

Call variation and vocalizations of the stealthy litter frog *Ischnocnema abdita* (Anura: Brachycephalidae)

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Abstract. *Ischnocnema abdita* is a small-sized litter frog belonging to the *I. verrucosa* species series and only known for mountainous areas of southeastern Espírito Santo State, Brazil, in the Municipalities of Santa Teresa (type locality), Cariacica and Mimoso do Sul. In this paper, we describe the calls and provide estimates of within-male variation of *I. abdita* from its type locality and from a recently discovered population in the region of Alto Caparaó, Municipality of Espera Feliz, Minas Gerais State, Brazil. Additionally, we also performed a GMYC analysis of molecular assignment that recovered the haplotypes of *I. abdita* from its type locality and from the new record (Alto Caparaó) under the same taxonomical entity. Our bioacoustical analysis revealed two distinct types of calls, herein referred as A and B calls. The A call was observed in both populations, whereas the B call was only recorded at Alto Caparaó. Despite the apparent similarity in the A calls from both localities, we observed differences in all traits analyzed. Moreover, each call trait expressed variation within males. The peak frequency never exceeded 5% variation and it was classified as static in both populations. Temporal parameters, such as call duration and interval between calls were classified either as dynamic or intermediate, with variations ranging from 1.8-66.1% within males. Although number of pulses per note was a dynamic trait at the type locality, it did not vary in both types of call recorded at Alto Caparaó.

Keywords. Systematics, Taxonomy, Bioacoustics, molecular assignment, general mixed Yule-coalescent (GMYC) model.

INTRODUCTION

Mate attraction has been associated with anuran vocalizations for more than a century (Courtis, 1907; Miller, 1909). The specificity of these calls is known for over fifty years (Blair, 1955, 1958; Martof, 1961) and their taxonomic role has been assessed several times (Wells,

1977; Gerhardt, 1982; Cocroft and Ryan, 1995; Robillard et al., 2006). With the recent advances in technology and the popularization of high quality recorders, ethological studies of mating calls increased rapidly in the past decades (Gerhardt, 1998; Rand, 2001; Gerhardt and Huber, 2002; Bruyninckx, 2015). Moreover, the degrees of call variation within and between species is now documented

for several species (Castellano and Giacoma, 2000; Tárano, 2001; Castellano et al., 2002; Kaefer and Lima, 2012; Klymus et al., 2012; Carvalho et al., 2015; Jansen et al., 2016; Miranda et al., 2016), including the genus *Ischnocnema* Reinhardt and Lütken (i.e., *I. guentheri* and *I. henselii* Kwet and Solé, 2005; *I. izecksohni* Taucce et al., 2012).

The genus *Ischnocnema* currently includes 33 species distributed in central and eastern Brazil and northern Argentina, most species being associated with the Brazilian Atlantic Forest (Canedo and Haddad, 2012; Frost, 2016). Although traditionally grouped into five species series (i.e., *I. guentheri*, *I. lactea*, *I. parva*, *I. ramagii* and *I. verrucosa*; Hedges et al., 2008), some species of the *I. guentheri*, *I. lactea*, *I. parva* and *I. verrucosa* series were clustered with different groups in the most recent phylogenetic study of the genus (Canedo and Haddad, 2012). Among these species, *Ischnocnema abdita* Canedo and Pimenta, 2010 and *I. bolbodactyla* (Lutz, 1925), which had previously been assigned to the *I. lactea* species series (Hedges et al., 2008; Canedo et al., 2010; Canedo and Pimenta, 2010), were relocated to the *I. verrucosa* series. Consequently, the *I. lactea* and *I. verrucosa* groups lost their morphological diagnoses (i.e., *I. lactea* species series was previously recognized by at least the outer digital discs of fingers moderate to large, whereas digital discs in species of the *I. verrucosa* series are small; Hedges et al. 2008). Currently, these two species series together are composed of 18 species (Canedo and Haddad, 2012; Padial et al., 2014), but the calls of only seven of them have been described up to date (i.e., *Ischnocnema verrucosa* series: *I. bolbodactyla*, *I. juipoca* (Sazima and Cardoso, 1978), *I. penaxavantinho* Giaretta et al., 2007; *I. lactea* series: *I. nigriventris* (Lutz, 1925), *I. randorum* (Heyer, 1985) and *I. vizottoi* Martins and Haddad, 2010).

Ischnocnema abdita is a small-sized litter frog belonging to the *I. verrucosa* species series and only known for mountainous areas of southeastern Espírito Santo State, Brazil, in the Municipalities of Santa Teresa (type locality), Cariacica and Mimoso do Sul (Canedo and Pimenta, 2010; Canedo and Haddad, 2012). Aside from its calling microhabitat (i.e. hidden at the base of bushes), there is no further information on the natural history of this species. The present study aims to (1) report a newly discovered population and new State record for *I. abdita* from the region of Alto Caparaó, Municipality of Espera Feliz, Minas Gerais State, Brazil; (2) investigate the taxonomic identity of this population through the analysis of molecular assignment; (3) describe and compare the calls of the newly discovered population and those from the type locality (i.e., Municipality of Santa Teresa, State of Espírito Santo); and (4) evaluate the degree of variation in bioacoustic traits within and between populations.

MATERIALS AND METHODS

Hypothesis test on molecular assignment

We sampled four individuals (MZUFV 15919, 15920, 15922 and 15923) from the Municipality of Espera Feliz, Serra do Caparaó region, Minas Gerais State (20°38'S, 41°53'W, 921 m a.s.l.) and used the mitochondrial partial sequence of 16S rRNA, amplified with the primers 16sAR (5'-CGCCTGTTTATCAAAAACAT-3'; Palumbi et al., 1991) and 16sWilk2 (3'-GACCTGGATTACTCCGGTCTGA-5'; Wilkinson et al., 1996), plus M13 tail. This marker was chosen based on its good performance as barcode for amphibians (Vences et al., 2005). The choice also considered availability of GenBank sequences for species of the *I. verrucosa* species group for comparative purposes. Our dataset comprised 17 sequences from five species, which also included samples of *I. abdita* from its type locality, plus an outgroup (*I. izecksohni*). Fragments were pre-aligned using ClustalW algorithm (Larkin et al., 2007) implemented with MEGA7 software (Kumar et al., 2016). Gaps open were penalized 10 times more than gaps extension (see Giribet and Wheeler, 1999) and final alignment was handmade. Establishment of primary homologies in the regions of ambiguous alignment of rRNA is not trivial (Gillespie, 2004); therefore, we opted for the exclusion of one of these regions with 85pb and final alignment had 511pb. Since *Ischnocnema abdita* from its type locality showed a distinct haplotype from that of *I. cf. abdita* from Caparaó and Espera Feliz, we did not discard the 'distinct species hypothesis'. Hence, we tested against the 'same species hypothesis'. Intraspecific distances were estimated only to *I. abdita* and *I. juipoca*, since only one individual represented each of the other species.

An ultrametric and full bifurcated mitochondrial gene tree was generated using a four-step procedure. In the first step, we excluded repeated haplotypes, maintaining only one copy. Then, we selected a model from a set of best models of DNA evolution estimated by jModelTest 2.1.8 for dataset (Darrriba et al., 2012). The selection was made using the corrected Akaike information criteria, in which all models with $\Delta AICc < 2$ were considered significantly supported (Burnham and Anderson, 2002; Burnham and Anderson, 2004). GTR+G was the second-best model selected ($\Delta AICc = 0.497$) and chosen to be applicable to all subsequent analyses. Third step was the estimation of a topology reliable and free of polytomies. For this purpose, we performed a maximum likelihood analysis on RAxML 8.2.4 (Stamatakis, 2014) choosing the 'best tree' under GTRGAMMA model, and performing a bootstrap support test with 1000 replicates. This analysis was repeated five times using distinct random seeds to verify topology, branch lengths and likelihood congruencies in estimated trees. One of these trees was chosen since all of them were virtually indistinguishable. In the last step, we made this gene tree ultrametric in MrBayes 3.2.6 (Ronquist et al., 2012) leaving mutation rates analysis adjustable. The search was made under five independent runs, 10 Markov chains with default heating value and 10^6 generations each run. Trees were sampled each 5000 generations and the final consensus tree was calculated from the last 75% retained trees. The convergence of runs was graphically evaluated in Tracer 1.6

software (Rambaut et al., 2014). This approach ensured a final tree with little variance in branch lengths.

The hypothesis test per se was made submitting the ultrametric tree to a maximum likelihood general mixed Yule-coalescent test, implemented in GMYC (Fujisawa and Barraclough, 2013) using single threshold method. This method was designed to single-locus and is intended to identify the limit between tokogeny and phylogeny, fitting the branches in a gene tree under models about these relationship patterns. GMYC performs a log-likelihood ratio (LR) test of fitted multiple species model against a null hypothesis of one species in gene tree (Fujisawa and Barraclough, 2013). Hypothesis was validated through calculations of intraspecific and interspecific K-2p distances (Kimura, 1980) in MEGA7 software (Kumar et al., 2016), with taxonomical units identified by GMYC as references. We expected interspecific distances greater than intraspecific ones (Hebert et al., 2003).

Bioacoustical analysis

We analyzed 36 calls of four individuals (MNRJ 34902, 34904, 34905 and 34906; paratypes) recorded at Estação Biológica de Santa Lúcia, Municipality of Santa Teresa, Espírito Santo State, Brazil, type locality of *Ischnocnema abdita* (19°57'S, 40°31'W; 650 m a.s.l.), on 14 and 16 January 2004, between 15:15 and 15:50. Calls were recorded with a Panasonic RQ-L31 cassette tape recorder coupled to a Leson SM-48 cardioid microphone. Vocalizations were digitized using software Avisoft SASLab Light, version 4.39, at a sampling rate of 22050 Hz and a resolution of 16 bits. We also recorded 95 calls of four individuals of *Ischnocnema abdita* from Serra do Caparaó region, Municipality of Espera Feliz, Minas Gerais State (20°38'S, 41°53'W, 921 m a.s.l.), on 10 March 2013. Although we collected some calling individuals at this locality (i.e., MZUFV 15919, 15920, 15921, 15922 and 15923), we were unable to relate them to any sound recording. Recordings were made with a Tascam DR-40 digital recorder, at sampling rate of 44100 Hz and a resolution of 24 bits. Voucher specimens are deposited at Museu Nacional do Rio de Janeiro (MNRJ), Municipality of Rio de Janeiro, Rio de Janeiro State, and at Museu de Zoologia João Moojen (MZUFV), Universidade Federal de Viçosa, Municipality of Viçosa, Minas Gerais State, Brazil.

Call measurements were made with the software Raven Pro 1.5 (Bioacoustics Research Program, 2014). Spectrogram was generated using window size = 512 samples, overlap = 70%; hop size = 3.49 ms; DFT size = 1024 samples and; and grid spacing = 43.1 Hz. Sound graphics were obtained using Seewave (Sueur et al., 2008) package of R platform (R Core Team, 2015) with the following settings: FFT = 512 samples and 70% overlap. Parameters measured were call duration (CD), call rate (CR), interval between calls (CI), number of notes per call (NN), note duration (ND), note rate (NR; given as notes/min), interval between notes (NI), number of pulses per note (PN), pulse rate (PR; given as pulses/sec), dominant frequency range (DF) and peak frequency (PF). Temporal parameters were measured directly from the oscillogram. Following Rocha et al. (2016), the DF represent the most energetic band of the call and is given

in range from the lowest value of “Frequency 5%” to the highest value of “Frequency 95%”. The PF was acquired through the parameter “Peak Frequency” and represents the frequency that is coincident with the peak of energy within the call. Further call terminology follows that of Toledo et al., 2015b. Results are presented as mean \pm standard deviation and range.

We performed t-tests for comparative issues. Normality and Levene's homogeneity test of variance were conducted for all sets of variables. When variables distribution deviate from normality curve, we \log_{10} -transformed them (results preceded by *), and when variables had heterogeneous variances, we conducted tests with separate variance estimates (results preceded by †). All statistical tests were conducted through Statistica v. 7.1.

Ischnocnema abdita is one of the species of the *I. lactea* and *I. verrucosa* series that have been reallocated between groups in recent papers (Heinicke et al., 2007; Hedges et al., 2008; Canedo and Haddad, 2012). Furthermore, the species *I. manezinho* Garcia, 1996 and *I. sambaqui* Castanho and Haddad, 2000, originally allocated tentatively in the *I. lactea* species series (Garcia, 1996; Castanho and Haddad, 2000), were not included in the phylogeny from Hedges et al. (2008) and are not currently assigned to any species series (Canedo and Haddad, 2012). Therefore, we decided to compare the call of *I. abdita* from the type locality with the species from both *I. lactea* and *I. verrucosa* groups plus the species *I. manezinho* and *I. sambaqui*. Moreover, the calls of *I. penaxavantino* and *I. sambaqui* were described as a single multi-pulsed note emitted at irregular intervals (Giaretta et al., 2007; Castanho and Haddad, 2000; respectively). However, we considered their calls as sequences of notes (notes = temporally discrete vocalization units composing the call; sensu Toledo et al., 2015b) based on their resemblance with other calls described for *Ischnocnema*.

Within-male variation

Estimates of within-male variation were made through coefficient of variation [(CV = SD/Mean) x 100]. CV was calculated for each male and results are expressed as means for each trait following previous authors (Gerhardt, 1991; Táranó, 2001; Carvalho et al., 2013; Miranda et al., 2016). Call traits with low variability (i.e. usually less than 5%) were classified as static; and those with relatively high variability (i.e. usually more than 12%) were classified as dynamic. Parameters with values between 5% and 12% were considered intermediate (Gerhardt, 1991; Miranda et al., 2016).

RESULTS

Molecular identification

The GMYC analysis returned six species, with a confidence interval from three to 10. Haplotypes of *Ischnocnema abdita* from its type locality, Caparaó and Espera Feliz were recovered under the same taxonomical entity (Fig. 1). The null hypothesis of no distinct species in the

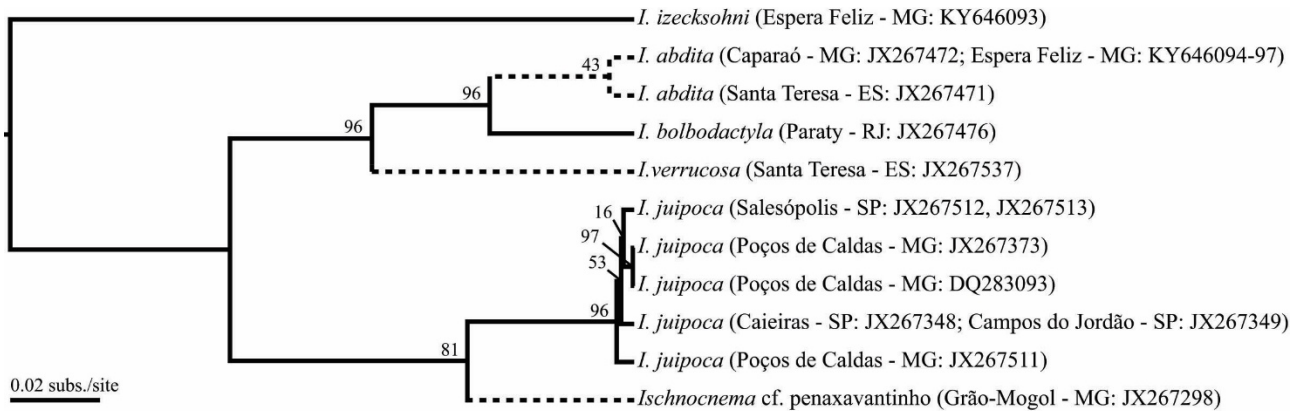


Fig. 1. Ultrametric gene tree of unique 16S haplotypes. GMYC identified lineages are alternating continuous and dashed branches. Numbers above nodes are the bootstrap supports estimated in maximum likelihood analysis. Information among parenthesis is the municipality where haplotypes were collected and GenBank accession numbers, respectively. ES = Espírito Santo State, MG = Minas Gerais State, SP = São Paulo State, RJ = Rio de Janeiro State.

global gene tree, including the outgroup, was not rejected (LR test: $P = 0.055$). This is probably a consequence of the low number of individuals sampled per species (Fujisawa and Barraclough, 2013). The six recovered entities were *I. izecksohni* (outgroup), *I. abdita*, *I. bolbodactyla*, *I. juipoca*, *I. cf. penaxavantino* and *I. verrucosa* (Fig. 1).

Intraspecific distances of *Ischnocnema abdita* were 0.003 (s.e. = 0.001, $n = 6$) and 0.004 of *I. juipoca* ones (s.e. = 0.002, $n = 7$). The mean of interspecific distances, excluding outgroup, was 0.125 ± 0.033 (minimum 0.068 between *I. abdita* and *I. bolbodactyla*; maximum 0.159 between *I. bolbodactyla* and *I. juipoca*); such value was approximately 32 times greater than the mean of intraspecific distances. This result supports the GMYC assignment.

Bioacoustical analysis

Santa Teresa (type locality)

A single type of call was observed in the recordings (Table 1). It is a call composed of a single type of pulsed note (Fig. 2A) emitted in sequences of 4–21 calls at mean rate of 6.2 ± 2.95 calls per min (CR = 2.9–8.5 calls/min; $n = 3$ individuals) and intervals of 9.0 ± 5.1 sec between calls (CI = 3.2–25.7 sec; $n = 30$ calls). Each note had three pulses on average (PN = 2.7 ± 0.4 , 2–3) emitted at mean rate of 36.3 ± 4.3 pulses per second (PR = 29.7–48.4 pulses/sec) and duration of 76.2 ± 13.5 , 54–101 ms (ND = CD). Dominant frequency ranged from 2781 to 4046 Hz with the peak frequency around 3500 Hz (PF = 3543 ± 126.9 , 3343–3812 Hz).

Espera Feliz (Fig. 3)

Recordings from this locality had two types of calls (Table 1). The most common type of call (hereafter referred as A call: 58.9%, $n = 56$ calls) was composed of a single type of pulsed note that is similar to the notes recorded at the type locality (Fig. 2B) and it is likely to be the advertisement call of *Ischnocnema abdita* (sensu Toledo et al., 2015b). This type of call was emitted in sequences of 1–16 calls at mean rate of 2.4 ± 1.1 calls per minute (CR = 1.2–3.8 calls/min; $n = 6$), and interval between calls was 43.6 ± 16.3 (CI = 14.7–76.9 sec; $n = 39$). Each note had mean duration of 41.5 ± 4.0 ms (ND = CD = 33–49 ms) and two pulses on average (PN = 2.01 ± 0.13 ; 2–3) emitted at a mean rate of 48.6 ± 4.9 pulses per second (PR = 40.8–60.6 pulses/sec). Dominant frequency ranged from 2670 to 4306 Hz, with peak frequency around 3200 Hz (PF = 3250 ± 163.5 ; 2971–3488 Hz).

The second and less common type of call (B call: 40.1%, $n = 38$) is usually composed of two pulsed notes, each note similar to the A call (Fig. 2C; Table 1). Although we also heard calls with three notes in the field, we were unable to record them. The B call was emitted in sequences of 1–13 calls emitted at mean rate of 2.5 ± 0.9 calls per minute (CR = 1.7–3.4 calls/min; $n = 3$). Mean duration of B call was 258.1 ± 34.7 ms (CD = 205–346 ms) and interval between calls was 29.9 ± 14.5 seconds on average (CI = 13.4–71.5 sec; $n = 22$). The first note was longer than the second one (ND₁ = 48.2 ± 13.6 , 31–68 ms; ND₂ = 35.7 ± 2.6 ; 30–42 ms) and had two or three pulses, whilst the second note always had two pulses. First note had lower pulse rate than the second (PR₁ = 50.7 ± 6 , 44.1–64.5; PR₂ = 56.2 ± 4.3 , 47.6–66.6 pulses/sec). Mean interval between notes was 174.3 ± 30 ms (NI = 137–266 ms) and note rate was 7.8 ± 1.04 notes per

Table 1. Call traits of *Ischnocnema abdita* from the Municipality of Santa Teresa, Espírito Santo State, Brazil (n = 36 calls from four individuals) and from the Municipality of Espera Feliz, Minas Gerais State, Brazil (n = 95 calls from four individuals). See materials and methods section for trait acronym definitions.

	Santa Teresa		Espera Feliz	
	A call		B call	
NN	1	1	2	
CD (ms)	76.2 ± 13.5 (54-101) n = 32	41.5 ± 4.0 (33-49) n = 54	258.1 ± 34.7 (205-346) n = 39	
CR (calls/min)	6.2 ± 2.95 (2.9-8.5) n = 3	2.4 ± 1.1 (1.2-3.8) n = 6	2.5 ± 0.9 (1.7-3.4) n = 3	
CI (s)	9.0 ± 5.1 (3.2-25.7) n = 30	43.6 ± 16.3 (14.7-76.9) n = 39	29.9 ± 14.5 (13.4-71.5) n = 22	
ND (ms)	76.2 ± 13.5 (54-101) n = 32	41.5 ± 4.0 (33-49) n = 54	48.2 ± 13.6 (31-68) 35.7 ± 2.6 (30-42) n = 39	
NR (notes/s)	-	-	7.8 ± 1.04 (5.8-9.7) n = 39	
NI (ms)	-	-	174.3 ± 30 (137-266) n = 39	
PN	2.7 ± 0.4 (2-3) n = 34	2.01 ± 0.13 (2-3) n = 56	2.4 ± 0.5 (2-3) 2 ± 0 (2-2) n = 39	
PR (pulses/s)	36.3 ± 4.3 (29.7-48.4) n = 32	48.6 ± 4.9 (40.8-60.6) n = 54	50.7 ± 6 (44.1-64.5) 56.2 ± 4.3 (47.6-66.6) n = 39	
DF (Hz)	2781-4046 n = 33	2670-4306 n = 56	2756-3962 2670-4134 n = 39	
PF (Hz)	3543 ± 126.9 (3343-3812) n = 30	3250 ± 163.5 (2971-3488) n = 53	3462 ± 144.2 (3186-3789) 3454 ± 129.4 (3186-3703) n = 38	

second on average (NR = 5.8–9.7 notes/sec). Dominant frequency ranged from 2756 to 3962 Hz in the first note and from 2670 to 4134 Hz in the second one. However, there was no significant difference in the peak frequency between notes of B call. (PF₁ = 3462 ± 144.2, 3186–3789; PF₂ = 3454 ± 129.4, 3186–3703 Hz).

Statistical analysis

The A call from the type locality (i.e., most common type of call, composed of a single type of pulsed note) was emitted at a higher rate (*t = 2.587; df = 7; P = 0.036) and with shorter intervals than the calls recorded at Espera Feliz (t = 11.188; df = 67; P < 0.001). Notes at the type locality had longer duration (t = 17.626; df =

84; P < 0.001), one more pulse per note (†t = 9.099; df = 36.6; P < 0.001) and pulses emitted at lower rate than at Espera Feliz (t = 11.741; df = 84; P < 0.001). Ultimately, the peak of energy was higher at the type locality (†t = 9.093; df = 73.1; P < 0.001).

The B call (i.e., less common type of call, composed of two pulsed notes) was observed only in the recordings from Espera Feliz. Within the call, the first note was longer (†t = 5.584; df = 40.9; P < 0.001) and had a lower pulse rate (*†t = 4.884; df = 66.2; P < 0.001). There were no significant differences in both notes' peak frequency (t = 0.252; df = 74; P = 0.801).

Despite the apparent similarity in the oscillogram and spectrogram of A and B calls from Espera Feliz (Fig. 2B–C), we observed several differences between the notes of the different calls (Table 1). The B call is emitted

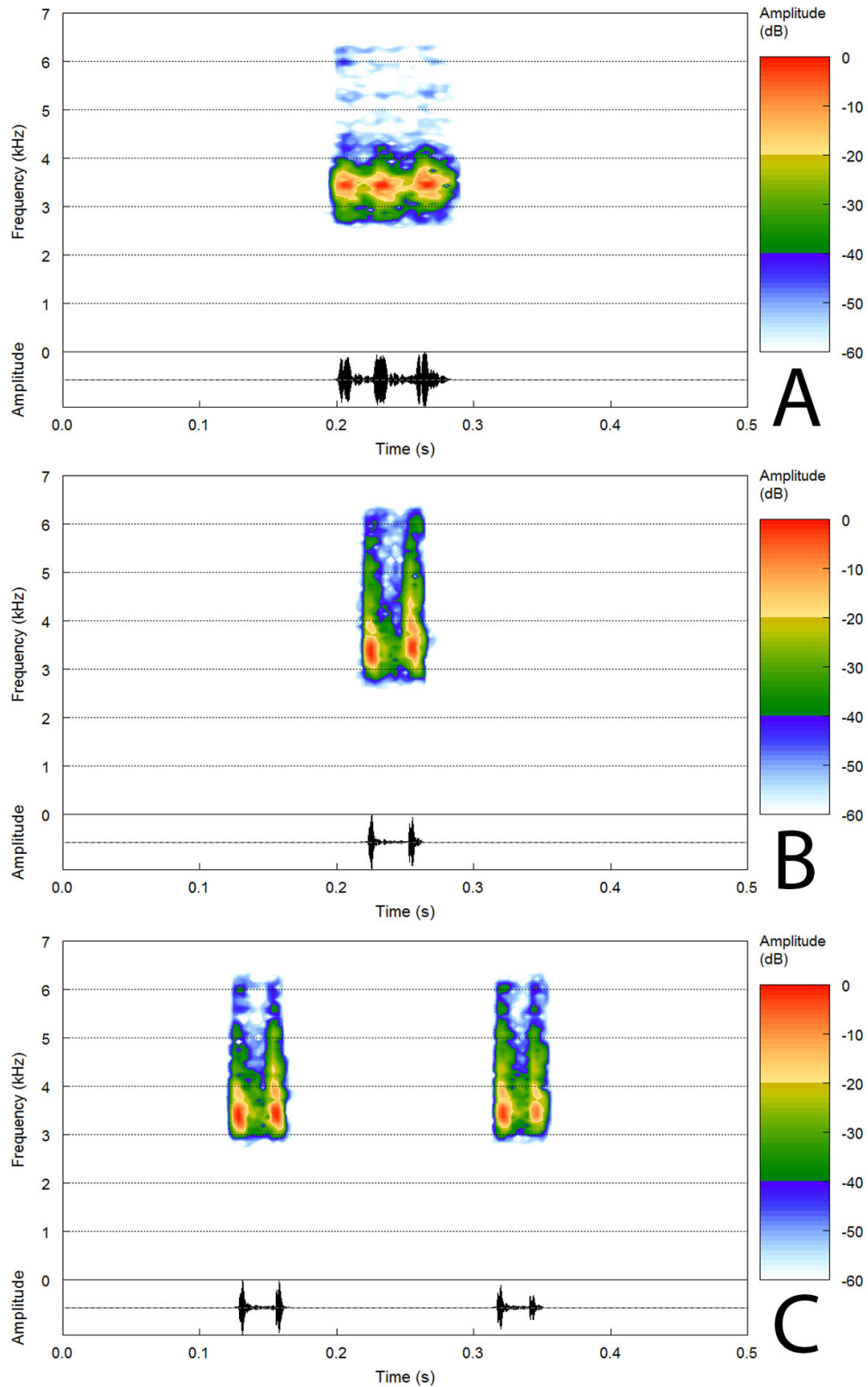


Fig. 2. Sonogram (above) and oscillogram of unvoiced calls of *Ischnocnema abdita* from (A) Municipality of Santa Teresa, Espirito Santo State, Brazil; (B) 'A' call and (C) 'B' call' from the Municipality of Espera Feliz, Minas Gerais State, Brazil.



Fig. 3. Unvouchered male of *Ischnocnema abdita* from Espera Feliz, Minas Gerais State, Brazil.

Table 2. Within-male variation in the call traits of *Ischnocnema abdita* from the Municipality of Santa Teresa, Espírito Santo State, Brazil (n = 4 males) and from the Municipality of Espera Feliz, Minas Gerais State, Brazil (n = 4 males). Results are expressed as mean coefficient of variation (CV) followed by range in parenthesis.

Locality - Call type	Call traits	Average CV (%)	Trait type
Santa Teresa	Call duration	16.1 (13.5-18.5)	Dynamic
	Interval between calls	54.9 (43.5-66.1)	Dynamic
	Pulses per note	17.8 (12.5-22.2)	Dynamic
	Pulse rate	10.6 (7.7-15.8)	Dynamic
Espera Feliz - A call	Peak frequency	1.8 (1.6-1.9)	Static
	Call duration	5.3 (1.8-8.3)	Intermediate
	Interval between calls	31.5 (27.4-35.8)	Dynamic
	Pulses per note	0%	Static
Espera Feliz - B call	Pulse rate	5.3 (1.8-8.2)	Intermediate
	Peak frequency	3.6 (1.7-4.9)	Static
	Call duration	9.1 (6.4-11.1)	Intermediate
	Interval between calls	49.6 (41.9-57.3)	Dynamic
Espera Feliz - B call	Note duration	7.0 (4.2-10.1) 6.5 (4.0-8.3)	Intermediate
	Interval between notes	13.6 (9.7-17.6)	Dynamic
	Pulses per note	0%	Static
	Pulse rate	7.3 (4.4-10.5) 6.6 (4.0-8.7)	Intermediate
	Peak frequency	2.2 (1.4-2.7)	Static
		1.4 (1.3-1.5)	

at similar rate than observed in the A call from Espera Feliz ($t = 0.101$; $df = 7$; $P = 0.922$), although with shorter intervals between calls ($t = 3.272$; $df = 59$; $P = 0.001$). The note from the A call has shorter duration than the first note from B call ($\dagger t = 2.982$; $df = 42.8$; $P < 0.005$), and longer than the second note from B call ($\dagger t = 8.218$; $df = 89.8$; $P < 0.005$). The note from the A call is composed of two pulses (three pulses observed once), whilst the first note from the B call has two or three pulses, and the second note was always composed of two pulses (similarly to the A call). On the other hand, mean pulse rate was the same for A call and first note of B call ($t = 1.808$; $df = 91$; $P = 0.07$), but lower than that of the second note of B call ($\dagger t = 7.873$; $df = 85.8$; $P < 0.001$). Ultimately, the peak frequency of A call was lower than those of both the first ($t = 6.403$; $df = 89.0$; $P < 0.001$) and the second ($\dagger t = 6.638$; $df = 88.1$; $P < 0.001$) notes of the B call.

Comparison between species

The high emission of the A call by *Ischnocnema abdita*, associated with its similarity to the advertisement call of *I. bolbodactyla* (sister species of *I. abdita* according to Canedo & Haddad, 2012), led us to compare this call with the advertisement calls described for the *I. lactea* and *I. verrucosa* groups, plus the species *I. manezinho* and *I. sambaqui* (see Materials and Methods for details). The A call of *I. abdita* is distinguished from the calls of *I. juipoca*, *I. penaxavantinho*, *I. sambaqui* and *I. manezinho* by having less notes per call (Table 3). It can be distinguished from the call of *I. randorum* by the shorter call duration and from those of *I. nigriventris* and *I. vizzotoi* by having a pulsed note structure. It can also be distinguished from the call of *I. bolbodactyla* by the longer note duration.

Within-male variation

To a certain degree, each call trait analyzed expressed variation (Table 2). Peak frequency never exceeded 4% variation, and was classified as static in both populations. Interval between calls had variations exceeding 65% in some individuals and was always classified as a dynamic trait. Call duration and number of pulses per note were classified as dynamic traits in the individuals from Santa Teresa. On the other hand, the number of pulses per note presented no variation (i.e., $CV = 0\%$) in both types of call from Espera Feliz and were classified as static. Call duration and pulse rate were intermediate parameters in both types of call from Espera Feliz. Furthermore, duration of both notes was also an intermediate trait in the B call from Espera Feliz.

Table 3. Comparative traits of the advertisement call of species from the *Isochnocnema lactea* and *I. verrucosa* species series, sensu Canedo and Haddad (2012) (RNA-Val, and 16S. See materials and methods section for definition of trait acronyms. *Cited as pulses.

Species	<i>I. abdita</i>	<i>I. bolbodactyla</i>	<i>I. juipoca</i>	<i>I. penaxavantinho</i>	<i>I. randorum</i>	<i>I. migriventris</i>	<i>I. vizzotoi</i>	<i>I. sambaqui</i>	<i>I. manezinho</i>
Species series	<i>I. verrucosa</i>	<i>I. verrucosa</i>	<i>I. verrucosa</i>	<i>I. verrucosa</i>	<i>I. lactea</i>	<i>I. lactea</i>	<i>I. lactea</i>	-	-
NN	1-3	1	10	15-18*	3-8	2-4	1	6-8*	11-13
CD (ms)	76.2 ± 13.5 (54-101)	32 ± 2 (29-37)	500	520-650	2000-5000	194-565	52.7 ± 10.2 (38-72)	592 ± 29.7 (508-633)	884.7 ± 57.2 (790-977)
CR (calls/min)	6.2 ± 2.95 (2.9-8.5)	-	-	6.6	-	-	9.5 ± 2.2	-	-
ND (ms)	76.2 ± 13.5 (54-101)	32 ± 2 (29-37)	20	2-10	40-100	28-88	-	48	17
NI (ms)	-	-	40	40	-	132 ± 12 (113-157)	-	-	-
PN	2.7 ± 0.4 (2-3)	3-4	Unpulsed	Unpulsed	3-8	Unpulsed	Unpulsed	Unpulsed	Unpulsed
DF (Hz)	2781-4046	2800-4400	2300-3500 (first 5 notes) 2500-4100 (last notes)	-	3800-5200	1955-3932	2780-3555	1800-2050	2400-2950
PF (Hz)	3543 ± 126.9 (3343-3812)	-	-	3800	-	2756-2928	-	-	-
Reference	This paper	Pombal & Cruz, 1999	Sazima & Cardoso, 1978	Giaretta et al., 2007	Heyer, 1985	Berneck et al., 2013	Martins & Haddad, 2000	Castanho & Haddad, 2000	Castanho & Haddad, 2000

DISCUSSION

We observed two distinct types of calls emitted by *Ischnocnema abdita*. The A call was observed in populations from both Santa Teresa (type locality) and Espera Feliz (new record) and was the most common type of call. Although we did not observe any behavior that could associate the described calls (i.e. A and B calls) with the attraction of mates, the high emission of the A call should possibly indicate that this type of call corresponds to the advertisement call of *I. abdita* (sensu Toledo et al., 2015b). Moreover, we observed significant differences in both temporal and spectral traits of the A call between localities. However, our molecular analysis supported the assignment of the newly discovered population to *I. abdita* in comparison with topotypical specimens.

Our analysis on within-male variation showed that spectral traits are less variable than temporal traits in both populations. Variation in temporal traits is often related to environmental condition (e.g., temperature) and the social context of the call (e.g., presence of a female or other males), whereas the variation in the spectral traits is linked to the calling apparatus of a frog (Gerhardt, 1991; Gerhardt & Huber, 2002). On the other hand, spectral parameters are frequently associated with species recognition (i.e., static traits), whereas temporal traits (i.e., dynamic traits) have a greater influence on the meaning and attractiveness of the signal (Ryan and Rand, 1990; Gerhardt, 1991, 1992; Tárano, 2001; Gerhardt, 2005).

Among the genus *Ischnocnema*, only three species had studies on intraspecific call variation (Kwet and Solé, 2005; Taucce et al., 2012; this paper) and they all found variation that agrees with previous studies (e.g., Castellano and Giacoma, 2000; Tárano, 2001; Castellano et al., 2002; Klymus et al., 2012; Carvalho et al., 2013; Grenat et al., 2013; Carvalho et al., 2015; Miranda et al., 2016). Intraspecific variability related to isolated and overlapping populations were described by Blair (1955) before Blair (1958) himself proposed the specificity of anuran mating calls. As stated by Castellano and Giacoma (2000), intraspecific call variation in frogs is likely the rule rather than the exception.

Despite the great overlap in the dominant frequency ranges, we observed significant differences in the peak frequency from both populations. Variation in spectral traits is often associated with the calling apparatus of a frog (i.e., vocal fold). Within the same species, for instance, larger individuals are prone to have larger vocal folds, which causes dominant frequencies to be lower (Gerhardt & Huber, 2002). Thus, the differences observed are likely related to morphological variation between populations. On the other hand, there is no information

on air temperature during the recordings and the cryptic behaviour of *Ischnocnema abdita* did not allow us to observe calling behaviour in the field. Therefore, we had no means of explaining the observed variation without further tests under the same environmental conditions and explicit social context.

Under the phylogenetic hypothesis from Canedo and Haddad (2012) (see also Padial et al., 2014), a general bioacoustical pattern for *Ischnocnema lactea* and *I. verrucosa* series is unclear. The *I. verrucosa* series includes species whose calls are either composed of a single type of pulsed note (e.g., *I. bolbodactyla*) or multiple unpulsed notes (e.g., *I. juipoca*). The same is observed for the *I. lactea* series, with representatives that display advertisement calls composed of a single type of unpulsed notes (e.g., *I. vizzotoi*) and of sequences of pulsed notes (e.g., *I. randerum*). These results somewhat agree with the current lack of diagnostic morphological characters for those groups (Canedo and Haddad, 2012).

This struggle to relate bioacoustical characters to synapomorphies is also observed in previous studies (e.g., Cannatella et al., 1998), whilst others have observed a strong phylogenetic signal in the calls (e.g., Robillard et al., 2006; Erdtmann and Amézquita, 2009). As shown by Robillard et al. (2006), the biomechanics involved in the sound production should also be taken into consideration in order to better understand homology between call units produced (see also McLister et al., 1995). Furthermore, our results point to intraspecific call variation that may be related to geographic and genetic structuration (see also Miranda et al., 2016). We encourage future researches to further investigate this, since geographic and genetic structuration may contribute to allopatric speciation (Turelli et al., 2001; Uyeda et al., 2009) and mating calls may evolve fast, leading to reproductive isolation in response to structuration (Panhuis et al., 2001).

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