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Advertisement call and genetic structure conservatism : good news for an endangered Neotropical frog

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Background. Many amphibian species are negatively affected by anthropogenic habitat change. Populations distributed over modified landscapes may be subject to local extinction or may be relegated to remaining, likely isolated and possibly degraded, patches of available habitat. Isolation without gene flow can lead to variability in phenotypic traits due to differences in local selective pressures, such as environmental structure, microclimate or site-specific species assemblages.

Methods. Here we tested this microevolution hypothesis by evaluating the acoustic parameters of 349 advertisement calls from 15 males representing six populations of the endangered species *Proceratophrys moratoi*. In addition, we analyzed the genetic distance between populations and the genetic diversity by haplotype network. We carried out a cluster analysis by Bray-Curtis index of similarity using acoustic data by UPGMA method. We correlated acoustic dissimilarities (calculated by Euclidean distance) with geographic and genetic distances among populations.

Results. Spectral traits of the advertisement call of *P. moratoi* presented lower coefficients of variation than did temporal traits both within and among males. Cluster analyses placed individuals without population and geographical distance congruence, but recovered the species topology in relation to sister species. The genetic distance was low among populations: it did not exceed 0.4% for the most distant populations, and was not correlated with acoustic distance.

Discussion. Both acoustic features and genetic sequences are highly conserved, suggesting that populations may be connected by recent migrations or that they are subject to stabilizing selective forces. Although future studies are needed, these findings contribute to a growing body of literature suggesting that this species would be a good candidate for a reintroduction program without negative effects on communication or even genetic heritage.

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Advertisement call and genetic structure conservatism: good news for an endangered Neotropical frog

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ABSTRACT

Background. Many amphibian species are negatively affected by anthropogenic habitat change. Populations distributed over modified landscapes may be subject to local extinction or may be relegated to remaining, likely isolated and possibly degraded, patches of available habitat. Isolation without gene flow can lead to variability in phenotypic traits due to differences in local selective pressures, such as environmental structure, microclimate or site-specific species assemblages.

Methods. Here we tested this microevolution hypothesis by evaluating the acoustic parameters of 349 advertisement calls from 15 males representing six populations of the endangered species *Proceratophrys moratoi*. In addition, we analyzed the genetic distance between populations and the genetic diversity by haplotype network. We carried out a cluster analysis by Bray-Curtis index of similarity using acoustic data by UPGMA method. We correlated acoustic dissimilarities (calculated by Euclidean distance) with geographic and genetic distances among populations.

Results. Spectral traits of the advertisement call of *P. moratoi* presented lower coefficients of variation than did temporal traits both within and among males. Cluster analyses placed individuals without population and geographical distance congruence, but recovered the species topology in relation to sister species. The genetic distance was low among populations: it did not exceed 0.4% for the most distant populations, and was not correlated with acoustic distance.

Discussion. Both acoustic features and genetic sequences are highly conserved, suggesting that populations may be connected by recent migrations or that they are subject to stabilizing selective forces. Although future studies are needed, these findings contribute to a growing body of literature suggesting that this species would be a good candidate for a reintroduction program without negative effects on communication or even genetic heritage.

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INTRODUCTION

The greatest threat to endangered amphibians is anthropogenic habitat change, which alters resource availability, environmental quality, and ecological processes (Metzger, 2001; Stuart et al., 2004). Such negative impacts have important implications for organisms that face new selective pressures created by habitat conversion (Forman, 1995). In addition, habitat fragmentation isolates populations and places many of them at risk of extinction due to demographic stochasticity, genetic depression, social dysfunction, and exogenous factors such as strong climatic variations and disasters (Simberloff, 1986). Therefore, it is predicted that species affected by these changes will (1) migrate to appropriate adjacent areas; (2) undergo local declines and extinctions; or (3) undergo local adaptation.

Surprisingly, many species thrive in modified sites even after profound human transformations. Because these landscapes often exhibit physical, climatic, and biological (e.g., species assemblages) shifts, the ability of the remaining species to persist is likely a consequence of remarkable phenotypic plasticity in traits such as behavior, morphology, and reproduction (Mayr, 1963; Pulido & Berthold, 2004; Merckx & Dyck, 2006).

One of the most important phenotypic traits in anuran evolutionary studies is the male advertisement call, because components of these calls are fundamental to species recognition, mate choice, and are thus under sexual selection (Ryan, 1991; Wycherley, Doran & Beebee, 2002; Smith, Osborne & Hunter, 2003; Kaefer & Lima, 2012; Grenat, Valetti & Martino, 2013). Moreover, calls are subject to natural selection over larger geographic ranges, mainly when physical barriers isolate populations (Simões et al., 2008; Kaefer, Tsuji-Nishikido & Lima, 2012; Tsuji-Nishikido et al., 2012). Because anurans do not tend to disperse over long distances (Blaustein, Wake & Sousa, 1994; Tozetti & Toledo, 2005; Lougheed et al., 2006), sexually related phenotypic traits are likely to be influenced by local environmental conditions (Bosch & de la Riva, 2004; Ey & Fischer, 2009).

The anuran advertisement call is a multidimensional signal which can be viewed as a collection of spectral and temporal acoustic traits that are influenced, for example, by body size, air temperature, and social context (Wells & Taigen, 1986; Gerhardt, 1991; Bee, 2002; Gerhardt & Huber, 2002; Wong et al., 2004; Toledo et al., 2015a). Therefore, sexual selection, habitat structure, and climatic conditions each might cause variation in call traits among populations (Jang et al., 2011; Faria et al., 2009 Kaefer & Lima, 2012; Kaefer, Tsuji-Nishikido & Lima,

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2012; Narins & Meenderink, 2014). In addition, other biotic processes, such as interspecific acoustic interactions, which generate distinctive background noise, can also affect call variation among populations (Littlejohn, 1976; Höbel & Gerhardt, 2003).

Different traits of advertisement calls may have distinct roles in anuran communication, and so may evolve by distinct selective pressures (Cocroft & Ryan, 1995; Erdtmann & Amézquita, 2009; Goicoechea, De La Riva & Padial, 2010). As a result, call traits should vary in unique and predictable ways. Gerhardt (1991) classified these traits as static or dynamic acoustic traits. Typically, spectral acoustic traits show low variability (static), and are related to conspecific recognition. Static traits consequently are subject to stabilizing or weakly directional selection by female choice. On the other hand, most temporal acoustic traits show higher variability (dynamic) and are considered to indicate a male's investment in reproduction; these may be subject to directional selection by females for values above species means (Gerhardt, 1991; Gerhardt & Bee, 2007).

However, acoustic variation is not always related to genetic variation at the population level (Heyer & Reid, 2003; Lougheed et al., 2006; Kaefer et al., 2013). In these cases, despite some phylogenetic signal recorded in vocalizations (Erdtmann & Amézquita, 2009; Goicoechea, De La Riva & Padial, 2010; Tobias, Evans & Kelley, 2011; Gingras et al., 2013), evolution of genotypes and phenotypes (as acoustic traits) may be decoupled (Lougheed et al., 2006) or asynchronous (Kaefer et al., 2013).

Herein, we expected that historical modifications of landscapes by agricultural crops have created barriers among persistent populations and affected phenotypic and genetic traits in an endangered Neotropical toad, *Proceratophrys moratoi*. We tested the hypothesis that unique selective pressures among these remaining populations have led to divergence in acoustic traits and increased genetic structure.

METHODS

Species

The genus *Proceratophrys* includes 40 South American toad species (Frost, 2015). *Proceratophrys moratoi*, originally described in the genus *Odontophrynus* (Jim & Caramaschii, 1980), is a member of the *P. cristiceps* species group (Giaretta et al., 2000), lacking palpebral appendages and postocular swellings. It was described from the municipality of Botucatu, state

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of São Paulo (Jim & Caramaschii, 1980), from where it is now extirpated (Brasileiro, Martins & Jim, 2008). Despite recent reports of new populations outside Botucatu (Brasileiro, Martins & Jim, 2008; Rolim et al., 2010; Maffei, Ubaid & Jim, 2011; Martins & Giaretta 2012) the species is endangered (EN) according to the current Brazilian red list (MMA, 2014).

Proceratophrys moratoi is endemic to the Brazilian Cerrado and occurs in open grasslands near small streams or swamps (Rolim et al., 2010; Maffei, Ubaid & Jim, 2011; Martins & Giaretta, 2012). Males call during the rainy season (generally from October to February). A single train of pulses repeated regularly characterizes the advertisement call of *P. moratoi*. Call duration is about 250 ms, and its frequencies range from 700 to 1900 Hz (Brasileiro, Martins & Jim, 2008; Martins & Giaretta, 2012).

Sites

We studied six populations of *P. moratoi*, which represent almost its entire known geographic distribution (Martins & Giaretta, 2012). We sampled in two southeastern Brazilian states (Figure 1): São Paulo (Avaré, Bauru, Itirapina and São Carlos) and Minas Gerais (Ituiutaba and Uberlândia). These localities belong to a region that is an important center of agricultural and livestock production (MMA, 2005) with a remarkable history of modification of land use and natural habitat devastation (Dean, 1995). With the exception of the populations from Itirapina, Bauru, and Uberlândia, which are in protected natural reserves, all populations are on modified landscapes.

Acoustic analyses

We analyzed 349 calls from 15 males of *P. moratoi* from six localities, 18 calls of two males of *Odontophrynus americanus* and 6 calls of one male of *Proceratophrys boiei*. Calls were recorded using the following combinations of microphones and recorders: (1) an Audiotechnica AT 835b microphone and a Marantz PMD-222 recorder, (2) a Dynamic microphone and an Uher 4000 recorder, (3) a Sennheiser ME67/K6 microphone and a Boss 864 recorder, (4) a Sennheiser ME67/K6 microphone and a Marantz PMD671 recorder, or (5) a Sennheiser ME66/K6 microphone and a M–audio Microtrack II recorder. All these recordings were made with sample rate of 44.1 or 48 kHz, and at 16 bits resolution. Acoustic recordings used in the present work are available in the Fonoteca Neotropical Jacques Vielliard, with collection numbers FNJV 10498,

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10577, 12222–24, 12228, the Smithsonian Institution website (http://vertebrates.si.edu/herps/frogs_boraceia/list.htm), and the personal collection of Ariovaldo A. Giaretta, which are detailed in the appendix of Martins & Giaretta (2012).

Acoustic analyses were conducted in Raven Pro 64 1.4 for Windows (Cornell Lab of Ornithology), with the following settings: FFT (Fast Fourier Transformation) = 1024; Overlap = 50 for spectral evaluations; and FFT = 256 and Overlap = 50 for temporal variables. Both temporal and spectral values were extracted from the spectrogram. We analyzed the following quantitative traits: frequency range, maximum frequency, minimum frequency, peak of dominant frequency, call duration, number of pulses per note, and pulse rate (pulses per second). Spectral measurements were obtained by selecting four variables in the source "choose measurements" in Raven: (1) Frequency 5% (Hz); (2) Frequency 95% (Hz) – these two measures include maximum frequency and minimum frequency, ignoring 5% below and above the total energy in the selected call; (3) Bandwidth 90% (Hz) – frequency range which included 90% of the energy distribution, i.e., the difference between Frequency 95% and Frequency 5%; (4) Max Frequency (Hz) – peak of dominant frequency (the frequency in which the power is maximum within the call). For temporal properties we made precise selections on calls in the spectrogram, and pulses were counted visually.

We calculated the variation in quantitative acoustic variables through coefficient of variation (CV; SD/mean) for both among-males and within-males level. As defined by Gerhardt (1991), CVs can be used to determine if a call trait is static (CV < 5% within males and < 12% among males) or dynamic (CV > 12% within males and among males).

Genetic analyses

Liver and muscle samples from 26 *P. moratoi* individuals from six populations were collected: four populations in the state of São Paulo: Itirapina (n = 5), São Carlos (n = 5), Bauru (n = 5), and Avaré (n = 2); and two populations in the state of Minas Gerais: Ituiutaba (n = 4) and Uberlândia (n = 5). This small sample size is in part due to the rarity of the species, and collection restrictions as it is endangered and apparently extinct in at least two populations. Tissues were preserved in 95% ethanol. Voucher specimens were deposited in the Coleção Científica Jorge Jim indexed in Museu Nacional, Rio de Janeiro, Brazil (CCJJ 7925, 7928-7938, 7944, 7950-7952, 7958). Tissue collection can be found in the Collection of tissue and

chromosome preparation Shirlei Maria Recco Pimentel, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil (SMRP 469.01-469.14, 469.26-469.42). Total genomic DNA was extracted according to Veiga-Menocello et al. (2014). We targeted a 650 bp region of the 16S mitochondrial gene using the primers 16Sar and 16Sbr (Palumbi et al., 1991). Fragments were purified with a purification kit (GE Healthcare Life Science, São Paulo, SP, Brazil) and sequences were obtained using the same primers and BigDye™ 3.1 cycle sequencing kits (Applied Biosystems Foster City, CA, USA) and read on an ABI 3700/Prism. Sequences were checked by eye using BioEdit v.5.0.9 and aligned with Muscle (Edgar, 2004). Genetic distances (*p* distances) were computed from mitochondrial loci using MEGA 5.1 (Tamura et al., 2011).

We verified haplotypes using DnaSP v. 5.10.01 (Librado & Rozas 2009). We obtained a haplotype network using the Median-joining network method (Bandelt, Forster & Rohl, 1999) with NETWORK 4.6.1.2.

Statistical analyses

We performed cluster analyses based on acoustic similarities using the Bray-Curtis index through the UPGMA method and bootstrap with 1000 randomizations (see Toledo et al., 2015b). We calculated the Euclidean distance values among populations for acoustic traits and after we correlated it with geographic and genetic distance values through Mantel tests with 1000 permutations. Geographic distance was estimated in Google Earth as the strait-line distance between two sites. We conducted statistical analyses in Past 2.17 (Hammer, Harper & Ryan, 2001)

RESULTS

Acoustic similarities and variability

The calling structure of all individuals presented the same pattern of a single periodic pulse train (Figure 2a). However, we found slight differences in spectral and temporal traits among calls from distinct localities (Table 1). Male calls from Avaré had the lowest frequencies while calls from Ituiutaba exhibited the highest frequencies. The individual from São Carlos presented the longest calls, whereas the male from Bauru emitted the shortest calls. Males from Itirapina emitted the calls with the highest pulse rates compared to other populations; male from

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Avaré had the lowest pulse rate. Temporal traits of the advertisement call presented high coefficients of variation among males (above 12%) and were considered dynamic (Figure 2b). Among the spectral traits, frequency range showed the highest coefficient of variation among males, while the other three spectral traits presented an intermediate variation (between 5 and 12%; Figure 2b). All call traits presented low variation within males, with CVs of less than 8% (Figure 2c), and the majority was considered static, with less than 5% variation.

Cluster analysis placed *O. americanus* and *P. boiei* as outgroups in relation to the focal *P. moratoi* individuals (Figure 3). Individuals were not grouped by population, except for males from Itirapina. The male from Avaré had the most distinct call and was grouped with two individuals from Uberlândia. However, other males from Uberlândia were placed in the other two major groups and individuals were not organized according to geographic distance among populations. This was confirmed after the absence of correlation between geographic distance and the acoustic distance between populations (r = - 0.23; $P = 0.73$;).

Haplotype network and genetic distance

We found seven haplotypes in the 26 partial sequences of the mitochondrial 16S gene (Figure 4). Most haplotypes (H1–H4) were shared among multiple populations, but three haplotypes (H5–H7) were found in one individual each and were limited to Uberlândia (H5, H6) or Ituiutaba (H7). Genetic distances of *P. moratoi* averaged 0.2% (0.0 – 0.4) between populations (Table 2) and 0.25% (0.0 – 0.5) within populations. Acoustic and geographic distance between populations was not correlated with genetic distance (r = - 0.32; $P = 0.86$; and r = - 0.32; P = 0.87).

DISCUSSION

We found the same structural pattern (a single periodic pulse train; Fig. 1) for all individuals as previously reported for the advertisement call of *P. moratoi* (Brasileiro, Martins & Jim, 2008; Martins & Giaretta, 2012).

Advertisement call variability showed by *P. moratoi* follows a general pattern among anurans, with spectral traits exhibiting lower variation than temporal ones, with the exception of frequency range (Gerhardt, 1991; Gerhardt & Huber, 2002). Low coefficients of variation in call traits we observed among males (showing highly stereotyped signals) could be a result of

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stabilizing selection (Kaefer & Lima, 2012; Kaefer, Tsuji-Nishikido & Lima, 2012), which is usually a result of generalized female choice (Jennions & Petrie, 1997). Alternatively, it could reflect the absence of selection and the presence of neutral/stochastic processes (Edrtmann & Amézquita, 2009; Kaefer et al., 2013; Toledo et al., 2015b). Because sexual signals carry information important in mate recognition (Ryan, 1991), spectral traits would not be expected to rapidly diverge. Temporal traits, which are generally dynamic, have been shown to vary due to social or environmental conditions (Bosch & de la Riva, 2004; Ey & Fischer, 2009). For example, some species respond to vocal interactions and chorus composition with rapid temporal adjustments in their calling behavior (Schwartz, 2001). Therefore, temporal traits such as pulse rate and call duration may be affected by social context. In species where females make choices based on temporal traits alone (Littlejohn, 1965), these traits would thus be predicted to mimic the patterns observed in spectral traits here However, mate choice by acoustic properties remains to be tested, since we do not know how females select males in *P. moratoi.*

Habitat structure, background noise, and other environmental differences are pivotal in the evolution of acoustic communication in frogs (Goutte, Dubois & Legendre, 2013; Schwartz & Bee, 2013). These factors certainly contribute to regional divergence in call traits among individuals (Amézquita et al. 2006). Each reproductive environment could present distinct species composition and acoustic qualities dramatically different. Based on this ecophenotypic hypothesis, we expected that local pressures would modulate call features in males of *P. moratoi* from distinct localities, since populations of this threatened species surrounded by human-transformed landscapes could show low connectivity. In contrast, cluster analyses failed to group individuals by populations (geographical context). Taken together, these results suggest minimal pressure for signal divergence, which could be explained the following, non-exclusive hypotheses: (1) the reproductive environment from the sampled localities is similar in respect to habitat structure and background noise; (2) the female choice drives to stable selection, which equalizes the general acoustic features of males from different localities; (3) the populations were recently connected, presenting traces of recent genetic flow; and (4) random evolutionary processes are acting on calls (Toledo et al., 2015b). We have not tested these hypotheses, but it is unlikely that populations were recently connected, as genetic distance was not correlated with acoustic distance among populations. Furthermore, although some studies have found a correlation between genetic and acoustic distances (Smith, Osborne & Hunter, 2003; Amézquita

et al., 2009), many others have shown that geographic variation in sexual signals and genetic distances among populations do not co-vary (Heyer & Reid, 2003; Lougheed et al., 2006; Pröhl et al., 2007). In these cases, is possible that evolution has been decoupled for genotypic and phenotypic features (Lougheed et al., 2006). The cluster analysis demonstrated that a phylogenetic signal in anuran advertisement call (as a phenotypic trait) might not evolve as fast as DNA differences appear, a result corroborated by Kaefer et al. (2013). Consequently, we suggest that a phylogenetic signal would appear only when higher taxonomic levels are compared, for example, different species, genera or families. In part, this result could be explained by the conservative nature of the molecular marker used (16S gene), which is commonly employed to separate different species (Fouquet et al. 2007; Brusquetti et al. 2014; Yang et al., 2014; Lourenço et al. 2015).

Acoustic and genetic variation seems to be conserved among individuals distributed across human-altered landscapes. Our results showed similar call types and genotypes (also presenting low genetic divergence) among different populations. Such reduced acoustic and possible genetic structure could be considered in future conservation actions: for example, these findings suggest that communication barriers (prezygotic) would pose no obstacle to reproduction (Dobzhansky, 1951; Tucker & Gerhardt, 2011) if a reintroduction program were initiated for this species. If the actual molecular marker used at the present work were able to represent all individuals genome, the genetic barrier (postzygotic) would pose no obstacle to reproduction too (Dobzhansky, 1951; Tucker & Gerhardt, 2011). The type locality of this species (Botucatu) is still preserved and a reintroduction could be considered after complementary genetic and natural history researches. The causes of this population decline are unknown and, therefore, a reintroduction initiative could also help to understand past declines (if the cause are still active) and help to prevent additional declines there (for example, of *Bokermannohyla izecksohni* – another species that could be threatened in Botucatu; L. F. Toledo & C. Z. Torres, unpublished data) and elsewhere. Thus, this work adds to the growing literature supporting *P. moratoi* as a potential candidate for conservation actions.

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FIGURES

Figure 1. Geographic distribution of *Proceratophrys moratoi*. State of São Paulo: 1) Avaré, 2) Bauru, 3) Botucatu (type locality – black dot), 4) Itirapina, 5) São Carlos; state of Minas Gerais: 6) Ituiutaba, and 7) Uberlândia. Map source: Google Earth.

Figure 2. Call and traits of the frog *Proceratophrys moratoi.* (a) Waveform of the call. The call is composed of a single pulse-train structure; (b) among-male and (c) within-male coefficients of variation of advertisement call traits. The horizontal continuous line represents the lower limit for dynamic traits (above 12%) in (b), and the dashed line represents the limit for static acoustic traits (below 5 % of variation) in (c). Dynamic and static traits according to Gerhardt (1991).

Figure 3. Dendrogram of two outgroup species (other Odontophrynidae) and 15 males of *Proceratophrys moratoi* from different localities resulting from a hierarchical cluster analysis based on similarity in call traits.

Figure 4. Haplotype network of *Proceratophrys moratoi* populations. The size and color of each ellipse indicate the frequency and geographic origin of the individuals.

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Table 1. Acoustic traits (mean ± SD, range) of seven populations of *Proceratophrys moratoi* from southeastern Brazil and two close species as outgroups. Data from the population of Botucatu were extracted from Brasileiro, Martins & Jim (2008).

Groups	Population (n = calls, M = males)	Frequency range (Hz)	Minimum frequency (Hz)	Peak of dominant frequency (Hz)	Maximum frequency (Hz)	Call duration (s)	Pulses per note	Pulses rate (p/s)
Proceratophrys moratoi	Avaré	291 ± 20	980 ± 20	1184 ± 23	1270 ± 23	0.297 ± 0.01	20 ± 0.9	69 ± 1.6
	(n = 8; M = 1)	(258 - 301)	(947 - 990)	(1163 - 1206)	(1249-1292)	(0.277 - 0.315)	(19 - 22)	(66 - 71)
	Bauru	423 ± 23	1029 ± 13	1314 ± 73	1452 ± 20	0.227 ± 0.02	21 ± 2.2	92 ± 2.8
	(n = 29; M = 1)	(387 - 474)	(990 - 1034)	(1077 - 1378)	(1421 - 1464)	(0.160- 0.260)	(15 - 24)	(81 - 96)
	Botucatu	730	928	1348 ± 86.6	1659	0.207 ± 17.6	17.5 ± 1.5	-
	(n = 59; M = 2)			(1153 – 1420)		(146 – 238)	(12 – 20)	
	Itirapina	353 ± 32	1092 ± 34	1317 ± 38	1445 ± 27	0.245 ± 0.02	23 ± 2.4	94 ± 3.2
	(n = 78; M = 3)	(281 - 388)	(1077 - 1206)	(1265 - 1421)	(1406 - 1507)	(0.183 - 0.288)	(17 - 27)	(85 - 103)
	Ituiutaba	433 ± 66	1129 ± 57	1440 ± 26	1562 ± 19	0.240 ± 0.01	19 ± 2	81 ± 4.9
	(n = 54; M = 2)	(301 - 517)	(1077 -1249)	(1378 - 1464)	(1550 - 1593)	(0.196 - 0.263)	(14-22)	(67 - 87)
	São Carlos	288 ± 20	1206 ± 0	1386 ± 24	1494 ± 20	0.307 ± 0.02	25 ± 1.4	83 ± 4.5
	(n = 26; M = 1)	(258 - 301)	(1206)	(1335 - 1464)	(1464 - 1507)	(0.274 - 0.382)	(23 - 28)	(71 - 89)
	Uberlândia	343 ± 95	1054 ± 70	1286 ± 90	1397 ± 92	0.262 ± 0.03	18 ± 1.3	71 ± 9.5
	(n = 141; M = 7)	(215 - 474)	(947 - 1206)	(1120 - 1464)	(1249 - 1550)	(0.186 - 0.316)	(15 - 22)	(60 - 97)
Outgroup	*Proceratophrys boiei*	577 ± 38	474 ± 0	637 ± 19	1051 ± 38	0.743 ± 0.05	32 ± 1.4	43.1 ± 1.1
	(n = 5; M = 1)	(517 - 603)	(474)	(603 - 646)	(990 - 1077)	(0.666 - 0.795)	(30 - 34)	(42 - 45)
	Odontophrynus americanus	287 ± 24	681 ± 24	825 ± 43	968 ± 38	0.664 ± 0.10	57 ± 4.6	86 ± 9.5
	(n = 15; M = 2)	(234 - 328)	(656 - 703)	(750 - 890)	(937 - 1031)	(0.508 - 0.816)	(49 - 65)	(79 - 103)

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492**Table 2.** Genetic distances (p-distance) based on 16S mitochondrial genes between individuals of
493six *Proceratophrys moratoi* populations in the upper matrix and the respective geographic distance
494(in km) in the lower matrix.

	Interpopulation variation (%)						Intrapopulation variation (%)	
	Itirapina	São Carlos	Bauru	Avaré	Uberlândia	Ituiutaba		
Itirapina	-	0.2	0.4	0.2	0.2	0.0	Itirapina	0.0
São Carlos	21.65	-	0.3	0.3	0.2	0.3	São Carlos	0.1
Bauru	114.54	116.59	-	0.2	0.4	0.4	Bauru	0.2
Avaré	129.93	141.36	59	-	0.4	0.3	Avaré	0.5
Uberlândia	354.97	331.88	375.50	437.27	-	0.3	Uberlândia	0.3
Ituiutaba	392.80	372.92	375.45	437.25	119.44	-	Ituiutaba	0.1

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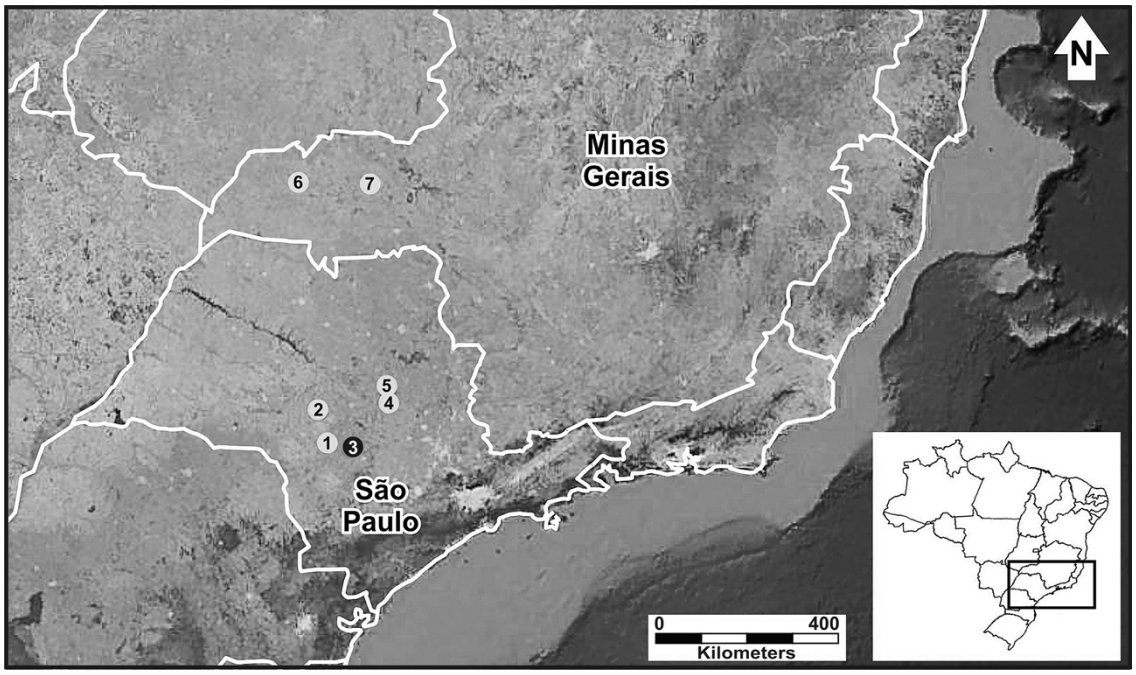
496 Figure 1



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498 Figure 2

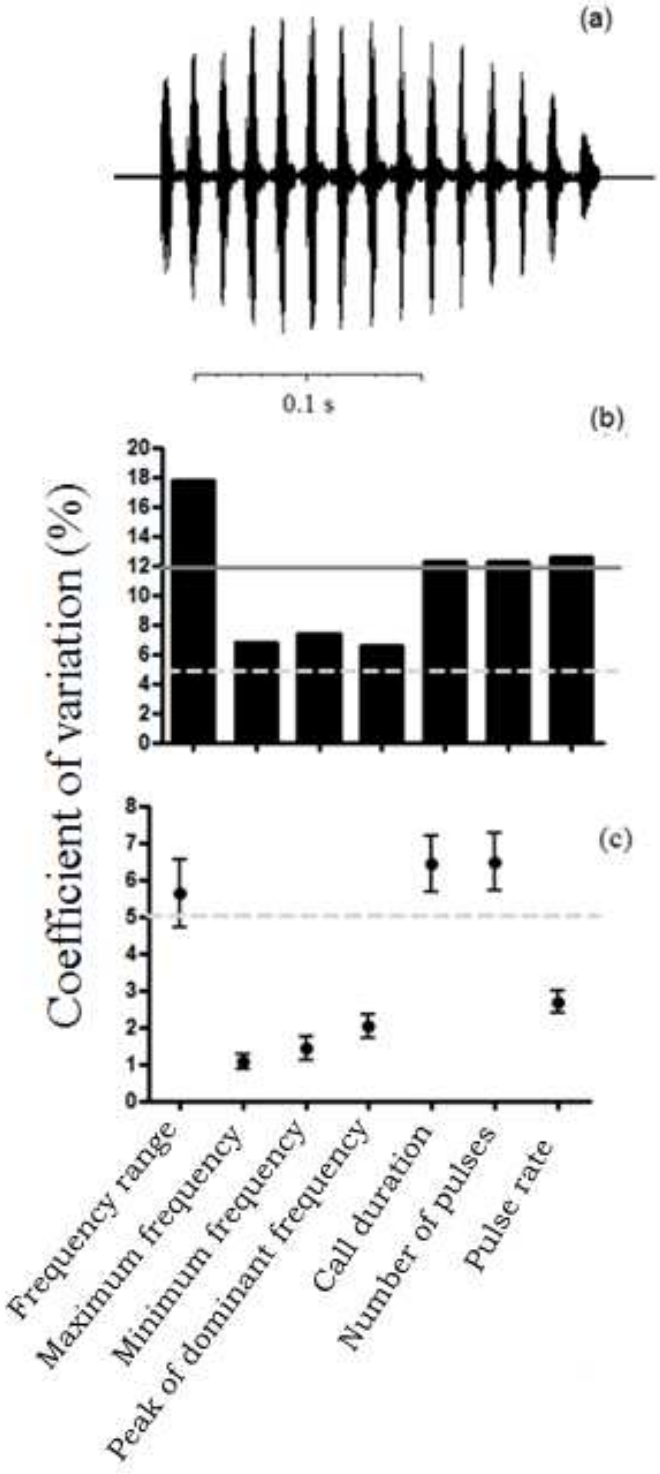

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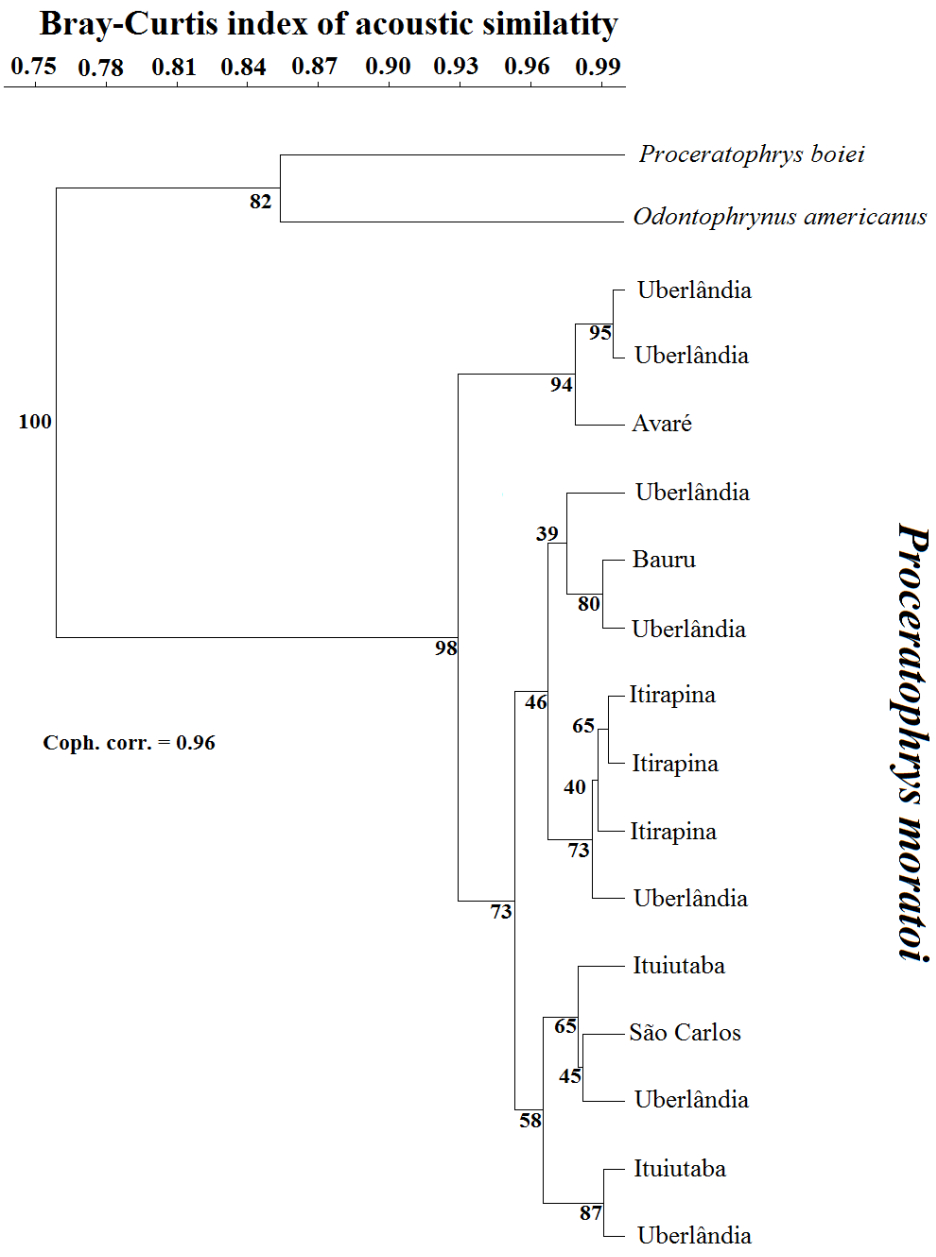
Figure 3



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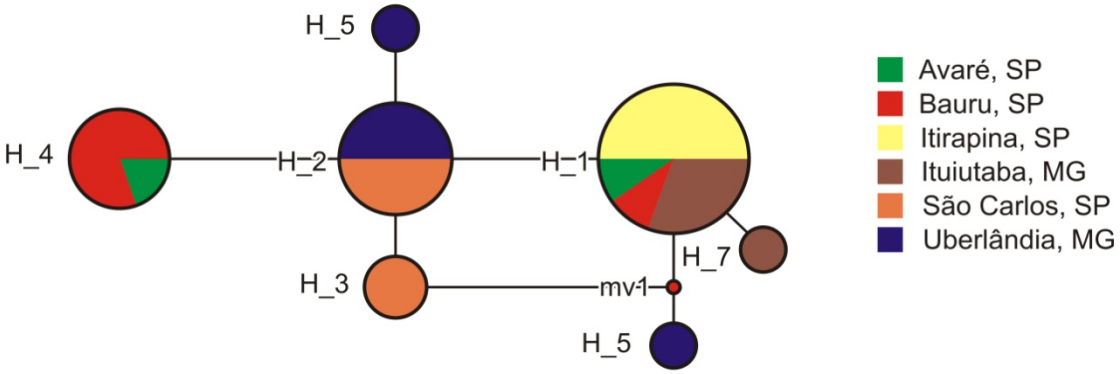
Figure 4