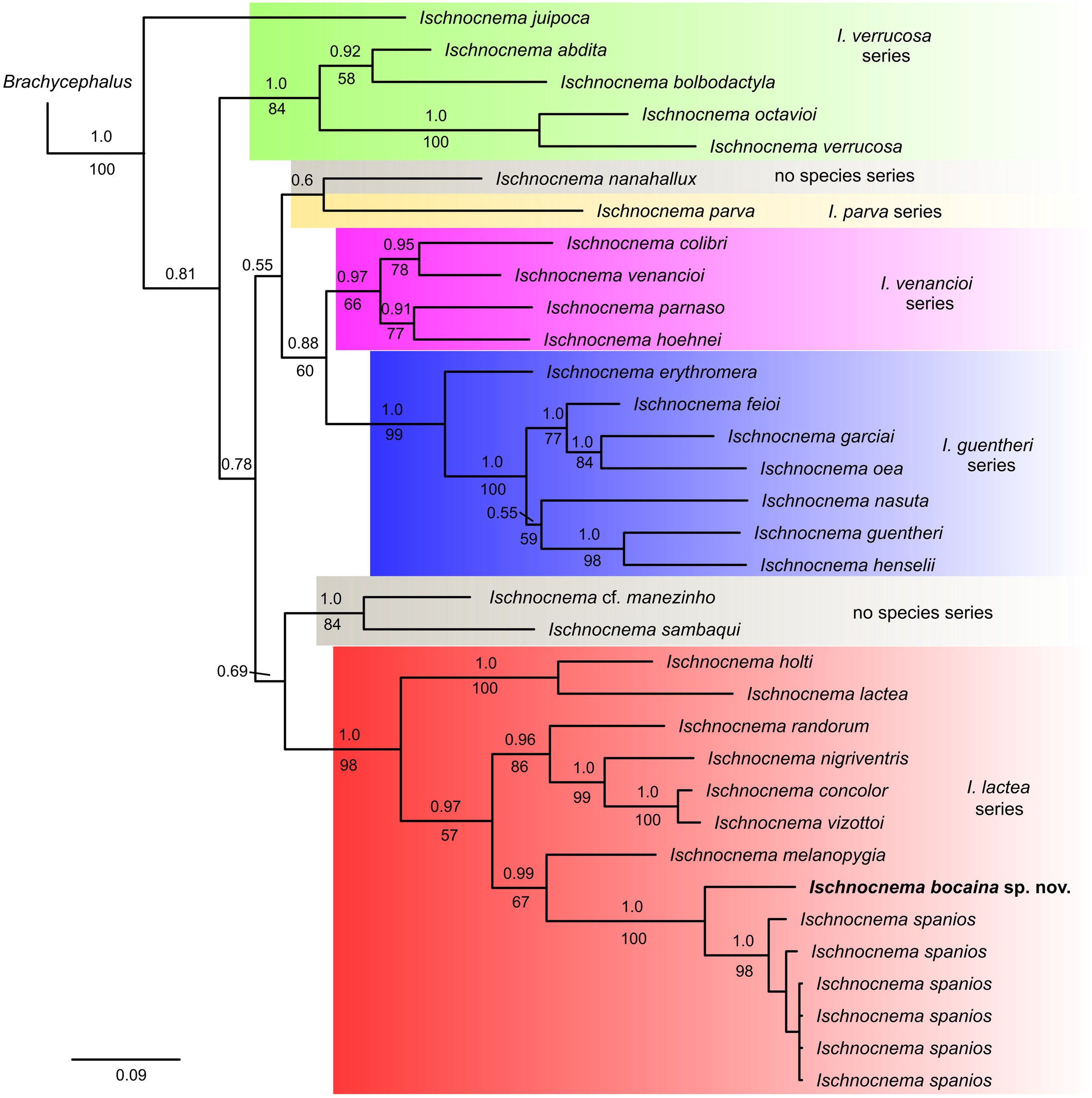

FIGURE 1. The 50% majority rule consensus tree from Bayesian inference of mitochondrial 16S rRNA gene (16S) showing the relationships within the *Ischnocnema lactea* species series. Numbers above branches indicate posterior probabilities and numbers below branches indicate maximum likelihood nonparametric bootstrap values. We only show bootstrap values above 50%.

FIGURE 2. Dorsal (left) and ventral (right) views of *Ischnocnema bocaina* **sp. nov.** holotype (MZUSP 138663). Scale bar = 10 mm. *Photos by B. F. Zaidan.*

TABLE 2. Call parameters comparing the members of the *Ischnocnema lactea* species series. Data are given as ranges (mean ± SD) where appropriate.

	I. bocaina **sp. nov.**		*I. nigriventris*		*I. lactea*	*I. randorum*	*I. vizottoi*
	Call 1*	Call 2	Call 1*	Call 2	Call 1*	Call 1*	Call 1*
Notes per call	9–18 (15.6 ± 2.3)	1	2–4	1	1	3–8	1
Call duration (ms)	1000–2010 (1760 ± 260)	13–14 (13.7 ± 0.0)	194–565	30–41	635–1060	2000–5000	38–72 (52.7 ± 10.2)
Note rate (notes/s)	8.1–9.0 (8.4±0.3)	–	–	–	–	1.3–2.6	–
Note rate acceleration (%)	-13.2 to 0.2 (-6.1 ± 4.4)	–	–	–	–	–	–
Call rise time (%)	91.6–99.4 (97.8–2.2)	–	–	–	–	–	–
Dominant Frequency (Hz)	2325–2756 (2576 ± 146)	2756–2842 (2834 ± 27)	1955–3932	2004–3685	2240–2756	3800–5200	3417–3763 (3611 ± 103)
Pulsed/non-pulsed	Non-pulsed	Non-pulsed	Non-pulsed	Non-pulsed	Pulsed	Pulsed	Non-pulsed

*Advertisement call

Natural history notes. The collectors found the two type specimens calling perched on trees about 50 cm above the ground, right after a heavy rain. The call activity started about one hour before dusk, together with two other *Ischnocnema* species from the *I. guentheri* and *I. parva* species series, but the new species ceased to call earlier, about one hour after dusk.

Geographic distribution. *Ischnocnema bocaina* **sp. nov.** is known only from the type locality: Estação Ecológica do Bananal, municipality of Bananal, state of São Paulo, southeastern Brazil (Fig. 6).

FIGURE 3. Lateral view of the head (above), ventral view of the foot (below, left), and ventral view of the hand (below, right) of *Ischnocnema bocaina* **sp. nov.** holotype (MZUSP 138663). Scale bar = 2 mm. *Photos by B. F. Zaidan.*

FIGURE 4. Live specimen of *Ischnocnema bocaina* **sp. nov.**, holotype (MZUSP 138663). *Photo by P. C. A. Garcia.*

Discussion

Since the proposition of the *Ischnocnema lactea* species series (Hedges *et al.* 2008), its content has changed significantly (see introduction) and the morphological definition of the series is outdated. However, we recovered *I. bocaina* within the *I. lactea* series as it is currently recognized with high support in both analyses, as the sister species of *I. spanios*. This result shows that the new species undoubtedly should be allocated to the *I. lactea* series. Nine of the eleven species of the *I. lactea* species series have their phylogenetic position known, but *I. gehrti* and *I. paranaensis* are still tentatively assigned to it. Both species are known only from their holotypes (*I. gehrti* holotype poorly preserved; Pombal Jr. & Cruz 1999), and a taxonomic revision and reevaluation of the morphological characters supporting the *I. lactea* species series is needed in order to clarify the phylogenetic affinities of these two species. Moreover, efforts towards the rediscovery of *I. gehrti* and *I. paranaensis* in their type localities and surroundings are paramount for getting molecular, acoustic, and new morphological data from these two poorly known species.

In addition to recovering the *I. lactea* series monophyletic, we also recovered the *I. venancioi* and *I. guentheri* series monophyletic, both with moderate to high support (0.97 of posterior probability and 66% of bootstrap to the former and 1.0 of posterior probability and 99% of bootstrap to the latter). Unlike previous studies (Canedo & Haddad 2012; Taucce *et al.* 2018a; b), we did not recover the *I. verrucosa* series monophyletic, and the relationships within the species series, despite overall similar, are not the same as previous studies. These differences are probably due to our small matrix (one mitochondrial fragment, final alignment of 554 bp) and, since we only wanted to position *I. bocaina* within the *I. lactea* series, discussing these relationships is beyond the scope of this paper. The only member of the *I. parva* series present in our phylogenetic analyses was *I. parva*.

The nuptial pad has been an important character when it comes to the taxonomy of *Ischnocnema.* It is a highly variable character in the genus, and the presence of a single large, conspicuous, glandular-appearing nuptial pad was proposed to be a putative synapomorphy of the clade composed by the *I. parva*, the *I. venancioi*, and the *I. guentheri* species series (Taucce *et al.* 2018b). In the *I. lactea* species series it can be absent (*I. spanios*), whitish and poorly-developed (*I. bocaina* sp. nov., *I. randorum*), double (*I. lactea*; Silva-Soares *et al.* 2018), and translucent (*I. nigriventris, I. holti*). The character state is unknown in *I.* concolor, but it is present in some males (Targino *et al.* 2009).

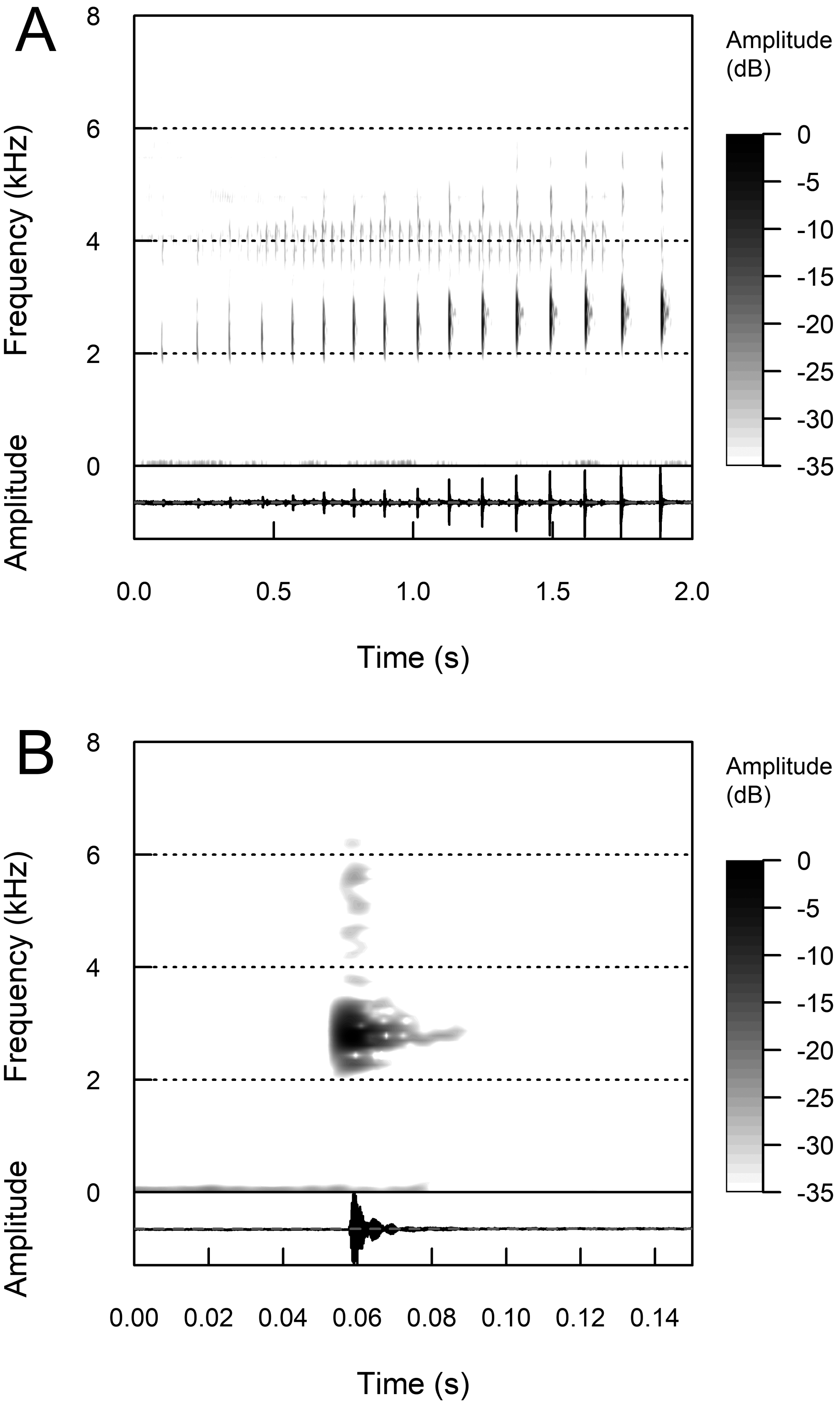

FIGURE 5. Spectrogram (above) and oscillogram (below) of the two types of calls of *Ischnocnema bocaina* **sp. nov.**: (A) advertisement call and (B) territorial call. Respectively recordings CBUFMG 1017 and 1016, 18 January 2008.

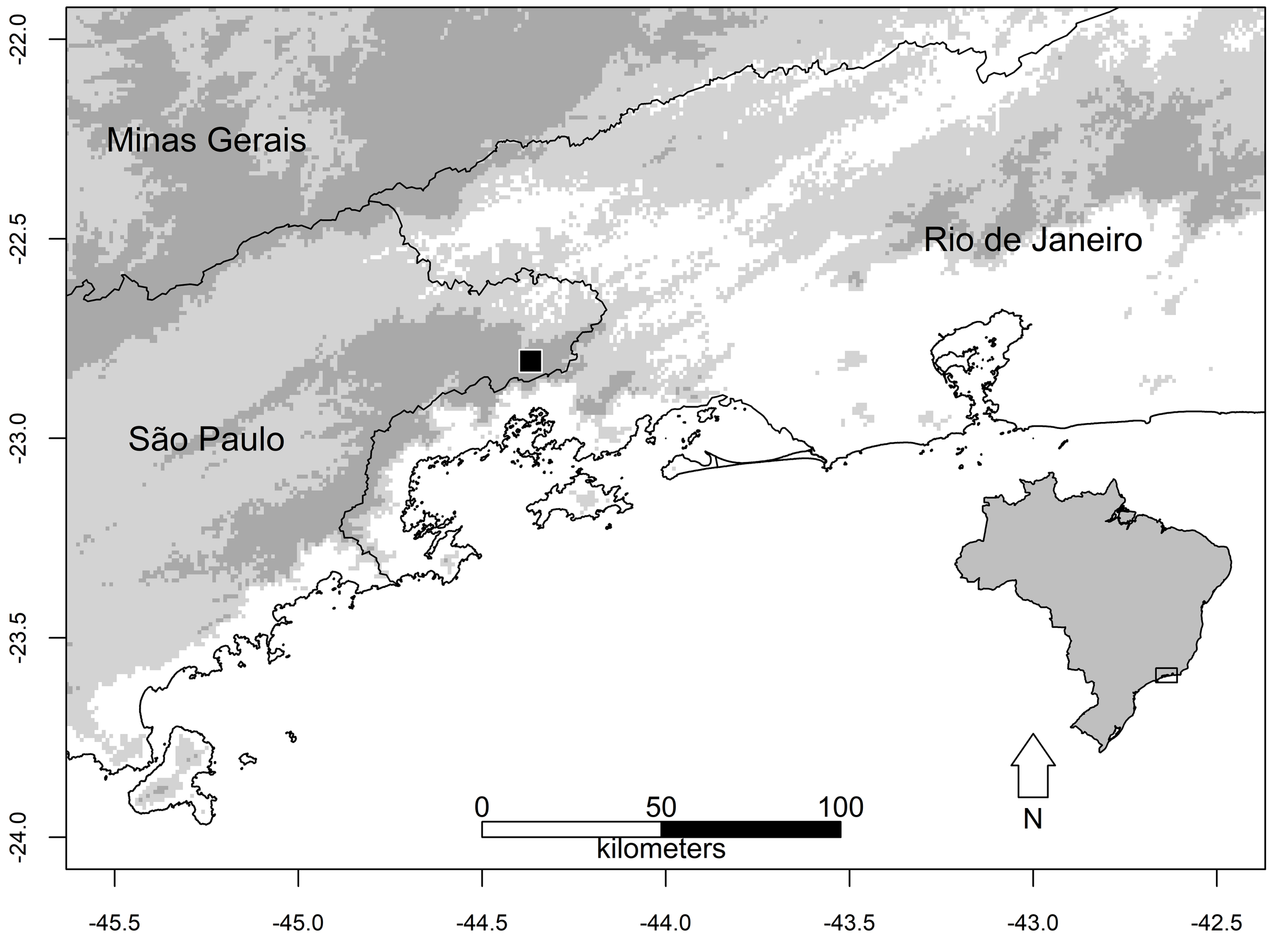

FIGURE 6. Map showing the type locality of *Ischnocnema bocaina* **sp. nov.** (indicated by a black square): Estação Ecológica do Bananal, municipality of Bananal, state of São Paulo, southeastern Brazil. Elevations above 500 and 1000 m are shaded gray.

Martins & Haddad (2010) state that it is absent in *I.* vizottoi, but we examined the type series and it is present and translucent in several males. When the nuptial pad is not large and conspicuous it may be difficult to see, especially when it is translucent, and we recommend care on checking this character on the specimens. It may be easier to see this character after drying the thumb of the specimens a little bit. Understanding the character states of the nuptial pads throughout the genus *Ischnocnema*, both macroscopically and microscopically, will be paramount for the understanding of the taxonomy and the phenotypic evolution of the genus.

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APPENDIX I. List of terminals and accession numbers of sequences used in the phylogenetic and genetic distance analyses.

Species	GenBank ID	In the phylogenetic matrix?	In the genetic distance matrix?
Brachycephalus pombali	HQ435700	yes	no
Brachycephalus ephippium	HQ435693	yes	no
Ischnocnema abdita	JX267472	yes	no
Ischnocnema bocaina	MK967680	yes	yes
Ischnocnema bolbodactyla	JX267476	yes	no
Ischnocnema colibri	MH538418	yes	no
Ischnocnema concolor	JX267366	yes	yes
Ischnocnema concolor	JX267493	no	yes
Ischnocnema erythromera	JX267340	yes	no
Ischnocnema feioi	MF957166	yes	no
Ischnocnema garciai	MF957163	yes	no
Ischnocnema guentheri	JX267503	yes	no
Ischnocnema henselii	JX267478	yes	no
Ischnocnema hoehnei	JX267507	yes	no
Ischnocnema holti	JX267306	yes	yes
Ischnocnema holti	JX267509	no	yes
Ischnocnema juipoca	JX267349	yes	no
Ischnocnema lactea	JX267342	yes	yes
Ischnocnema lactea	JX267308	no	yes
Ischnocnema lactea	JX267514	no	yes
Ischnocnema lactea	JX267515	no	yes
Ischnocnema lactea	JX267516	no	yes
Ischnocnema lactea	JX267517	no	yes
Ischnocnema lactea	JX267518	no	yes
Ischnocnema cf. *manezinho*	JX267481	yes	no
Ischnocnema melanopygia	JX267292	yes	yes
Ischnocnema melanopygia	JX267519	no	yes
Ischnocnema nanahallux	KC569986	yes	no

......continued on the next page

APPENDIX I. (Continued)

Species	GenBank ID	In the phylogenetic matrix?	In the genetic distance matrix?
Ischnocnema nasuta	JX267311	yes	no
Ischnocnema nigriventris	JX267483	yes	yes
Ischnocnema nigriventris	JX267482	no	yes
Ischnocnema nigriventris	JX267359	no	yes
Ischnocnema octavioi	JX267521	yes	no
Ischnocnema oea	JX267313	yes	no
Ischnocnema parnaso	MH538421	yes	no
Ischnocnema parva	KY399230	yes	no
Ischnocnema randorum	JX267361	yes	yes
Ischnocnema randorum	JX267381	no	yes
Ischnocnema sambaqui	JX267531	yes	no
Ischnocnema spanios	JX267490	yes	yes
Ischnocnema spanios	JX267489	yes	yes
Ischnocnema spanios	JX267488	yes	yes
Ischnocnema spanios	JX267487	yes	yes
Ischnocnema spanios	JX267486	yes	yes
Ischnocnema spanios	JX267485	yes	yes
Ischnocnema venancioi	JX267321	yes	no
Ischnocnema verrucosa	JX267538	yes	no
Ischnocnema vizottoi	JX267350	yes	yes
Ischnocnema vizottoi	JX267352	no	yes

APPENDIX II. Specimens examined (all from Brazil).

Ischnocnema holti: MINAS GERAIS: Itatiaia: MZUSP 97343–97371

Ischnocnema nigriventris: SÃO PAULO: Paranapiacaba, municipality of Santo André: CFBH 23478–23479; Parque das Neblinas, municipality of Bertioga: CFBH 28951–28952.

Ischnocnema randorum: SÃO PAULO: Boracéia, municipality of Salesópolis: MZUSP 23665–23670, 36865, 37555, 49644 (paratypes).

Ischnocnema spanios: SÃO PAULO: Carlos Botelho: MZUSP 135942; Jaceguava: MZUSP 135942; Mogi das Cruzes: MZUSP 133910–133913; Paranapiacaba, municipality of Santo André: MZUSP 142792–142795, 145271–145272, 145255, 145275–145277, 145263, 153754–153764; Furnas, muncipality of Piedade: MZUSP 136443–136449.

Ischnocnema vizottoi: SÃO PAULO: Campos do Jordão: CFBH 8205–8221 (paratypes).