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FULL PAPER



Revisiting the generic position and acoustic diagnosis of *Odontophrynus salvatori* (Anura: Odontophrynidae)

Felipe de Medeiros Magalhães^{1,3}, Reuber A. Brandão², Adrian Antonio Garda³ & Sarah Mângia^{1,4}

¹ Programa de Pós-Graduação em Ciências Biológicas, Universidade Federal da Paraíba–UFPB, Centro de Ciências Exatas e da Natureza, Cidade Universitária, CEP 58000-000, João Pessoa, Paraíba, Brazil

² Laboratório de Fauna e Unidades de Conservação (LAFUC), Departamento de Engenharia Florestal, Universidade de Brasília, CEP 70910-900, Brasília, Distrito Federal, Brazil

³ Laboratório de Anfíbios e Répteis (LAR), Departamento de Botânica e Zoologia, Universidade Federal do Rio Grande do Norte, Campus Universitário. Lagoa Nova, CEP 59078-900, Natal, Rio Grande do Norte, Brazil

⁴ Universidade Federal de Mato Grosso do Sul, Instituto de Biociências, Cidade Universitária, CEP 79070-900, Campo Grande, Mato Grosso do Sul, Brazil

Herein we evaluate the phylogenetic position, and revisit the generic allocation of *Odontophrynus salvatori*, which has for long been considered controversial because it exhibits intermediate morphological features between *Odontophrynus* and *Proceratophrys*. By assessing a fragment of the 16S mitochondrial gene from topotypical specimens, we confirm that *O. salvatori* is a member of the genus *Proceratophrys* and sister to *P. moratoi*, also forming a clade with *P. concavitympanum* and *P. ararype*. Therefore, we formally transfer *O. salvatori* to the genus *Proceratophrys salvatori* (Caramaschi 1996) comb. nov.]. Additionally, the calls of *Proceratophrys salvatori* and *P. moratoi*, formally compared for the first time, are shown to exhibit similar structures: they both emit single multi-pulsed notes that differ mainly in pulse repetition rate and dominant frequency. Finally, we summarise occurrence records for *P. salvatori* and *P. moratoi* and provide a new record of *P. moratoi* in Mato Grosso State, extending its distribution about 490 km to the north-west.

Keywords: Advertisement call, geographic distribution, phylogenetic position, Proceratophrys, species diagnosis, taxonomy

INTRODUCTION

dontophrynus salvatori Caramaschi 1996 was described based on two adult males collected at Chapada dos Veadeiros National Park, Alto Paraíso de Goiás Municipality, Goiás State, Brazil (Caramaschi, 1996). Compared to its congeners, this species is morphologically more similar to "Odontophrynus" moratoi (Jim & Caramaschi, 1980), which was reallocated to the genus Proceratophrys based on multilocus molecular data (Amaro et al., 2009), being sister to the clade formed by P. concavitympanum and P. ararype (Mângia et al., 2018). Despite its currently undoubtful phylogenetic position within Proceratophrys, P. moratoi was tentatively allocated in the genus Odontophrynus by having the optic ramus of the squamosal bone not touching the maxillae (not seen in species of Proceratophrys; Jim & Caramaschi, 1980), while also lacking some external morphological characteristics exhibited by species in this genus. At that time, such combination of features rendered it an intermediate position between Odontophrynus and Proceratophrys and posed some doubts about its correct generic allocation until the work of Amaro et al. (2009). Accordingly, the same osteological condition was observed in *Odontophrynus salvatori*, leading Caramaschi (1996) to allocate this species as a congener of "*Odontophrynus*" *moratoi* (as originally published). However, the hypothesis that *O. salvatori* is a member of *Proceratophrys* has been for long suggested either because of the phylogenetic position of *P. moratoi* (the most likely sister taxa of *O. salvatori*, Caramaschi, 1996; Amaro et al., 2009) and its overall larval morphology, which resembles mostly *Proceratophrys* rather than *Odontophrynus* (Rossa-Feres & Jim, 1996; Brandão & Batista, 2000). Nevertheless, *O. salvatori* was never included in any molecular phylogenetic analysis and its generic position is still considered uncertain.

Advertisement calls of frogs are primarily involved in mating recognition and have been employed as an important taxonomic tool to diagnose morphologically cryptic anuran species (Köhler et al., 2017), especially within *Proceratophrys* (Cruz & Napoli, 2010; Mângia et al., 2010). Both *Odontophrynus salvatori* and *P. moratoi* have had their calls described (Brasileiro et al., 2008; Bastos et al., 2011; Martins & Giaretta, 2012), but were never compared, as they were not considered congeners. Additionally, Bastos et al. (2011) did not report pulse repetition rate of *O. salvatori* calls, a key

Correspondence: Felipe de Medeiros Magalhães (felipemm17@gmail.com)

acoustic diagnostic feature among species of the family Odontophrynidae (Martino & Sinsch, 2002; Cruz & Napoli, 2010; Nascimento et al., 2019). Because there are few morphological characters that diagnose *O. salvatori* from *P. moratoi* (Caramaschi, 1996), acoustic comparisons (including pulse rate) are highly desirable to improve their diagnosis and test the utility of calls in anuran taxonomy.

Herein, we present the phylogenetic position of *Odontophrynus salvatori* based on a segment of the 16S rRNA mitochondrial gene (16S mtDNA) from topotypical specimens, clarifying the position of this species within Odontophrynidae (sensu Pyron & Wiens, 2011). Additionally, we revisited the previously published calls of *O. salvatori* and *Proceratophrys moratoi* and provide new acoustic information from *O. salvatori* topotypical specimens aiming to evaluate acoustic diagnosis between these species based on standardised analysis. Finally, we summarise occurrence records for both *O. salvatori* and *P. moratoi* and *P. moratoi* and provide a new record for *P. moratoi* in Mato Grosso State.

METHODS

Voucher specimens of molecular samples used in this study are housed at the Herpetological collection from Universidade de Brasília, Brasília Municipality, Brazil (CHUNB), Amphibian collection from Universidade Estadual Paulista, São José do Rio Preto Municipality, São Paulo State, Brazil (DZSJRP) and Amphibian collection from Universidade Federal de Uberlândia, Uberlândia municipality, Minas Gerais State, Brazil (AAG-UFU). Some specimens only have field number tags associated (Reuber Albuquerque Brandão-RAB, or Guarino Colli-GRCOLLI). We sequenced two adult specimens (RAB3148, 3149) and a tadpole (DZSJRP 3206.1) of Odontophrynys salvatori collected at the type locality (Caramaschi, 1996) in Chapada dos Veadeiros National Park, Alto Paraíso de Goiás Municipality, Goiás State, central Brazil (14°9'35.60"S, 47°36'14.14"W; 1180 m above sea level [a.s.l.], DATUM WGS84). To assure taxonomic identification, we also sequenced a specimen collected at Nova Xavantina Municipality, Mato Grosso State (14°38'49.42"S, 52°4'37.21"W; 312 m a.s.l. DATUM WGS84) that morphologically resembles Proceratophrys moratoi (GRCOLLI20295). We also provide additional sequences of Proceratophrys species from the Cerrado biome: P. branti from Mateiros municipality, Tocantins State (CHUNB27366, 27376 and 23378), P. cururu from the Espinhaço mountain range, Minas Gerais State (RAB3225 and 3290), topotypical P. goyana from Chapada dos Veadeiros, Goiás State (RAB3285), and P. cristiceps from western Bahia State (AAG-UFU1984). We extracted genomic DNA from specimen's livers and tadpole tail muscle using the salt precipitation method described by Bruford et al. (1992). We amplified a ~550 base pair (bp) segment of the 16S mtDNA using primers pair 16Sar-L (CGCCTGTTTATCAAAAACAT) and 16Sbr-H (CCGGTCTGAACTCAGATCACGT) of Palumbi et al. (1991) and PCR protocols described in Amaro et al. (2009). We sent PCR products to Macrogen Inc. (Seoul, South Korea) for purification and sequencing. We checked sequencing quality and edited chromatograms in Geneious v1.8.7 (Kearse et al., 2012).

To confirm the phylogenetic position of O. salvatori, we assembled 16S mtDNA segments available in GenBank (mainly those originally provided by Amaro et al., 2009), encompassing representatives from all genera in the family Odontophrynidae (sensu Pyron & Wiens, 2011), such as Proceratophrys (42 individuals, including our 11 sequenced individuals), Odontophrynus (16 individuals), and Macrogenioglottus (2 individuals), plus Thoropa miliaris and Cycloramphus acangatan as outgroups, totaling 62 terminals (see Supplementary Materials Appendix I for GenBank accession numbers). We aligned sequences using the default settings of MAFFT algorithm (Katoh et al., 2002) also available in Geneious v.1.8.7, resulting in a 516 bp final alignment (including gaps). We estimated the 16S gene tree with BEAST v1.10.4 software (Suchard et al., 2018), implementing the GTR+I+G nucleotide substitution model as suggested by jModeltest version 2.1.6 (Darriba et al., 2012), and birthdeath speciation model as the tree prior. We ran BEAST analysis for 10 million generations sampling every 1000 steps. We assessed run convergence (Effective Sample Size > 200) with Tracer v1.7, generated the maximum credibility tree with TreeAnnotator v1.10.4 (https:// beast.community/treeannotator), and drew it using FigTree v1.4.2 (Rambaut, 2014). Additionally, we used the Tamura-Nei (Tamura & Nei, 1993) corrected p-distances implemented in Molecular Evolutionary Genetics Analysis (MEGA) v7.0 software (Kumar et al., 2015) to compute between-group mean genetic distances of O. salvatori in comparison to species among the three genera within Odontophrynidae, which is shown in Table 1. Prior to this analysis, we trimmed our alignment to fit the shortest sequence available, resulting in a 448 bp alignment employed to calculate distances.

We recorded advertisement calls of two Odontophrynus salvatori topotypical males on 16 December 2012 (recording vouchers: RAB3147, SVL 26.5 mm; and RAB3149, SVL 25.5 mm) using a Marantz PMD 660 coupled with a Sennheiser ME66 directional microphone. To evaluate if there are acoustic diagnostic traits between O. salvatori and Proceratophrys moratoi, we gathered call information previously published for these species (Brasileiro et al., 2008; Bastos et al., 2011; Martins & Giaretta, 2012) and also reanalysed available recordings deposited at Fonoteca Neotropical Jacques Vielliard (FNJV) in a standardised approach (Supplementary Materials Appendix II). We also compare calls of O. salvatori and P. moratoi with those of P. concavitympanum and P. ararype (data from Santana et al., 2010; Mângia et al., 2018), considering their close phylogenetic relationship (see results). We summarised call parameters in Table 2. We analysed advertisement calls in Raven Pro 1.5 (Center for Conservation Bioacoustics, 2014) with the following spectrogram settings: Hann window type, Fast Fourier Transform window width = 256 samples, frame = 100,



Figure 1. Maximum clade credibility 16S rRNA mitochondrial gene tree as inferred from a Bayesian analysis in BEAST. Circles on nodes denote significant posterior probability (pp = 0.95-1.0), and are coloured according to genus. All nodes recovered with non-significant support (pp < 0.95) are omitted. Scale indicates rate of base substitutions per site. Arrow indicates the phylogenetic position of *P. salvatori*. Sequences provided by us are highlighted in bold.

Table 1. Tamura-Nei corrected pairwise distances (average p-values in %) of *P. salvatori* and phylogenetically related *Proceratophrys* species estimated from a 448 base pair segment of the 16S rRNA mitochondrial gene. The remaining species of *Proceratophrys* and species of *Odontophrynus* were pooled to facilitate visualisation. For these, we reported the minimum and maximum values of genetic distance.

	Species	1	2	3	4	5	6	7	8
1	P. salvatori	-							
2	P. moratoi	2.4	-						
3	P. ararype	5.7	5.7	-					
4	P. concavitympanum	6.8	7.8	4.5	-				
5	P. aff. concavitympanum	6.8	6.7	1.6	4.8	-			
6	pooled remaining Proceratophrys	6.9–11.6	6.7-12.2	6.7-10.8	7.3–11.5	7.0–11.5	-		
7	pooled Odontophrynus	11.1–12.7	12.4–14.3	10.8-13.4	11.1–13.1	10.7-13.6	9.3–15.8	-	
8	Macrogenioglottus alipioi	12.3	13.8	12.8	12.3	12.4	10.3-14.5	4.7-8.4	-

overlap = 50 %, and DFT size = 256 samples. All other settings followed the 'default' of Raven. We constructed audio spectrograms with the R package Seewave 1.7.3 (Sueur et al., 2008) in R 3.6.1 platform (R Development Core Team, 2018) using the following settings: Hanning window, 256 points resolution (Fast Fourier Transform), and 70 % of overlap. We follow the terminology for call descriptions proposed by Köhler et al. (2017). Recordings were stored as uncompressed wav files at the Arquivos Sonoros da Universidade Federal do Rio Grande do Norte (ASURFN679–680).

RESULTS

The maximum clade credibility gene tree unequivocally recovered Odontophrynus salvatori as a member of the genus Proceratophrys and sister of P. moratoi with high support (posterior probability [pp] = 1.0; Fig. 1). Within the genus Proceratophrys, these two species are sister to the P. concavitympanum + P. ararype clade (pp = 1.0), as previously reported (Amaro et al., 2009; Mângia et al., 2018). Overall, our tree topology is generally similar to previous phylogenetic hypotheses (Amaro et al., 2009; Pyron & Wiens, 2011), with Proceratophrys being the sister clade to Odontophrynus + Macrogenioglottus, also with significant node support (pp = 1.0). Therefore, we formally place O. salvatori in the genus Proceratophrys [Proceratophrys salvatori (Caramaschi 1996) comb. nov.]. Additionally, P. salvatori exhibited 2.4 % genetic divergence in comparison to its closer relative (P. moratoi), and at least 5.7 % and 11 % in comparison to its other congeners and pooled Odontophrynus species, respectively (Table 1).

The advertisement call of *Proceratophrys salvatori* topotypical males (Fig. 2) is emitted in series of single multi-pulsed notes (complete pulses with silent intervals between each one; n = 65 analysed notes). Notes lasting from 0.297–0.413 s (mean 0.366 s; SD = 0.02) with 17–25 pulses (mean 21.2; SD = 1.5), and pulse repetition rate from 54–61 pulses per second (mean 58; SD = 2.1). Dominant frequency varied from 1688–1875 Hz (mean 1849 Hz; SD = 59). Our description matches that of Bastos et al. (2011), also based on calls from topotypical specimens, except for the lower mean dominant frequency reported (mean 1572 Hz; SD = 226). Accordingly, our data from the advertisement call reanalysis of some *P. moratoi* populations agrees with previous descriptions (Appendix II).

Based on the standardised analysis of advertisement calls, we identified that *Proceratophrys salvatori* calls are emitted with a lower pulse repetition rate and at a higher dominant frequency if compared to those of *P. moratoi* (see Table 2). Interestingly, although the dominant frequency of *P. salvatori* calls is higher than that of *P. moratoi*, these species do not differ in male size (Table 2). Considering mean values from different works, note duration is slightly longer in *P. salvatori* (mean ranging from 0.317–0.366 s) if compared to *P. moratoi* calls (mean ranging from 0.202–0.280 s), although raw values overlap (Table 2). Additionally, the lower pulse repetition rate and higher dominant frequency promptly



Figure 2. Advertisement call of *P. salvatori* (ASUFRN680, air temperature 23 °C, air humidity 78%) recorded at Chapada dos Veadeiros National Park, Alto Paraíso de Goiás Municipality, Goiás State. A) Call sequence with 4 consecutive notes; and B) spectrogram C) power spectrum and D) oscillogram of one highlighted note.

distinguishes the calls of *P. salvatori* from those of *P. concavitympanum* and *P. ararype* (Table 2).

DISCUSSION

As expected by previous non-molecular evidence (Caramaschi, 1996; Brandão & Batista, 2000), Proceratophrys salvatori was recovered as the sister taxa of P. moratoi. Both species lack ocular-dorsal ridge of warts, a feature that distinguishes them from remaining congeners, except from P. vielliardi (Brandão et al., 2013), and some populations of P. cristiceps (Mângia et al., 2020). Within Odontophrynidae, P. salvatori and P. moratoi share with their congeners the lack dorsal and tibial glands, interdigital membrane, and nuptial pads and exhibit tubercles on the thenar surfaces of hands and feet (Jim & Caramaschi, 1980; Caramaschi, 1996), characteristics not shared with species of Odontophrynus. Conversely, there is not much evidence that helps discriminate between these two species based on external morphology. For instance, Caramashi (1996) ranked as diagnostic characters the shorter squamosal bone on the optic branch (comparatively longer in P. moratoi; Jim & Caramaschi, 1980), dorsal coloration pattern and their presumably allopatric distribution (but see below). Recent works showed that dorsal coloration patterns are highly variable within Proceratophrys (Mângia et al., 2020), and a re-evaluation of this feature with a comprehensive sampling and genetic background is highly desirable to recognise species-specific traits.

Pulse repetition rate has been reported as an important acoustic component that discriminates species in *Proceratophrys* (e.g., Cruz & Napoli, 2010; Malagoli et al., 2016; Nascimento et al., 2019). Accordingly, the calls

Table 2. Summary of advertisement call parameters of *P. salvatori* and related species from all localities analysed and from literature (see also Supplementary Materials Appendix II). SVL reported represents the minimum and maximum values for adult males of each species. mm = millimetres; s = seconds; Hz = Hertz.

Species	male SVL (mm)	Note duration (s)	Pulses/note	Pulse rate (Pulses/s)	Dominant Frequency (Hz)
P. ararype	35.6–42.2ª	0.374-0.648	38–65	96-103	1034–1378
P. concavitympanum	39.6-51.6 ^b	0.178-0.500	19–51	100-119	754–1116
P. moratoi	24.7–31°	0.146-0.335	12–26	69–103	1153–1594
P. salvatori	25.5-27.8 ^d	0.198-0.420	15–25	54-61	1572-1875

SVL data from: ^a Mângia et al., 2018; ^b Santana et al., 2010; ^c Jim & Caramaschi, 1980, Brasileiro et al., 2008; ^d our data, Caramaschi, 1996



Figure 3. Distribution on topographic map of *P. moratoi* and *P. salvatori*. Abbreviations for Brazilian states: BA (Bahia), DF (Distrito Federal), GO (Goiás), MG (Minas Gerais), MS (Mato Grosso do Sul), MT (Mato Grosso), PR (Paraná), RJ (Rio de Janeiro), SP (São Paulo). The orange shading in South America inset represents the boundaries of Cerrado biome. Locality numbers from 1–25 refers to those in Table 3.

of P. moratoi and P. salvatori are mainly distinguished by pulse rate (values not overlapping) and dominant frequency (values barely overlap), also distinguishing the latter species from their phylogenetically closely related congeners (P. concavitympanum and P. ararype; Table 2). Moreover, it is well known that dominant frequency of anuran calls is constrained by male body size (being negatively correlated; Tonini et al., 2020), a pattern also observed for Proceratophrys (Nascimento et al., 2019). Nevertheless, we did not find differences in male body size between P. salvatori and P. moratoi, although calls of P. salvatori are 300Hz higher pitched if compared to its sister species (but see Bastos et al., 2011). Conversely, differences in dominant frequency between the calls of P. salvatori and P. concavitympanum and P. ararype are likely explained by male body size (Table 2). Our results provide additional diagnostic features that aid in the recognition of these morphologically similar species, reinforcing the importance of acoustic traits as an essential tool for taxonomic resolutions in *Proceratophrys* (Nascimento et al., 2019).

Despite both species being restricted to the Cerrado biome, *Proceratophrys salvatori* was only reported to occur within the Central Brazilian and Veadeiros Plateaus (both composing the highlands of Central Brazil in Goiás state, Azevedo et al., 2016; Martins-Ferreira & Campos, 2017), mostly at sites above 1000 m above sea level (Caramaschi, 1996; Brandão & Batista, 2000; Table 3). Conversely, *P. moratoi* occurs mostly below 900 m a.s.l. across the Canastra range and mountainous regions within Cerrado patches in São Paulo State (Fig. 3), with only two altitudinal records surpassing 1000 m a.s.l. in Minas Gerais State. Based on its currently known distribution, our new record in Mato Grosso State extends *P. moratoi* occurrence area in approximately 490 km north-west from the nearest locality in south-eastern **Table 3.** Summary of known distribution records for *P. moratoi* and *P. salvatori*. Alt = altitude in meters above sea level. Lat = latitude; Long = longitude. * species type locality.

#	Municipality	Locality	State	Alt	Lat	Long	Source		
	Proceratophrys moratoi								
1	Botucatu*	Rubião Junior	SP	865	-22.883	-48.5	Jim & Caramaschi, 1980		
2	Avaré	Fazenda Recreio	SP	675	-22.887	-48.947	Maffei et al., 2011		
3	Lençois Paulista	-	SP	740	-22.82	-48.88	Arruda et al., 2017		
4	Bauru	Jardim Botânico Municipal de Bauru	SP	550	-22.347	-49.016	Rolim et al., 2010		
5	Itirapina/Brotas	ESEC de Itirapina	SP	740	-22.215	-47.911	Brasileiro et al., 2008		
6	São Carlos	-	SP	815	-22.017	-47.939	Carvalho-Jr et al., 2010		
7	São Roque de Minas	Serra da Canastra	MG	1140	-20.151	-46.654	Haddad et al., 1988		
8	Uberaba	-	MG	740	-19.831	-47.71	Neves et al., 2019		
9	Perdizes	-	MG	1030	-19.339	-47.284	Martins & Giaretta, 2012		
10	Fronteira	-	MG	460	-20.263	-49.229	Neves et al., 2019		
11	Comendador Gomes	-	MG	650	-19.725	-49.196	Neves et al., 2019		
12	Uberlândia	-	MG	830	-19.015	-48.266	Martins & Giaretta, 2012		
13	Tupaciguara	-	MG	830	-18.662	-48.622	Neves et al., 2019		
14	Monte Alegre de Minas	-	MG	745	-18.869	-48.847	Martins & Giaretta, 2012		
15	Centralina	-	MG	710	-18.648	-49.155	Neves et al., 2019		
16	Ituiutaba	-	MG	550	-18.949	-49.43	Martins & Giaretta, 2012		
17	Paranaiguara	-	GO	410	-18.76	-50.61	Arruda et al., 2017		
18	Campo Alegre de Goiás	-	GO	940	-17.553	-47.828	Passos & Paredero, 2019		
19	Nova Xavantina	-	MT	300	-14.688	-52.422	Present study		
	Proceratophrys salvatori								
20	Alto Paraíso de Goiás*	Parque Nacional Chapada dos Veadeiros	GO	1280	-14.133	-47.533	Caramaschi, 1996		
21	Brasília	APA Cafuringa, Poço Azul	DF	1085	-15.582	-48.047	Brandão & Batista, 2000		
22	Brasília	ESEC Águas Emendadas	DF	1060	-15.545	-47.566	Brandão & Batista, 2000		
23	Brasília	ESEC Jardim Botânico de Brasília	DF	1120	-15.914	-47.885	Brandão & Batista, 2000		
24	Pirenópolis	Parque Estadual dos Pirenéus	GO	1175	-15.75	-48.834	Brandão & Batista, 2000		
25	Silvânia	FLONA de Silvânia	GO	865	-16.653	-48.609	Bastos et al., 2011		

Goiás State, also representing the lowest altitudinal record for this species, which previously was 410 m (Arruda et al., 2017).

The genetic divergence between Proceratophrys salvatori and P. moratoi is slightly lower than the threshold of 3 % estimated for Neotropical amphibians for the same 16S segment (Fouquet et al., 2007). Moreover, new records reported have been gradually expanding the distribution of P. moratoi northwards from the type locality across the Cerrado Biome (Table 3), narrowing the occurrence gap between P. salvatori from ~980 km (when initially described; Caramaschi, 1996) to currently 130 km (Fig. 3), thus raising doubts on the specific limits of these species. Nevertheless, they seem to show a discontinuous distribution along the borders of Central Brazilian highlands in Goiás State (Fig. 3). This high altitudinal Plateau is known to harbour high levels of flora and fauna endemism (Alves et al., 2014; Colli-Silva et al., 2019), including micro-endemic species of frogs such as Boana ericae, Bokermmanohyla sapiranga, B. pseudopseudis, Leptodactylus tapiti, P. rotundipalpebra,

and *Scinax rupestris* (Sazima & Bokermann, 1978; Brandão et al., 2012; Santoro & Brandão, 2014; Araújo-Vieira et al., 2015). Accordingly, studies focusing on endemic plants associated with montane savannas have been showing that the Central Brazilian and Veadeiros Plateaus likely acted as microrefugia during glacial cycles of the Pleistocene (Bonatelli et al., 2014; Perez et al., 2016). Therefore, it is plausible that *P. salvatori* diversified recently across these altitudinal gradients in Central Brazil, explaining the relatively lower genetic divergence compared to *P. moratoi*. In this sense, future studies should address diversification scenarios and test for their specific limits by sampling more specimens and genes under a phylogeographic framework.

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