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Unraveling the variability and genetic structure of barker frog *Physalaemus cuvieri* (Leiuperinae) populations from different regions of Brazil

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ABSTRACT. The barker frog *Physalaemus cuvieri* is widely distributed in South America and is found in all regions of Brazil. Significant intraspecific morphological variation in this species has been reported. To determine the genetic structure of the natural Brazilian populations of *P. cuvieri*, 10 different populations geographically separated by 99.41 to 2936.75 km were evaluated using 10 polymorphic microsatellite loci. In addition, mitochondrial DNA data were analyzed to determine genetic distance between the populations. The genetic variation was found to be significant in most of the populations (H_E ranged from 0.40 to 0.59, and allelic richness ranged from 2.07 to 3.54). An F_{ST} value of 0.27 indicated that high genetic structure was present among the *P. cuvieri* populations. STRUCTURE

analyses grouped the 10 populations into nine clusters and indicated that only two of the populations were not genetically differentiated. The genetic distance calculated from the mitochondrial DNA data showed values <0.03 for seven of the populations.

Key words: *Physalaemus*; Barker frog; Population structure; Microsatellite; Mitochondrial DNA

INTRODUCTION

Physalaemus was recently included in the subfamily Leiuperinae (Pyron and Wiens, 2011). Currently, there are 45 species in this genus, and they are widely distributed throughout South America. The *Physalaemus cuvieri* group (*sensu* Nascimento et al., 2005) is composed of the following species: *P. albonotatus*, *P. centralis*, *P. cicada*, *P. cuqui*, *P. cuvieri*, *P. ephippifer*, *P. erikae*, *P. fischeri*, and *P. kroyeri* (Frost, 2013).

P. cuvieri is found in all regions of Brazil, the Misiones Province of Argentina, eastern Paraguay, the departments of Beni and Santa Cruz in Bolivia and, possibly, the lowlands of southern Venezuela (Frost, 2013). In Brazil, *P. cuvieri* inhabits a large area (Global Amphibian Assessment, 2009). It has been reported that significant intraspecific morphological variations, including size, the presence of warts on the skin, finger size and the presence of an inner tarsal tubercle and inguinal and parotoid glands, exist in the species (Nascimento et al., 2005). Distinct breeding periods in *P. cuvieri* populations in the northeastern and southeastern regions of Brazil have also been found (Barreto and Andrade, 1995).

The *P. cuvieri* group contains closely related species that also demonstrate intraspecific morphological variation, and these related species have been considered to be sibling species of *P. albonotatus* and *P. centralis* (Barrio, 1965; Frost, 2013). Therefore, the identification of a species based exclusively on morphological characteristics is not reliable (Barrio, 1965). Although new species are known to emerge, their identification is often difficult.

Previous groups have investigated *P. cuvieri* populations. Random amplified polymorphic DNA markers have been used to reveal complex spatial variation in *P. cuvieri* populations from the Cerrado region of the State of Goiás, Brazil, which consists of significant spatial heterogeneity coupled with low genetic distance similarity (Telles et al., 2006). In addition, cytogenetic studies have found chromosomal variation at both the intra- and interpopulation level for the number and localization of nucleolar organizer regions in *P. cuvieri* (Silva et al., 1999; Quinderé et al., 2009). Lastly, microsatellite markers have been used to assess the genetic structure of closely related species within the *P. cuvieri* group (Conte et al., 2011). Although previous studies have found evidence of genetic diversity between *P. cuvieri* populations, microsatellite studies, including those determining the genetic structure of *P. cuvieri* populations, have not been reported in the literature.

Ten polymorphic microsatellite loci from a GA-CA-enriched library have been isolated from *P. cuvieri* (Conte et al., 2009). These loci have enabled studies on the genetic structure of the populations of this species. In the present study, we used these polymorphic microsatellite loci to assess the genetic diversity and genetic structure of 10 *P. cuvieri* populations from various regions of Brazil. The populations were separated geographically by 99.41 to 2936.75 km, and their mitochondrial DNA was examined. This study aimed to contribute to our knowledge of this anuran species.

MATERIAL AND METHODS

Population sampling and DNA extraction

P. cuvieri specimens (N = 160 adults) were sampled from 10 locations in Brazil at distances ranging from 99.41 to 2936.75 km (Figure 1 and Table 1). Specimens were collected in the summer seasons from 1999 to 2007. The 10 populations were chosen to represent multiple Brazilian regions, including those known to exhibit cytogenetic variation. The abbreviations used are as follows: MA1 = São Pedro da Água Branca, Maranhão; MA2 = Urbano Santos, Maranhão; TO3 = Porto Nacional, Tocantins; MG4 = Uberlândia, Minas Gerais; SP5 = Vitória Brasil, São Paulo; SP6 = Palestina, São Paulo; SP7 = Nova Itapirema, São Paulo; MT8 = Chapada dos Guimarães, Mato Grosso; BA9 = Vitória da Conquista, Bahia; and RS10 = Passo Fundo, Rio Grande do Sul. Genomic DNA from tissue samples (liver, heart and/or muscle) was extracted using the Genomic Prep Cells and Tissues DNA Isolation kit (Amersham Pharmacia Biotech, USA) by the Tris, NaCl, EDTA, and SDS method (Martins and Bacci Jr., 2001).

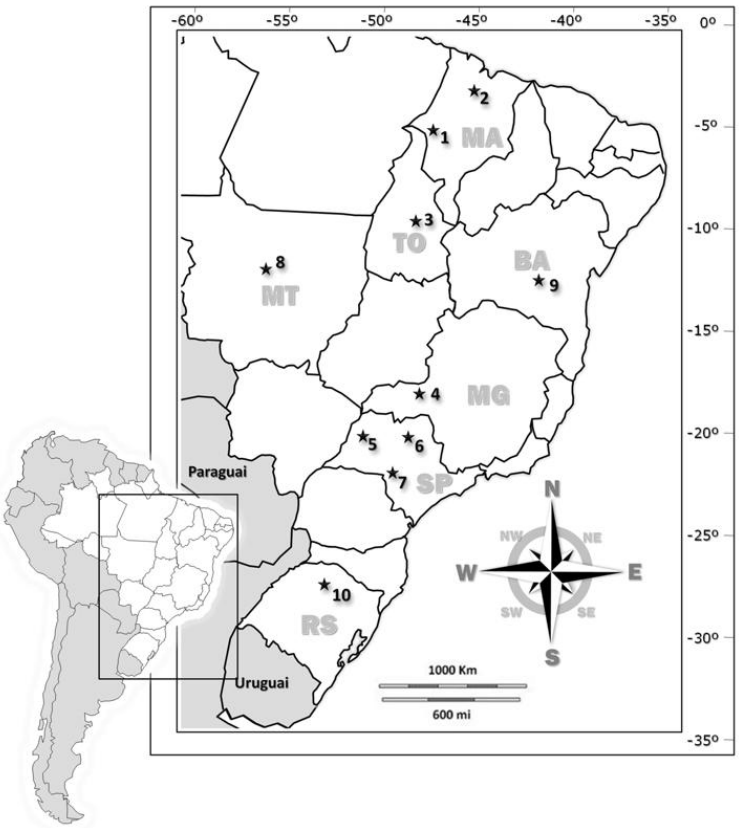

Figure 1. Map of Brazil displaying the ten locations in which natural populations of *Physalaemus cuvieri* were surveyed. MA1 = São Pedro da Água Branca, MA; MA2 = Urbano Santos, MA; TO3 = Porto Nacional, TO; MG4 = Uberlândia, MG; SP5 = Vitória Brasil, SP; SP6 = Palestina, SP; SP7 = Nova Itapirema, SP; MT8 = Chapada dos Guimarães, MT; BA9 = Vitória da Conquista, BA; and RS10 = Passo Fundo, RS.

Microsatellite analysis

Ten previously identified *P. cuvieri* microsatellite loci were used for genetic analyses of *P. cuvieri* populations (Conte et al., 2009) and included the following: P1A10, P3A12, P6A8, P9C1, P12D1, P13A5, P17B10, P20D4, P21D10, and P22C9. The PCR products were visualized on 3% agarose gels, and DNA amplification products were separated by electrophoresis on 6% denatured polyacrylamide gels and silver stained (Creste et al., 2001). DNA fragment sizes were determined by comparison with a 10-bp DNA ladder (Invitrogen, USA).

Mitochondrial DNA sequencing

The mitochondrial 16S ribosomal gene was chosen as a region of conserved sequence. The 16S gene was amplified from the samples using the following primers: 12SL13, Titus I (H), Hedges 16L2a, Hedges16H10, 16Sar-L, and 16Sbr-H (for primer sequences, see Goebel et al., 1999). The PCR amplified products were purified with a GFX PCR and Gel Band DNA Purification kit (GE Healthcare, England) and used directly as the template for sequencing. Sequencing was performed in an automatic ABI/Prism DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator kit (Applied Biosystems) according to manufacturer instructions. DNA sequences were bidirectionally sequenced and edited using the Bioedit version 7.0.1 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using ClustalW.

Data analysis

Expected (H_E) and observed (H_O) heterozygosities and private alleles were estimated using the genetic data analysis (Lewis and Zaykin, 2000). The FSTAT software (Goudet, 1995) was used to estimate pairwise F_{ST} among the populations through Nei's F-statistics. The Weir and Cockerham estimator of F_{IS} (f - inbreeding coefficient) was used to investigate possible deviations from Hardy-Weinberg equilibrium and to determine the mean of allelic richness. Genetic differentiation among populations was estimated using the F_{ST} statistic (Wright, 1951).

Clustering of the *P. cuvieri* populations was performed using the STRUCTURE software (Pritchard et al., 2000). The settings used were as follows: a burn-in length of 100,000, MCMC repeats of 100,000 and 10 iterations for each K. The possible Ks ranged from 2 to 10. A distinct cluster number (K) was identified (Evanno et al., 2005). This number examines the second order rate of change of the log probability of the data with respect to the number of clusters.

The Geographic Distance Matrix Generator (Ersts, 2007) was used to calculate all pairwise distances among the 10 populations from a list of geographic coordinates.

The genetic distance between the 10 populations of *P. cuvieri* was determined using mitochondrial DNA sequences from two specimens from the MA1 and MT8 populations and three specimens from the remaining eight populations. In total, 28 sequences were assessed using MEGA version 4 (Tamura et al., 2007). The sequences of the following four *Physalaemus* species were obtained from GenBank and used as outgroups: *P. gracilis* (Frost et al., 2006), *P. nattereri, P. barrioi*, and *P. signifier* (Ron et al., 2006).

RESULTS

Microsatellite data analysis

The H_E per *P. cuvieri* population ranged from 0.402 in SP7 to 0.597 in RS10. The H_O per population ranged from 0.417 in TO3 to 0.803 in MA1. Allelic richness varied from 2.070 to 3.543 (Table 1). The overall inbreeding coefficient was f = -0.11 (95%CI). The dominance of negative f values per population (Table 1) revealed an excess of heterozygotes. This finding might have been due to favorable heterozygote selection during sampling and may indicate an absence of inbreeding. Private alleles were present in two loci from the RS10 population and in one locus from the SP5 population.

Table 1. *Physalaemus cuvieri* populations, the sampling locations in Brazil and the specimen identification numbers.

Sampling location/ Latitude/Longitude	Sample size	Region	Voucher accession No.	Population	H_O	H_E	f	Allelic richness
São Pedro da Água Branca, MA 5°00'02.85"S, 48°17'26.97"W	13	Northeast	MNRJ 24233, MNRJ 24234, MNRJ 24255 to MNRJ 24261 and MNRJ 24263 to 24266	MA1	0.803	0.481	-0.720	2.263
Urbano Santos, MA 3°12'30.07"S, 43°24'16.27"W	15	Northeast	ZUEC 13091, 13095, 13097, 13098, 13102, 13104, 13105, 13106, 13107, 13108, 13110, 13111, 13124, 13126, 13127	MA2	0.569	0.435	-0.322	2.707
Porto Nacional, TO 10°42'27.90"S, 48°25'01.35"W	21	North	ZUEC 13374, 13376 to 13379, 13355 to 13359, 14691 to 14697, 14699 to 14702	TO3	0.417	0.571	0.273	3.543
Uberlândia, MG 18°54'39.99"S, 48°20'25.76"W	16	Southeast	ZUEC 13366 to 13372, 14705 to 14713	MG4	0.505	0.484	-0.046	2.974
Vitória Brasil, SP 20°11'49.65"S, 50°29'22.82"W	14	Southeast	ZUEC 14667 to 14680	SP5	0.508	0.543	0.064	2.920
Palestina, SP 20°23'29.69"S, 49°25'5806"W	17	Southeast	ZUEC 14634 to 14642, 14730 to 14732, 14643 to 14647	SP6	0.502	0.420	-0.200	2.583
Nova Itapirema, SP 21°06'00.12"S, 49°31'59.92"W	12	Southeast	ZUEC 12355 to 12357, 14681 to 14689	SP7	0.616	0.402	-0.571	2.070
Chapada dos Guimarães, MT 15°27'10.24"S, 55°44'20.94"W	17	Middle West	ZUEC 14616, 14617, 14619 to 14633	MT8	0.441	0.480	0.080	2.905
Vitória da Conquista, BA 14°51'53.12"S, 40°50'05.82"W	16	Northeast	ZUEC 14714 to 14729	BA9	0.462	0.512	0.100	2.960
Passo Fundo, RS 28°13'35.90"S, 52°28'42.71"W	19	South	ZUEC 14648 to 14666	RS10	0.634	0.597	-0.063	3.327

MA = Maranhão; TO = Tocantins; MG = Minas Gerais; SP = São Paulo; MT = Mato Grosso; BA = Bahia; RS = Rio Grande do Sul; ZUEC = Zoology Museum (Prof. Dr. Adão José Cardoso), Universidade Estadual de Campinas, SP, Brazil; MNRJ = Museu Nacional do Rio de Janeiro, Universidade Federal do Rio de Janeiro, SP, Brazil; and BC = Laboratory register number in the Department of Anatomy, Cell Biology and Physiology, Universidade Estadual de Campinas, SP, Brazil. The means of the observed (H_O) and expected (H_E) heterozygosities, breeding index (f), and allelic richness in *P. cuvieri* populations are also shown.

The overall F_{ST} value indicated a genetic variation of 27.1% between the *P. cuvieri* populations. Pairwise F_{ST} values ranged from 0.076 between TO3 and BA9 and up to 0.428 between the MA2 and SP6 populations (Table 2).

STRUCTURE analysis revealed a ΔK max of 56.31 and K = 9 (data not shown) and clustered the 10 *P. cuvieri* populations into 9 groups. As depicted in Figure 2, the groups are distinguished by colors. Population clustering probabilities are listed in Table 3, and the letters A to I represent the clusters. Of 160 specimens, 91 (56.8%) were assigned to a cluster with ≥0.90 probability. In the SP5 and SP6 populations, 18 specimens of 91 (19.7%) were grouped within the same cluster. Only the SP7 population had all 12 specimens (100%) assigned to a single cluster.

Table 2. F_{ST} pairwise comparisons between the ten *Physalaemus cuvieri* populations.

Population	MA1	MA2	TO3	MG4	SP5	SP6	SP7	MT8	BA9	RS10
MA1	-									
MA2	0.288	-								
TO3	0.158	0.236	-							
MG4	0.278	0.323	0.193	-						
SP5	0.310	0.350	0.227	0.193	-					
SP6	0.350	0.428	0.273	0.238	0.080	-				
SP7	0.352	0.405	0.220	0.386	0.308	0.392	-			
MT8	0.289	0.347	0.115	0.329	0.298	0.405	0.143	-		
BA9	0.263	0.299	0.076	0.284	0.287	0.366	0.239	0.092	-	
RS10	0.256	0.283	0.215	0.248	0.214	0.322	0.323	0.258	0.237	-

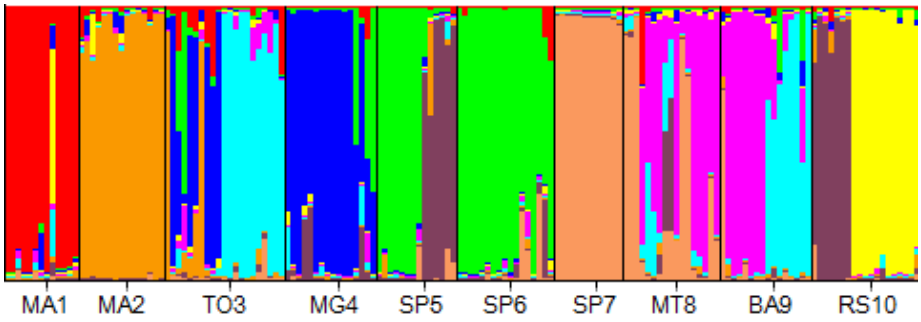

Figure 2. Graph of the STRUCTURE analysis for K = 9. Each vertical line represents a *Physalaemus cuvieri* specimen, and the color composition indicates the probability of the specimen belonging to each of the 9 clusters defined by the program.

Table 3. Results of the clustering analysis (STRUCTURE) for K = 9 (mean posterior probability for each population).

Population	N	Inferred clusters									P < 0.90
		A	B	C	D	E	F	G	H	I	
MA1	13	**9**/0.863	0.007	0.023	0.048	0.013	0.021	0.008	0.007	0.010	4
MA2	15	0.022	0.005	0.016	0.017	0.007	0.007	**10**/0.916	0.005	0.006	5
TO3	21	0.054	0.056	**1**/0.249	0.010	0.041	**3**/0.454	0.111	0.008	0.018	17
MG4	16	0.014	0.108	**9**/0.767	0.012	0.011	0.027	0.006	0.051	0.005	7
SP5	14	0.006	**6**/0.551	0.009	0.005	0.008	0.010	0.035	**2**/0.351	0.026	6
SP6	17	0.024	**12**/0.863	0.006	0.007	0.013	0.006	0.007	0.027	0.046	5
SP7	12	0.005	0.006	0.004	0.003	0.006	0.006	0.004	0.005	**12**/0.961	-
MT8	17	0.022	0.005	0.009	0.011	**1**/0.468	0.085	0.009	0.060	**2**/0.329	14
BA9	16	0.009	0.031	0.017	0.010	**6**/0.485	**3**/0.374	0.012	0.007	0.056	7
RS10	19	0.008	0.008	0.008	**10**/0.596	0.007	0.008	0.018	**5**/0.334	0.013	4

Numbers in bold represent the specimens assigned to one of the 9 clusters with a probability ≥0.90. The last column shows specimens below the assignment of 0.90. N = total sample size.

Mitochondrial DNA analysis

The genetic distance analysis of the 10 *P. cuvieri* populations showed the smallest value (0.004) among SP7 to SP5 and SP6 and the largest value (0.077) between MT8 and TO3. Despite having been sampled from very distant locations (more than 2000 km), seven populations (MG4, SP5, SP6, SP7, MT8, BA9, and RS10) displayed a genetic distance <0.03 between them. The population TO3 showed high genetic distance (>0.03) when compared with all the other populations. High genetic distances were also noted between MA1 or MA2

and the remaining populations (Table 4). The GenBank accession numbers are listed in Table 5. Two sequences from MA2 and one from SP6 are currently in submission (Lourenço LB, Targueta CP, Baldo D, Nascimento J, et al., unpublished data).

Table 4. Genetic distance determined from the mitochondrial DNA data between ten *Physalaemus cuvieri* populations.

Population	MA1	MA2	TO3	MG4	SP5	SP6	SP7	MT8	BA9	RS10	OUT-1	OUT-2	OUT-3	OUT-4
MA1		587.19	673.75	1564.29	1769.00	1746.03	1798.00	1422.00	1399.00	2631.85	-	-	-	-
MA2	0.016		992.89	1808.12	2028.26	2080.34	2098.46	1899.97	1316.18	2936.75	-	-	-	-
TO3	0.071	0.075		903.44	1052.03	1045.33	1357.76	942.42	928.77	1956.25	-	-	-	-
MG4	0.062	0.067	0.076		289.21	203.19	288.39	874.35	906.29	1033.52	-	-	-	-
SP5	0.061	0.066	0.075	0.013		109.40	135.42	766.32	1188.18	905.08	-	-	-	-
SP6	0.063	0.067	0.076	0.015	0.005		99.41	861.79	1103.55	943.96	-	-	-	-
SP7	0.063	0.067	0.075	0.014	0.004	0.004		904.81	1163.67	878.43	-	-	-	-
MT8	0.063	0.067	0.077	0.017	0.013	0.015	0.013		1560.70	1437.55	-	-	-	-
BA9	0.061	0.064	0.073	0.020	0.024	0.025	0.025	0.025		1931.00	-	-	-	-
RS10	0.061	0.066	0.071	0.014	0.012	0.014	0.013	0.014	0.016		-	-	-	-
OUT-1	0.171	0.170	0.177	0.179	0.179	0.179	0.179	0.175	0.179	0.178		-	-	-
OUT-2	0.176	0.174	0.174	0.176	0.176	0.176	0.176	0.175	0.173	0.173	0.186		-	-
OUT-3	0.179	0.178	0.180	0.176	0.174	0.175	0.175	0.173	0.174	0.173	0.126	0.181		-
OUT-4	0.185	0.179	0.177	0.184	0.185	0.184	0.184	0.179	0.182	0.185	0.185	0.100	0.186	

Outgroups are designated as: OUTG-1 = *P. nattereri*; 2 = *P. gracilis*; 3 = *P. signifer*; 4 = *P. barrioi*. The four outgroup samples are presented below diagonal. The geographic distances (km) between the populations are presented above diagonal.

Table 5. GenBank accession numbers for all 28 16S sequences obtained from the *Physalaemus cuvieri* populations and the four outgroup samples.

Population	Sequence	GenBank
MA1	92.11_16S	HQ592376
	92.09_16S	HQ592375
MA2	92.17_16S	In submission
	92.19_16S	In submission
	92.30_16S	HQ592368
TO3	92.76_16S	HQ592362
	92.77_16S	HQ592363
	92.86_16S	HQ592364
MG4	92.88_16S	HQ592365
	92.89_16S	HQ592366
	92.90_16S	HQ592367
SP5	92.97_16S	HQ592369
	92.99_16S	HQ592370
	92.100_16S	HQ592371
SP6	92.101_16S	HQ592357
	92.102_16S	HQ592358
	92.143_16S	In submission
SP7	92.64_16S	HQ592355
	92.158_16S	HQ592356
	92.164_16S	HQ592354
MT8	92.172_16S	HQ592349
	92.180_16S	HQ592348
BA9	92.184_16S	HQ592372
	92.185_16S	HQ592373
	92.199_16S	HQ592374
RS10	92.139_16S	HQ592359
	92.140_16S	HQ592360
	92.216_16S	HQ592361
OUTG-1	OUTG-1	DQ337208.1
OUTG-2	OUTG-2	DQ283417.1
OUTG-3	OUTG-3	DQ337209.1
OUTG-4	OUTG-4	DQ337213.1

Outgroup samples are designated as follows: OUTG-1 = *P. nattereri*; 2 = *P. gracilis*; 3 = *P. signifer*; 4 = *P. barrioi*.

DISCUSSION

The *P. cuvieri* microsatellite markers in this study have been previously used to examine a number of alleles, null alleles, H_O and H_E and deviations from Hardy-Weinberg equilibrium (Conte et al., 2009). The dominance of the negative *f* values described here is similar to that of previous studies on anurans. Negative inbreeding index values for *R. temporaria* populations have been found and indicate a favorable heterozygote selection (Schmeller and Merilä, 2007). In another study of *Rana temporaria* and *Bufo bufo* populations, the average *f* values equaled zero, indicating an absence of inbreeding in these species (Seppä and Laurila, 1999). Hence, there was an apparent excess of heterozygotes, which was probably due to sampling. The lack of evidence of inbreeding in most of the populations sampled indicates that they are panmictic populations.

The private alleles identified in RS10 and SP5 may indicate that these two populations are more genetically isolated than the other populations examined. The RS10 population was separated into two subgroups that consisted of 7 specimens sampled in 2006 and 12 sampled in 2007. Although we found that the RS10 population formed an isolated group and suggested the presence of private alleles, other southern populations of the species were not sampled to confirm this result. A similar result was found when analyzing these populations together with other populations from the *Physalaemus* group using SSR data (Conte et al., 2011). However, the hypothesis that these specimens belong to a different species was not investigated here, and it certainly cannot be discarded.

In the present study, the high global F_{ST} value (0.27) indicated high levels of genetic differentiation between the *P. cuvieri* populations examined. This finding is supported by several reports that have assessed the genetic structure in anuran species, including the following: Driscoll (1998a), studying *Geocrinia alba* and *G. vitellina*, and Driscoll (1998b), studying *Geocrinia lutea* and *Geocrinia rosea*, with both studies using allozymes; and Barber (1999), studying *Hyla arenicolor* using mitochondrial DNA, and Kraaijeveld-Smit et al. (2005), studying *Alytes muletensis* using microsatellite data.

Most of the F_{ST} pairwise comparisons in this study indicated relatively high differentiation between the populations, since 42 of the pairwise comparisons were significantly different from zero. This range of pairwise F_{ST} values is similar to the range observed in other studies that have been performed in South and Central America. In Amazonian frogs, a mean pairwise $F_{ST} = 0.280$ has been found, indicating a strong, positive relationship between genetic divergence and differences between populations of *P. petersi* (Funk et al., 2009). Studies of two species from Central America, *Dendropsophus ebraccatus* and *Agalychnis callidryas*, showed that pairwise F_{ST} comparisons were significant in most populations, indicating restricted gene flow even between the populations within the regions of Costa Rica and Panama (Robertson et al., 2009).

To our knowledge, there is no other genetic study of *P. cuvieri* populations with which to compare these results. However, the F_{ST} data derived from other anuran species are in agreement with the data described here. The majority of previous studies that have investigated the genetic structure of anuran populations have addressed populations that were up to a few kilometers apart. On a larger geographic scale, the genetic differentiation between *P. cuvieri* populations has frequently been high. Our 10 polymorphic microsatellite loci showed genetic structuring of the natural *P. cuvieri* populations from regions of Brazil that were separated

from each other by a distance of 100 km (F_{ST} = 0.397) to 2900 km (F_{ST} = 0.283).

The 10 *P. cuvieri* populations were clustered into nine groups. The SP5 and SP6 populations (109.40 km apart) were grouped together in the STRUCTURE analysis. However, although SP7 was located close to SP6 (99.4 km apart), they were distinguished from each other and from the remaining populations. A similar distinction occurred in the RS10 population, and the TO3 population was also divided into two sets of specimens. The TO3 population consisted of 10 specimens that were captured in an open pasture area in 2004 and 11 that were captured in a forest in 2007. The sampling areas for TO3 were 50 m apart from each other. The 10 TO3 specimens captured in 2004 were grouped with MG4, and the 11 specimens captured in 2007 clustered together with individuals from BA9, indicating that these specimens share similar alleles.

The genetic distances determined from the mitochondrial DNA data revealed a value > 0.03% among MA1/MA2, TO3 and among the remaining populations. A threshold value of approximately 0.03 appears to be appropriate to distinguish between intra- and interspecific divergence between neotropical anurans (Fouquet et al., 2007a). Amphibians are often characterized by high levels of genetic differentiation. Intraspecific pairwise divergence of the mitochondrial 12S and 16S genes has been found to extend up to almost 6% (Vences et al., 2005). In most comparisons between conspecific populations, the values are lower. Divergences of approximately 4 to 5% usually indicate distinct species (Fouquet et al., 2007b). Defining a new species based on the genetic distances found in a single DNA fragment is problematic (Veith, 1996). However, above a certain threshold, the distance found could be used as an indicator for identifying candidate species (Fouquet et al., 2007a).

For several species, studies that have captured and recaptured animals have shown that neighboring amphibian populations can interact through migration and gene flow (Berven and Grudzien, 1990). However, some amphibians exhibit strong fidelity to their habitats and have low dispersive capabilities (Funk et al., 2005). Such behavior can contribute to the restriction of gene exchange between amphibian populations (Newman and Squire, 2001; Burns et al., 2004).

Several studies have shown that amphibians are highly philopatric (Berven and Grudzien, 1990; Seppä and Laurila, 1999; Manier and Arnold, 2006), and some amphibian populations tend to become isolated (Shaffer et al., 2000). Consequently, amphibians are highly vulnerable to environmental degradation, which can lead to a reduction in their population size (Ficetola and De Bernardi, 2004). Small and isolated populations lose genetic variability (Johansson et al., 2007), and increased interpopulation distances promote relative isolation among local populations (Rowe et al., 2000).

The data described in the present study reveal the high fidelity of the *P. cuvieri* populations to their breeding pond based on the geographic scales used. This finding suggests that *P. cuvieri* is strongly philopatric and has low vagility. The majority of the previous studies on the genetic structure of anuran populations have addressed populations that were a few kilometers apart. On a larger geographic scale, high levels of genetic differentiation between *P. cuvieri* populations have been frequently observed. The 10 polymorphic microsatellite loci investigated here showed genetic structuring within the natural populations of *P. cuvieri* that were isolated from regions of Brazil and were separated by distances ranging from 99.41 km (F_{ST} = 0.615) to 2936 km (F_{ST} = 0.552).

In addition to revealing interpopulation genetic differentiation between the populations tested, the microsatellite analysis revealed important variation within populations. These results are in agreement with previously published studies that have demonstrated morpho-

logical and cytogenetic polymorphisms within species at the inter- and intrapopulational level.

Physalaemus cuvieri species may represent a complex of subspecies or species. This report confirms the need for more detailed molecular studies to better understand the variations that we found here.

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