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Study of closely related species within the *Physalaemus cuvieri* group (Anura): contribution of microsatellite markers

M. Conte[1,4], M.I. Zucchi[2], G.V. Andrade[3], A.P. Souza[4] and S.M. Recco-Pimentel[1]

[1]Departamento de Anatomia, Biologia Celular e Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brasil
[2]Polo Regional Centro Sul, Agência Paulista de Tecnologia dos Agronegócios, Piracicaba, SP, Brasil
[3]Departamento de Biologia, Centro de Ciências da Saúde, Universidade Federal do Maranhão, São Luís, MA, Brasil
[4]Departamento de Biologia Vegetal, Instituto de Biologia, Universidade Estadual de Campinas, Campinas, SP, Brasil

Corresponding author: S.M. Recco-Pimentel
E-mail: shirlei@unicamp.br

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ABSTRACT. Various species of the *Physalaemus cuvieri* group of frogs are difficult to distinguish morphologically, making molecular analysis an attractive alternative for indentifying members of this group, which is considered to be at risk because of loss of habitat. The genetic structure of natural populations of *P. ephippifer* and *P. albonotatus* species was investigated and analyzed, together with that of five previously studied populations of *P. cuvieri*. Nine microsatellite loci were used in the analyses. The overall G_{ST} value (0.46) revealed high genetic variation among the populations, as expected for different species. Bayesian analysis implemented by the STRUCTURE software clustered the seven populations into seven groups (K = 7). All the *P. albonotatus* and *P. ephippifer* specimens were grouped into a single cluster, both

species showing clear differentiation from *P. cuvieri*. The different grouping based on these microsatellites of some *P. cuvieri* individuals from Porto Nacional and from Passo Fundo suggests that they could be a new species, indicating a necessity for taxonomic reevaluation. Despite the intrinsic difficulties in analyzing closely related species, the nine microsatellite loci were found to be adequate for distinguishing these three species of the *P. cuvieri* group and their populations.

Key words: Related species; Frogs; Microsatellites; *Physalaemus*; Population structure

INTRODUCTION

The *Physalaemus* genus of the family Leiuperidae consists of 45 species (Frost, 2009) distributed into seven groups: "albifrons", "cuvieri", "deimaticus", "gracilis", "henselii", "olfersii", and "signifer" (Nascimento et al., 2005). Of these 45 species, nine belong to the *P. cuvieri* group and are named *P. albonotatus*, *P. centralis*, *P. cicada*, *P. cuqui*, *P. cuvieri*, *P. ephippifer*, *P. erikae*, *P. fischeri*, and *P. kroyeri*. The *P. cuvieri* group is widely distributed from southern to northern South America, in the east of the Andes from Argentina to Venezuela, in the open Cerrado, Caatinga, Chaco, and Llanos Domains (Nascimento et al., 2005).

The *P. cuvieri* group contains cryptic species with intraspecific morphological variation. Therefore, the identification of species, such as *P. cuvieri*, *P. ephippifer* and *P. albonotatus* (analyzed in this present study) and *P. centralis*, based exclusively on morphological characteristics is not reliable (Barrio, 1965). In addition, new species are supposed to occur and their identification is often difficult.

The correct species delimitation and identification is important for defining diversity and conservation strategies. The speciation process is not always accompanied by morphological changes, and thus, the true number of biological species is likely to be greater than currently known, as most of the species are determined on purely morphological traits (Bickford et al., 2007). Given the increasing worldwide destruction and disturbance of natural ecosystems, and considering that most species remain undescribed, efforts to catalogue and explain biodiversity need to be prioritized.

DNA markers have been proven useful for detecting and differentiating morphologically similar species (Bickford et al., 2007) and have been used as molecular markers, mainly to investigate the genetic structure of natural populations (Jones and Ardren, 2003; Lai and Sun, 2003). Microsatellites as genetic markers can be species-specific, but fortunately, the presence of highly conserved flanking regions has allowed cross-amplifications in species that diverged as long as 470 million years ago (Zane et al., 2002). They can be used as heterologous molecular markers in closely related species. These markers offer great potential for studies on parentage (Myers and Zamudio, 2004), gene flow (Austin et al., 2004; Kraaijeveld-Smit et al., 2005; Arens et al., 2007), and maintenance of genetic diversity (Funk et al., 2005; Wilkinson et al., 2007; Allentoft et al., 2009). High degrees of conservation in primer-binding sites have occasionally been shown among certain taxa (Hamill et al., 2007).

In this study, we report the comparison among two species of the *P. cuvieri* group (*P. ephippifer* and *P. albonotatus*, one population each) along with five populations of *P. cuvieri*

using microsatellite markers to analyse population genetic structure of these closely related species of the *Physalaemus* genus.

MATERIAL AND METHODS

Population sampling and DNA extraction

The specimens analyzed in this study were sampled in seven localities of Brazil (Figure 1). In total, 111 individuals were collected in the breeding season under a permission issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA, Proc. #02010.002895/03-84). One population of each species, *P. ephippifer* (PA12) and *P. albonotatus* (MT13) (Table 1), was analyzed together with five populations of *P. cuvieri* from Urbano Santos, Maranhão State (MA2), Porto Nacional, Tocantins State (TO3), Uberlândia, Minas Gerais State (MG4), Passo Fundo, Rio Grande do Sul State (RS10), and Crateús, Ceará State (CE11). The populations MA2, TO3 and MG4 were previously analyzed by Conte et al. (2009), and also, these three populations plus CE11 (*P. cuvieri*), PA12 (*P. ephippifer*) and MT13 (*P. albonotatus*) were cytogenetically studied (Quinderé et al., 2009). TO3 especially showed several remarkable chromosomal differences among *P. cuvieri* populations. To complete the sampling in this study, we added the RS10 population (analyzed by Conte M, unpublished results). These authors showed that TO3 and RS10 populations were divided into two sets of individuals each, representing a different sampled season. The accession numbers of voucher specimens are shown in Table 1.

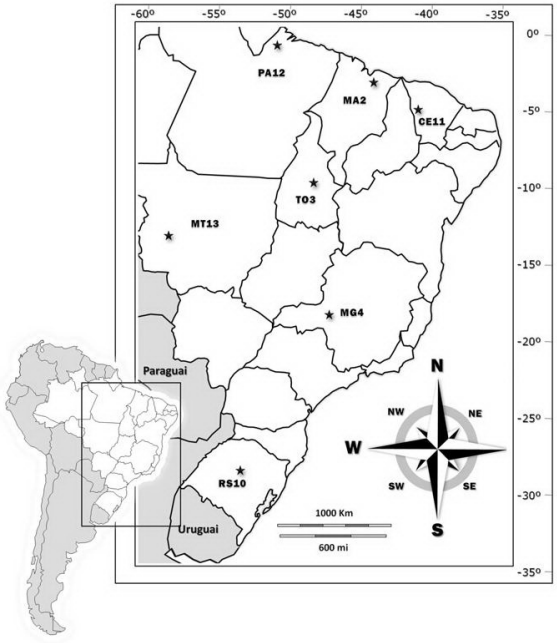

Figure 1. Map of Brazil displaying the sampling localities of *Physalaemus* populations. PA12 = *P. ephippifer*; MT13 = *P. albonotatus*; the five remaining populations (MA2, TO3, MG4, RS10, CE11) = *P. cuvieri*.

Table 1. Sampling localities of *Physalaemus* populations and total number of specimens analyzed.

Species	Sampling localities	Sample size	Region of Brazil	Biome	Population symbol	Museum accession No.
P. cuvieri	Urbano Santos, Maranhão (3°12'30.07"W; 43°24'16.27"S)	15	Northeast	Cerrado	MA2	ZUEC 13091; 13095; 13097; 13098; 13102; 13104; 1315; 13106; 13107; 13108; 13110; 13111; 13124; 13126, and 13127
	Porto Nacional, Tocantins (10°42'27.90"W; 48°25'01.35"S)	21	North	Cerrado	TO3	ZUEC 13374; 13376 to 13379; 13355 to 13359; 14691 to 14697, and 14699 to 14702
	Uberlândia, Minas Gerais (18°54'39.99"W; 48°20'25.76"S)	16	Southeast	Cerrado	MG4	ZUEC 13366 to 13372; 14705 to 14713
	Passo Fundo, Rio Grande do Sul (28°13'35.90"W; 52°28'42.71"S)	19	South	Pampas	RS10	ZUEC 14648 to 14666
	Crateús, Ceará (5°11'49.02"W; 40°40'10.52"S)	14	Northeast	Caatinga	CE11	ZUEC 13077 to 13079; 13081 to 13083 and 13085 to 13090
P. ephippifer	Belém, Pará (1°27'14.24"W; 48°30'28.99"S)	15	North	Amazonia	PA12	ZUEC 13703 to 13705; 13708; 13709 and 13730 to 13739
P. albonotatus	Lambari d'Oeste, Mato Grosso (15°50'00"W; 57°35'00"S)	11	Middle-east	Pantanal/Cerrado	MT13	UFMT 4462 to 4472

ZUEC = Zoology Museum of the State University of Campinas; UFMT = Museum of the Federal University of Mato Grosso.

Genomic DNA was extracted from liver, muscle and heart tissues of the *Physalaemus* specimens using the Genomic Prep Cells and Tissues DNA Isolation kit (Amersham Pharmacia Biotech) and the TNES method (Tris, NaCl, EDTA, SDS; Martins and Bacci Jr., 2001).

Microsatellite analysis

Nine specific microsatellite loci, previously developed for *P. cuvieri* and named P1A10, P3A12, P6A8, P9C1, P12D1, P13A5, P17B10, P20D4, and P21D10 (Conte et al., 2009), were used to genotype the individuals in the present study. The choice of the nine microsatellite markers used was based on data from previous analyses regarding number of alleles, null alleles, observed (H_O) and expected (H_E) heterozygosities, and deviation from the Hardy-Weinberg equilibrium (Conte et al., 2009).

Polymerase chain reaction (PCR) amplifications were performed according to Conte et al. (2009). PCR products were visualized on 3% agarose gels. Amplified DNA fragments were separated by electrophoresis on 6% denatured polyacrylamide gels using a 10-bp ladder (Invitrogen) as size standard, and silver stained (Creste et al., 2001).

Data analysis

The FSTAT software (Goudet, 1995) was used to estimate overall G_{ST}, G_{ST} pairwise (Nei, 1973) and Cockerham's estimator of F_{IS} (f - inbreeding coefficient) in order to investigate possible deviations from the Hardy-Weinberg equilibrium. Means of expected and observed heterozygosities were calculated using GDA (Lewis and Zaykin, 2000).

The Bayesian approach has been extensively used in population analyses. In the STRUCTURE software (Pritchard et al., 2000), K populations were assumed and each population is characterized by allelic frequency group in each locus. This software is able to cluster the individuals in populations that are as close as possible of the Hardy-Weinberg equilibrium, without *a priori* information about sampling sites. This feature is important to recognize cryptic species (Falush et al., 2007). The STRUCTURE software was used to group the individuals in clusters. The analysis uses Bayesian model-based clustering algorithm and attempts to identify genetically distinct populations on the basis of patterns of allele frequencies. The allele frequencies correlated among populations were used and STRUCTURE was applied in overlapping subsets of seven populations at a time. Runs were performed with a burn-in length of 100,000 and MCMC repeats of 100,000, with 10 iterations for each K. The range of possible Ks was from 2 to 7. Identification of number of distinct clusters (K) was performed following the procedure described by Evanno et al. (2005). In order to define the genetic relationships among populations, the neighbor-joining analysis (Saitou and Nei, 1987) was performed using the DARwin 5.0 software (Perrier and Jacquemoud-Collet, 2006).

RESULTS

Of the 10 microsatellite loci previously developed for *P. cuvieri* (Conte et al., 2009), nine cross-amplified were suitable for assessing genetic variability and genetic structure among the populations analyzed of *P. cuvieri*, *P. albonotatus* and *P. ephippifer*.

The overall H_E mean per population ranged from 0.09 in MT13 (*P. albonotatus*) to 0.58

in RS10 (*P. cuvieri*) populations. The H_O varied in a range from 0.15 in MT13 to 0.59 in RS10 (*P. cuvieri*), as shown in Table 2. The inbreeding coefficient (*f*) measures the correlation of genes among individuals belonging to a same population, and it determines random mating within the samples. The overall *f*-value was -0.12 (95%CI). The predominance of negative *f*-values, calculated by each population (Table 2), suggests that this was largely due to heterozygote excess.

Table 2. *Physalaemus* populations analyzed with microsatellites.

Population	H_E	H_O	*f*
MA2	0.41	0.56	-0.38
TO3	0.54	0.45	0.18
MG4	0.46	0.53	-0.14
RS10	0.58	0.59	-0.03
CE11	0.30	0.31	-0.05
PA12	0.34	0.47	-0.37
MT13	0.09	0.15	-0.61

PA12 = *P. ephippifer*; MT13 = *P. albonotatus*; other five populations (MA2, TO3, MG4, RS10, CE11) = *P. cuvieri*; H_E = expected heterozygosity; H_O = observed heterozygosity; *f* = breeding index.

The overall G_{ST} value of 0.46 (95%CI) indicated a high genetic differentiation among the sampled populations, and in the G_{ST} pairwise, the values ranged from 0.17 between TO3 and PA12 to 0.77 between PA12 and MT13 (Table 3).

Table 3. Genetic differentiation among populations measured as pairwise G_{ST} values (lower).

Population	MA2	TO3	MG4	RS10	CE11	PA12	MT13
MA2	0						
TO3	0.24	0					
MG4	0.36	0.20	0				
RS10	0.31	0.23	0.28	0			
CE11	0.34	0.30	0.37	0.40	0		
PA12	0.45	0.17	0.44	0.35	0.54	0	
MT13	0.72	0.64	0.68	0.63	0.74	0.77	0

The clustering analysis using STRUCTURE revealed K = 7, indicating that the dataset contained 7 distinct genetic units (Figure 2). The population clustering probabilities (P > 0.90) are listed in Table 4, and letters A to G symbolize the clusters. The closely related species *P. ephippifer* (PA12) and *P. albonotatus* (MT13) were assigned to a single cluster each, cluster A and G, respectively (Table 4). The *P. cuvieri* population grouping was: MA2 had 11 of 15 individuals assigned with all 14 individuals from CE11 to cluster C; TO3 was divided into two sets, with 9 individuals assigned to cluster E and 6 to cluster F together with 11 individuals from MG4. Moreover, RS10 had two distinct sets: cluster B with 10 individuals and cluster D with 6 individuals. The clustering of the 7 *Physalaemus* populations is represented in Figure 2, in which the groups are distinguished by colors.

The neighbor-joining tree (10,000 bootstraps) generated by the DARwin software (Figure 3) revealed that the clustering was similar to that with STRUCTURE (Figure 2). The first clade in the Figure 3 representation contained individuals from the PA12 population (*P. ephippifer*). The next clade has part of TO3 individuals, followed by a set of individuals from RS10. The most apart clade has individuals from MT13. Subsequently, there is a clade consisting of a set of individuals from the RS10 population and lower there is another branch composed of MA2 and CE11 individuals. Ultimately, the lowest positioned clade shows individuals from the MG4 population and part of TO3.

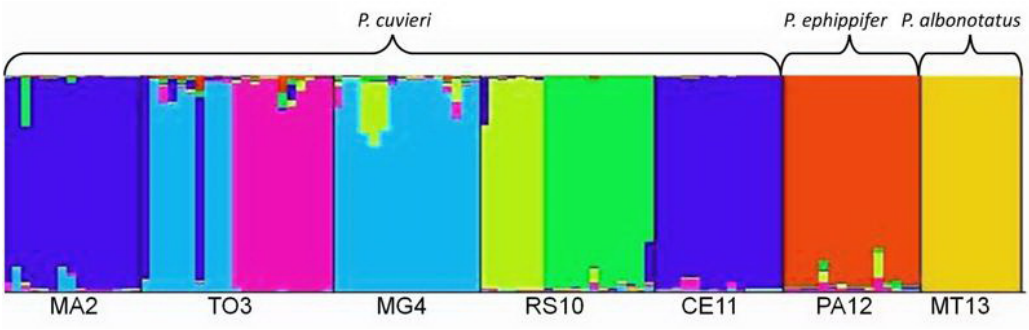

Figure 2. Color scheme: graphical output from STRUCTURE for K = 7. Clustering of *Physalaemus* populations, with K = 7. The color composition displays the probability of belonging to each of the seven clusters defined by STRUCTURE.

Table 4. Results of STRUCTURE analysis for seven *Physalaemus* populations (K = 7) showing mean probabilities for each population assigned to a cluster.

Population	N	Inferred cluster by STRUCTURE							P < 0.90
		A	B	C	D	E	F	G	
MA2	15	0.006	0.023	**11**/0.933	0.005	0.008	0.023	0.002	4
TO3	21	0.018	0.012	**1**/0.1	0.009	**9**/0.498	**6**/0.361	0.002	5
MG4	16	0.005	0.011	0.007	0.059	0.016	**11**/0.9	0.002	5
RS10	19	0.006	**10**/0.599	0.026	**6**/0.353	0.006	0.007	0.003	3
CE11	14	0.004	0.005	**14**/0.964	0.004	0.013	0.006	0.004	-
PA12	15	**13**/0.95	0.012	0.004	0.015	0.014	0.004	0.002	2
MT13	11	0.002	0.002	0.002	0.002	0.002	0.002	**11**/0.989	-

Numbers in bold refer to the individuals assigned to one of the seven clusters, with probability >0.90. The last column on the right side refers to the remaining individuals assigned to other clusters. PA12 = *P. ephippifer*; MT13 = *P. albonotatus*; other five populations (MA2, TO3, MG4, RS10, CE11) = *P. cuvieri*. N = sample size.

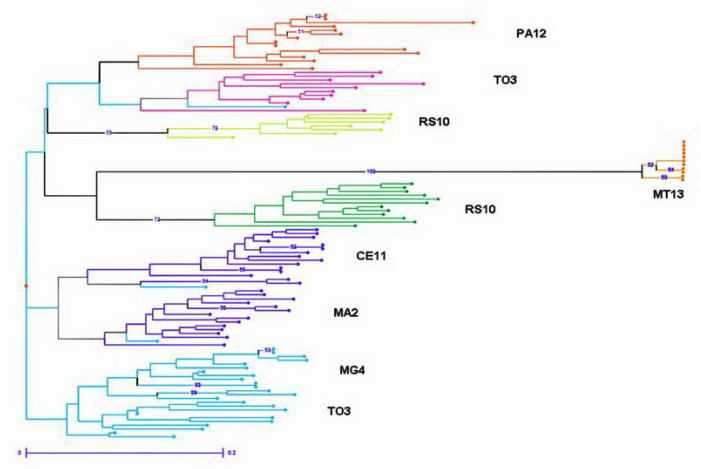

Figure 3. Neighbor-joining dendrogram. Numbers in the dendrogram indicate bootstrap probability (%) based on 100,000 replicates. Only probabilities over 50% were represented. Color representation of the five clusters and identification of specimens are as following: Red = PA12; pink = part of TO3; yellow = part of RS10; orange = MT13; green = remainder of RS10; dark blue = CE11 and MA2; pale blue = remainder of TO3.

DISCUSSION

The results described herein demonstrated that the 9 microsatellite loci (Conte et al., 2009) are suitable to separate the closely related species studied in this analysis and, besides, these markers were useful to determine the genetic structure of these closely related *Physalaemus* species. In addition, these markers allowed the discrimination of two populations (TO3 and RS10) of *P. cuvieri*.

The negative *f*-values found in six populations suggested heterozygote excess within these populations, probably due to favorable heterozygote selection or an effect of sampling. Still, these negative values show that these populations are panmictic. Some authors found similar results for this coefficient (i.e., Schmeller and Merilä, 2007; Allentoft et al., 2009).

In accordance with our expectations, the overall G_{ST} value (0.46) was high and indicated high genetic differentiation among populations. A smaller F_{ST} value (0.27) was recently estimated for 10 *P. cuvieri* populations (Conte M, unpublished results). This F_{ST} = 0.27 in *P. cuvieri* is coherent when compared with the G_{ST} = 0.46 reported in the present study for distinct yet closely related species analyzed together. In a previous study, Chiari et al. (2006) reported microsatellite and cytocrome *b* analyses of *Dyscophus antongilii* and *D. guineti*, in which the values of F_{ST} and R_{ST} were 0.606 and 0.546, respectively. These results suggested a clear genetic differentiation between the two *Dyscophus* species. Morgan et al. (2008), using microsatellite and mitochondrial DNA data, analyzed two species of frogs, *Pseudophryne pengilleyi* and *P. corroboree*, from distinct localities. The authors reported F_{ST} values more significant between geographically more distant populations, and the global Φ_{ST} showed that 18.7% of the molecular variation was partitioned among these populations. The present data of *Physalaemus* reflect a strong genetic differentiation among the sampling localities, showing that the microsatellites clearly distinguished the species involved in the present analysis.

The cytogenetic analysis of populations analyzed here (MA2, TO3, MG4, and CE11 - *P. cuvieri*, PA12 - *P. ephippifer* and MT13 - *P. albonotatus*) found a nucleolar organizer region (NOR) pattern that allowed the clustering of the populations from MA2, CE11 and MG4, but the TO3 did not group with any other population. The PA12 NOR was located in the same chromosome of these four *P. cuvieri* populations, but in a distinct region of pair 8. *Physalaemus ephippifer* (PA12) also differed by the presence of heteromorphic ZZ/ZW sex chromosomes. Ultimately, the *P. albonotatus* (MT13) karyotype clearly distinguished this species from other species of the *P. cuvieri* group (Quinderé, 2007). For the RS10 population, there are no cytogenetic data.

The neighbor-joining tree obtained from the analysis with the DARwin software, similar to the clustering obtained by STRUCTURE, were also useful in discriminating species of the *cuvieri* group. In the present study, these analyses discriminated PA12 and MT13 from each other and from other *P. cuvieri* populations analyzed. Even though they have been considered sibling species (Frost, 2009), they were clearly separated in all the genetic analyses, including STRUCTURE.

These two analyses also clearly separated the TO3 and RS10 (both *P. cuvieri*) populations in two groups each. Interestingly, even though all TO3 specimens were collected in the same locality, 10 individuals were captured from February to April 2004, in an open pasture area, and 11 were sampled in a forest, in April 2007. The former sample of TO3 population clustered with MG4, suggesting that set of individuals could really be *P. cuvieri*, since MG4 behavior was similar to that of

other populations of this species studied by Conte M (unpublished results). The second part did not show any grouping with the populations analyzed here, suggesting that these individuals probably belong to other species. Similar results were found for the RS10 population. Seven individuals were sampled in 2006 and 12 specimens were collected in 2007. These specimens were also divided into two sets of individuals by Bayesian analysis, and none of these sets showed any group with the *Physalaemus* species and populations analyzed here. The previous cytogenetic data (Quinderé et al., 2009) and the microsatellite analysis of the TO3 population (Porto Nacional County, TO) indicate that these individuals probably belong to other species, although the individuals are morphologically identical to and identified as *P. cuvieri*. We suggest a taxonomic review of this population, as well as the individuals from the RS10 population. These results reinforce that microsatellite analysis can be useful in detecting problems in species delimitation.

One crucial point in delimiting cryptic species is to distinguish between broad admixture and narrow contact zone or even restricted hybridization (Fouquet et al., 2007). There are several but perhaps incorrect assumptions about cryptic species, indicating that their speciation was so recent that morphological or other diagnosable traits have not yet evolved. Strong divergent natural or sexual selections are thought to be primary drivers behind rapid morphological divergence with little accompanying genetic differentiation (Bickford et al., 2007). Thus, it is difficult to explain or identify the mechanisms involved in cryptic species differentiation. There are few studies on population genetic structure of related species (Rosenberg et al., 2001; Parker et al., 2004; Engel et al., 2005; Ellis et al., 2006; Chiari et al., 2006), but they are rare for anuran sibling species.

The difficulty of morphological distinction between individuals from *P. cuvieri* demonstrates that very closely related species may go undetected in suitable habitats for decades or longer, even when those areas have been extensively studied. According to Luhring (2008), management decisions dependent on the perceived absence of a cryptic species should be made with the utmost caution, a high volume of targeted survey efforts, and long-term data sets, because hidden biodiversity can be obscured to the most qualified of experts.

The results obtained here showed that microsatellite markers were useful in determining the genetic structure of three related species of the genus *Physalaemus*. The levels of interspecific variation detected among these species, make microsatellite markers suitable for studies of population differentiation of these anuran species. The separation between TO3 and RS10 deserve special attention, and additional studies are warranted to better understand these results.

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