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.

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 habitat change due to anthropogenic activities. Populations distributed over modified landscapes may be subject to local extinction or may be relegated to the remaining—likely isolated and possibly degraded—patches of available habitat. Isolation without gene flow could lead to variability in phenotypic traits owing to differences in local selective pressures such as environmental structure, microclimate, or site-specific species assemblages.

Methods. Here, we tested the microevolution hypothesis by evaluating the acoustic parameters of 349 advertisement calls from 15 males from six populations of the endangered amphibian species *Proceratophrys moratoi*. In addition, we analyzed the genetic distance among populations and the genetic diversity by the haplotype network. We performed cluster analysis on acoustic data based on the Bray-Curtis index of similarity, using the UPGMA method. We correlated acoustic dissimilarities (calculated by Euclidean distance) with geographical 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 congruence in population or geographical distance, but recovered the species topology in relation to sister species. The genetic distance among populations was low; 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 could be connected by recent migrations, and that they are subject to stabilizing selective forces. Although further studies are required, these findings add to a growing body of literature suggesting that this species would be a good candidate for individuals reintroduction program without negative effects on communication or genetic heritage.

INTRODUCTION

The greatest threat to endangered amphibians is habitat change caused by anthropogenic activities, 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 exerted by habitat conversion (Forman, 1995). In addition, habitat fragmentation causes isolation of populations, and places them at risk of extinction towing 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 would (1) migrate to appropriate adjacent areas; (2) undergo local decline and extinction; or (3) undergo local adaptation.

Surprisingly, several species thrive in modified sites even after profound anthropogenic transformation. Because these landscapes often exhibit physical, climatic, and biological (e.g., species assemblage) shifts, the ability of the remaining species to persist is likely a consequence of 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 evolutionary studies of anurans is the male advertisement call, because components of these calls are fundamental to species recognition and 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 populations are isolated by physical barriers (Simões et al., 2008; Kaefer, Tsuji-Nishikido & Lima, 2012; Tsuji-Nishikido et al., 2012). Because anurans tend to not disperse over long distances (Blaustein, Wake & Sousa, 1994; Tozetti & Toledo, 2005; Lougheed et al., 2006), sexual phenotypic traits in anurans are likely influenced by local environmental conditions (Bosch & de la Riva, 2004; Ey & Fischer, 2009).

The anuran advertisement call is a multidimensional signal that 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 might all cause variation in call traits among populations (Jang et al., 2011; Faria et al., 2009 Kaefer & Lima, 2012; Kaefer, Tsuji-Nishikido & Lima, 2012;

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

Different traits of advertisement calls may have distinct roles in anuran communication, and therefore, 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. Consequently, static traits 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 thought 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 being recorded in vocalizations (Erdtmann & Amézquita, 2009; Goicoechea, De La Riva & Padial, 2010; Tobias, Evans & Kelley, 2011; Gingras et al., 2013), the evolution of genotypes and phenotypes (as acoustic traits) may be decoupled (Lougheed et al., 2006) or asynchronous (Kaefer et al., 2013).

Herein, we speculated that historical modification of landscapes by agricultural crops has 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

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), according to the current Brazilian red list, the species is endangered (EN) (MMA, 2014).

Proceratophrys moratoi is endemic to the Brazilian Cerrado and is found 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). The advertisement call of *P. moratoi* is characterized by a single train of regularly repeated pulses. Call duration is approximately 250 ms, and the frequency ranges 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 the populations in two southeastern Brazilian states (Figure 1): São Paulo (Avaré, Bauru, Itirapina and São Carlos) and Minas Gerais (Ituiutaba and Uberlândia). These regions represent an important center of agricultural and livestock production (MMA, 2005) with a remarkable history of land use modification and natural habitat devastation (Dean, 1995). With the exception of the populations from Itirapina, Bauru, and Uberlândia, which were in protected natural reserves, all populations inhabited modified landscapes.

Acoustic analyses

We analyzed 349 calls from 15 males of *P. moratoi* from 6 localities, 18 calls from 2 males of *Odontophrynus americanus* and 6 calls from 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 an M–audio Microtrack II recorder. All recordings were made with sample rate of 44.1 or 48 kHz, and at 16-bit resolution. Acoustic recordings used in the present study are available in the Fonoteca Neotropical Jacques Vielliard, with collection

numbers FNJV 10498, 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 performed 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 that 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 visually counted the pulses.

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 attributable 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 using a purification kit (GE Healthcare Life Science, São Paulo, SP, Brazil); sequences were obtained using the same primers and BigDye™ 3.1 cycle sequencing kits (Applied Biosystems Foster City, CA, USA), and were 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 values for Euclidean distance among populations for acoustic traits, and correlated them with the values for geographical and genetic distance through Mantel tests with 1000 permutations. Geographical distance was estimated in Google Earth as the straight-line distance between two sites. We conducted statistical analyses in Past 2.17 (Hammer, Harper & Ryan, 2001)

RESULTS

Acoustic similarities and variability

The structure of calls from 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, whereas calls from Ituiutaba had the highest frequencies. The individual from São Carlos presented the longest calls, whereas the individual from Bauru emitted the shortest calls. Males from Itirapina emitted calls with the highest pulse rates; the call from the male from 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, whereas 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 lower than 8% (Figure 2c), and the majority was considered static, with <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 the 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 geographical distance among populations. This was confirmed by the absence of a correlation between geographical distance and the acoustic distance between populations (r = - 0.23; $P = 0.73$).

Haplotype network and genetic distance

We found 7 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 geographical distance between populations was not correlated with genetic distance (r = - 0.32; $P = 0.86$; and r = - 0.32; P = 0.87).

DISCUSSION

In the present study, we found a common structural pattern (a single periodic pulse train; Fig. 1) for all individuals, which is consistent with the findings of previous reports on the advertisement call of *P. moratoi* (Brasileiro, Martins & Jim, 2008; Martins & Giaretta, 2012).

The variability in the advertisement call of *P. moratoi* follows a general pattern among anurans; spectral traits exhibit lower variation than do temporal ones, with the exception of frequency range (Gerhardt, 1991; Gerhardt & Huber, 2002). The low coefficients of variation in call traits we observed among males (showing highly stereotyped signals) could be attributed to

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 important information for mate recognition (Ryan, 1991), spectral traits would not be expected to diverge rapidly. Temporal traits, which are generally dynamic, have been shown to vary with 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 wherein females make choices based on temporal traits alone (Littlejohn, 1965), these temporal traits would be predicted to minimize the patterns observed in spectral traits. However, we observed that mate choice by acoustic properties remains to be tested, since we do not know how female *P. moratoi* individuals select males.

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 considerably different acoustic qualities. Based on this ecophenotypic hypothesis, we expected that local pressures would modulate call features in *P. moratoi* males from distinct localities, because populations of this threatened species surrounded by human-transformed landscapes could show low connectivity. In contrast, cluster analyses failed to group individuals by population (geographical context). Taken together, these results indicate minimal pressure for signal divergence, which could be explained by the following, non-exclusive hypotheses: (1) the reproductive environment in the sampled localities is similar with respect to habitat structure and background noise; (2) the female choice drives 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 act on the calls (Toledo et al., 2015b). We did not test 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 reported a

correlation between genetic and acoustic distances (Smith, Osborne & Hunter, 2003; Amézquita et al., 2009), many others have shown that geographical 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, it is possible that evolution has been decoupled for genotypic and phenotypic features (Lougheed et al., 2006). Cluster analysis demonstrated that a phylogenetic signal in anuran advertisement call (as a phenotypic trait) might not evolve as rapidly as DNA differences appear, a finding corroborated by Kaefer et al. (2013). Consequently, our results suggest that a phylogenetic signal would be apparent only when higher taxonomic levels are compared, for example, different species, genera, or families. This finding could be explained, in part, by the conservative nature of the molecular marker used—the 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).

Although our dataset is limited, we observed that acoustic and genetic variation appears to be conserved among individuals distributed across human-altered landscapes. Our preliminary 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. Nevertheless, playback experiments are required for testing this hypothesis first. From the genetic point of view, if the actual molecular marker used in the present study could represent the genomes of all individuals, 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, natural history, and experimental research. The causes of the decline of this population are unknown, and therefore, a reintroduction initiative could also aid in understanding past decline (if the cause is still active) and help to prevent further decline here (for example, of *Bokermannohyla izecksohni*—another species that could be threatened in Botucatu; L. F. Toledo & C. Z. Torres, unpublished data) and elsewhere. Thus, the findings of the present study add to the growing body of literature supporting *P. moratoi* as a potential candidate for conservation actions, although additional work is necessary before an action plan could be initiated

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476 **FIGURES**

477

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

481

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

487

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

491

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

494

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).

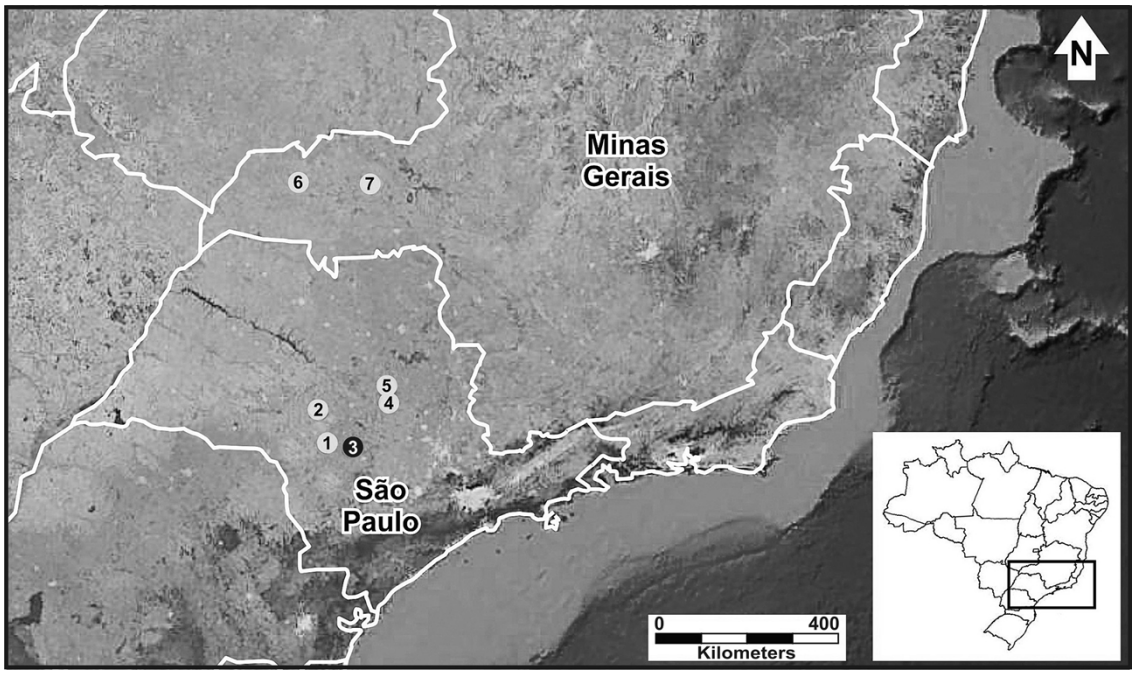
Groups	Population (n = calls, M = males)	Frequency range (Hz)	Minimum frequency (Hz)	Peak of dominant frequency (Hz)	Maximum frequency (Hz)	Call duration (s)	Pulses per note	Pulses rate (p/s)
Proceratophrys moratoi	Avaré	291 ± 20	980 ± 20	1184 ± 23	1270 ± 23	0.297 ± 0.01	20 ± 0.9	69 ± 1.6
	(n = 8; M = 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)

498**Table 2.** Genetic distances (p-distance) based on 16S mitochondrial genes between individuals of
499six *Proceratophrys moratoi* populations in the upper matrix and the respective geographic distance
500(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

501

502 Figure 1



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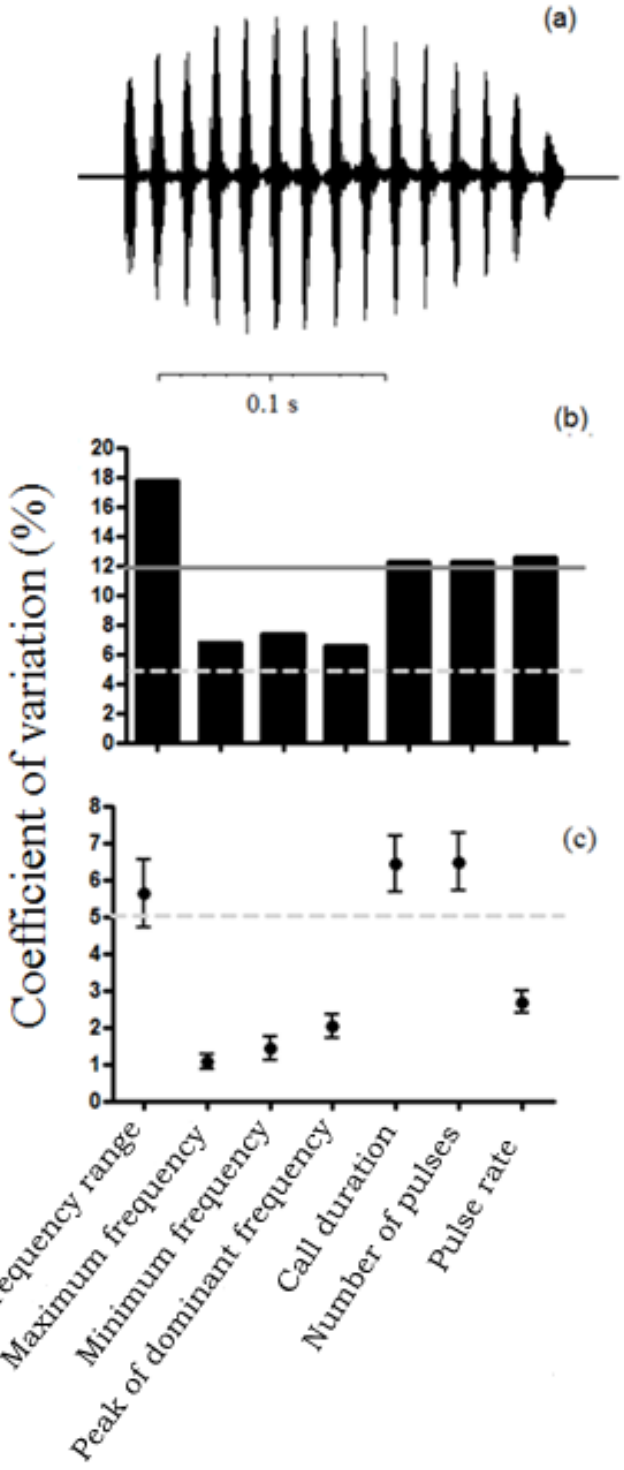

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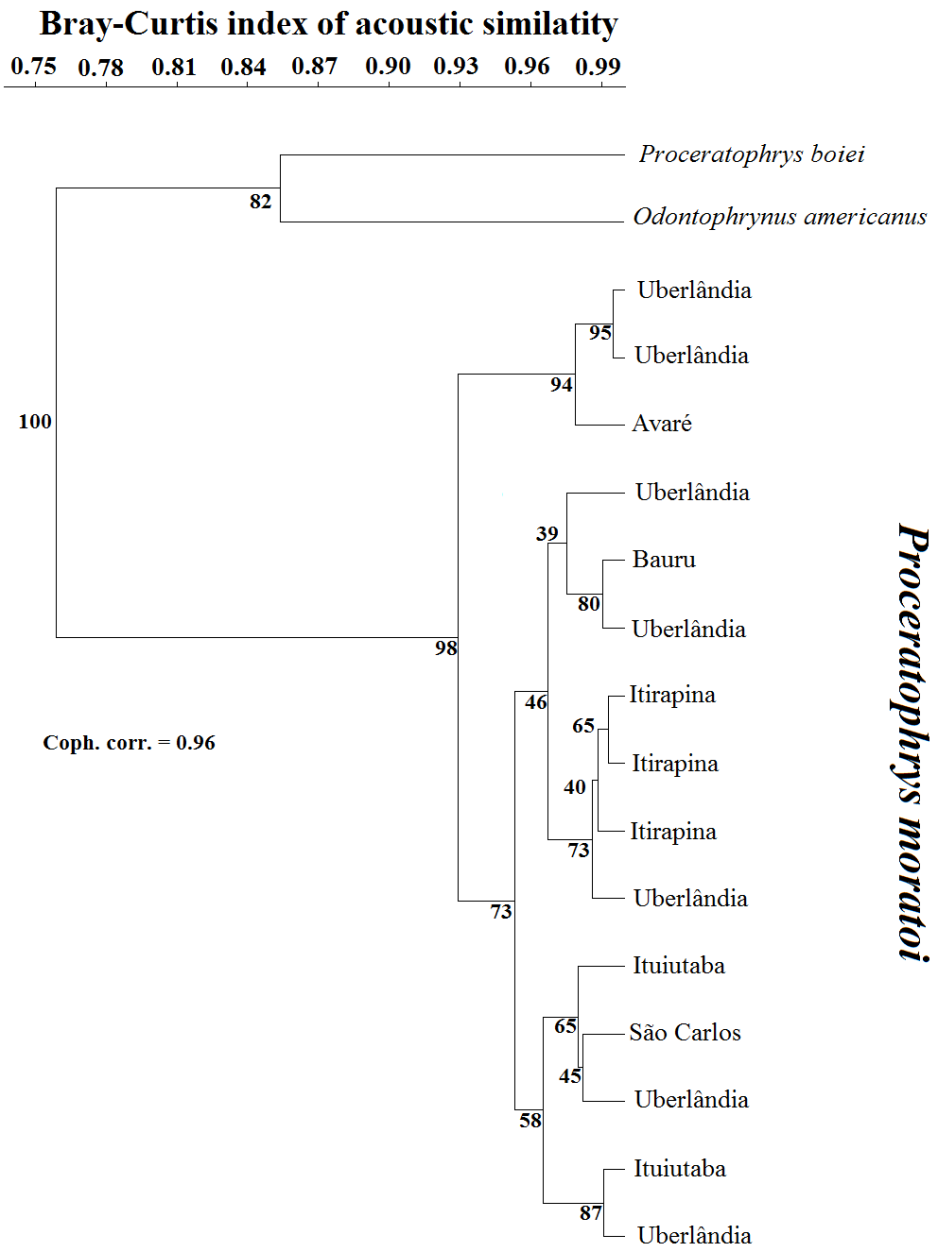

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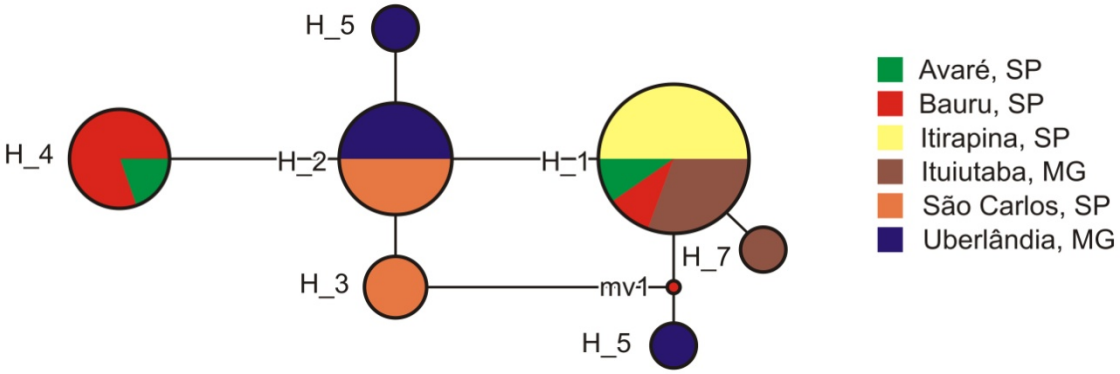

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