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# The pioneering use of ISSR (Inter Simple Sequence Repeat) in Neotropical anurans: preliminary assessment of genetic diversity in populations of *Physalaemus cuvieri* (Amphibia, Leiuperidae)

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**ABSTRACT**

The greatest diversity of anurans in the world is in Brazil and one of the major challenges is to reconcile the accelerated economic development with strategies that aim to maintain this diversity in forest fragments, often representing ESUs of some biomes. This study aimed to obtain data that will support conservation projects through the pioneering use of ISSR analysis in Neotropical anurans, estimating the intra- and interpopulation genetic diversity of four populations of *P. cuvieri* (Paraná and São Paulo regions). Of the 65 loci scored 58 were polymorphic, with 0.797 intrapopulation variation and 0.203 interpopulation variation. The index of interpopulation genetic differentiation ($F_{ST}$) proved to be high among the population of Marmeleiro-PR and the three populations of SP ($F_{ST} > 0.288$); genetic dissimilarity was related to the geographical distance. The ISSR proved to be efficient and useful molecular markers in comparison with other markers most widely used for preliminary diagnosis of genetic diversity in populations of amphibians, and could be applied as a tool for future conservation projects, since they could identify potential ESUs and influence decisions on the preservation of fragments.

**Key words:** ESU; $F_{ST}$; Gene flow; Genetic structure; Molecular marker

## INTRODUCTION

Amphibians are considered relatively poor dispersers and highly philopatric (Blaustein et al., 1994). Usually, anuran populations exhibit a high degree of spatial structure, mainly when interpopulation distances exceed several kilometers (Shaffer et al., 2000). Amphibians have become a focus for studies on the effect of habitat fragmentation on genetic diversity and population differentiation (Reh and Seitz, 1990; Hitchings and Beebee, 1998).

With more than 800 described species, Brazil has the highest species diversity of frogs in the world, with many endemic species (SBH, 2011). The barker frog *Physalaemus cuvieri* Fitzinger, 1826 occurs in Argentina, Brazil, Paraguay, Bolivia, Guyana and possibly in the southern plains of Venezuela. In Brazil *P. cuvieri* occurs in environments with distinct climatic characteristics, where the amount and distribution of rainfall and range of temperature differs, as in the Caatinga, Cerrado (Brazilian savannah) and Forest (Nimer, 1989). *Physalaemus cuvieri* is a very small Leiuperidae (about 3 cm of rostrum–anal length). It breeds in permanent, semi-permanent and temporary bodies of water; and the eggs are laid in foam nests attached to grass stems at the margin of the pond. Males of *Physalaemus cuvieri* call on the water surface, floating by the inflation of vocal sacs and lungs. Aggressive interactions among males are frequently observed, mainly during the period of high activity levels (Barreto and Andrade, 1995). Studies using genetic molecular markers (microsatellite and RAPD) to evaluate the variation and population genetic structure of *P. cuvieri* have shown relatively low dispersion rates, high habitat fidelity and specificity (Telles et al., 2006; Conte et al., 2011).

Currently in Brazil one of the greatest scientific challenges is to reconcile strategies that avoid the loss of an important part of biodiversity with economic development, mainly related to agricultural activities (Ewers, 2005). The Atlantic Forest and Cerrado were the most degraded biomes during this process of human occupation, making small and isolated fragments be the only current representatives of native vegetation as well as its characteristic fauna (Ranta et al., 1998; Oliveira and Marquis, 2002). These small fragments are still very important for maintaining the diversity of amphibians, since they serve as a refuge for animals during the dry season, as a day shelter during the breeding season, a foraging area and even as dispersal corridors (Silva and Rossa-Feres, 2007; Silva and Rossa-Feres, 2011). According to the same authors, these fragments collaborate with the maintenance of regional rainfall regimes, essential for the survival of most species of anurans.

One important component of biodiversity is genetic variability; with the popularization of molecular techniques it has become a tool for determining more accurately whether a population does or does not have the minimum attributes for maintenance or if it is at risk of extinction (Nei et al., 1975; Leberg, 1992). Thus molecular techniques have been used for several studies of anurans, and the most commonly used markers are allozymes (Spasic-Boskovic et al., 1999; Bisconti et al., 2011), RAPD (Telles et al., 2006; Silva et al., 2007),

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mitochondrial DNA (Vences et al., 2005; Funk et al., 2007) and microsatellites (Martínez-Solano and García-París, 2005; Conte et al., 2011).

ISSR (*Inter Simple Sequence Repeat*) analysis has recently been used, since it produces excellent results and also has very low cost compared to other techniques. In ISSR, fragments are amplified via PCR, obtaining dominant markers and using only one primer (anchored microsatellite sequences) composed of three or four repeating units of a microsatellite (often 18-20 base pairs) that may or may not have one to four degenerate nucleotides anchored in the 3 'or 5' ends (Zietkiewicz et al., 1994). The amplified genomic segment is the one that lies between two blocks of microsatellite. Thus a previous knowledge of the region to be amplified is not necessary, nor a time-consuming and expensive step of genomic library construction (or cloning and sequencing) as with microsatellite markers. The greater reproducibility is an advantage that makes the ISSR more promising than RAPD, due to the use of longer primers which allows the use of high annealing temperature (45-60 °C) leading to higher stringency (Reddy et al., 2002), and increasing the repeatability of experiments (for review, see Semagn et al., 2006).

The extinction of genetically unique populations has largely taken place since 1900 as a result of habitat destruction, pollution and overexploitation. The main agents that cause a species to lose genetic variability are the isolation of populations and the reduction of the population effective size. These reductions may result in a decline in fitness and eventual extinction (Carvalho and Hauser, 1998). The present study provides preliminary data of the pioneering use of the ISSR technique in Neotropical anurans to estimate the intra- and interpopulation genetic diversity in populations of *P. cuvieri*, and verify the effectiveness of this technique to generate data that may support proposals for the conservation of threatened forest fragments.

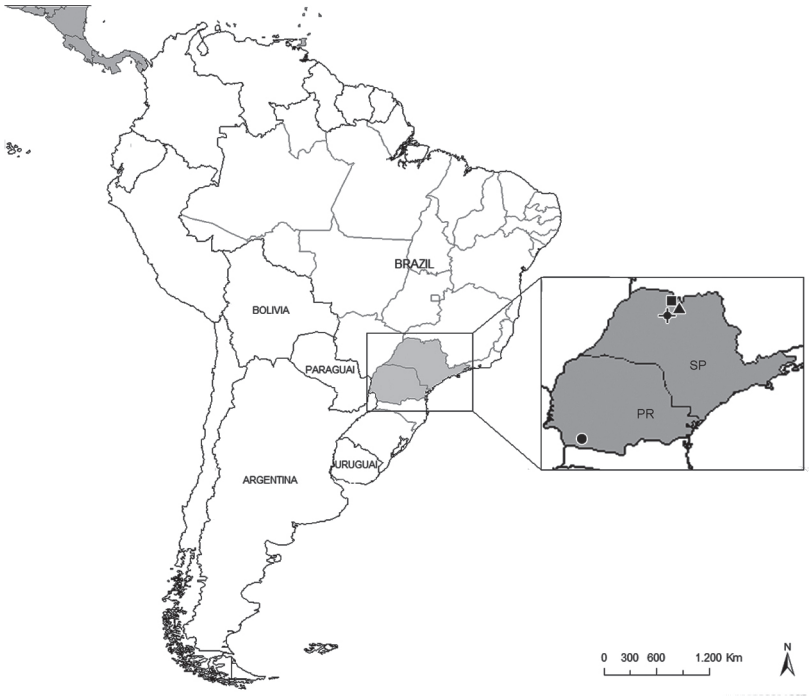


**Figure 1:** Map of sampling sites of *P. cuvieri* populations in Brazil: Marmeleiro-PR (●), Nova Itapirema-SP (✦), Eng. Schmidt-SP (▲) and Talhado-SP (■).

## MATERIAL AND METHODS

Three populations of *P. cuvieri* from São Paulo (SP): Engenheiro Schmidt (20º51'58.45"S; 49º18'29.07"W), Nova Itapirema (21º05'60.00"S; 49º31'60.00"W), Talhado (20º40'51.01"S; 49º17'34.57"W), and a population from Paraná (PR): Marmeleiro (26º08'58.43"S; 53º01'31.77"W) were sampled (Fig. 1). Since this is a preliminary study for presenting new and useful information on the utility of ISSR marker to assess the genetic variability in Neotropical anurans, only five male individuals were collected from each population (we suggest increasing the sample size and the number of populations for inferences on the genetic structure and for conservation purposes); they were anesthetized and sacrificed in the laboratory by benzocaine saturation. Fragments of liver were removed and stored in 100% ethanol for DNA extraction, which was performed with *GenElute™ Mammalian Genomic DNA Miniprep Kit* (Sigma-Aldrich) following the manufacturer's instructions.

For the amplification of ISSR fragments, the following primers were previously selected and used $(GGAC)_3A$, $(GGAC)_3C$, $(GGAC)_3T$ and $(GGAC)_4$. The conditions for DNA amplification via PCR were as recommended by Fernandes-Matioli et al. (2000), with modifications. The amplification reaction mixture consisted of Tris-KCl, 2 mM $MgCl_2$, 0.92 mM primer, 0.38 mM dNTP, 1 U/ reaction Taq DNA polymerase, DNA (10 ng) and enough water to make up a volume of 13 µL. The amplification reactions were performed in Eppendorf Mastercycler Gradient thermocycler scheduled for 4 cycles of 45s at 94 °C, 1 min at 51 °C and 1 min at 72 °C, followed by 29 cycles of 45s at 94 °C, 1 min at 48 °C and 1 min at 72 °C. After the last cycle of amplification, the reaction mixture was cooled and maintained at 4 °C. Negative controls without DNA were included in each set of amplifications. After amplification, samples consisting of 7 µL of PCR reaction mixture were subjected to electrophoresis on 1.4% agarose gels and stained with ethidium bromide (0.2 µg/mL). Electrophoresis was performed in TBE buffer (Tris-borate), 5 $V.cm^{-1}$, for 4 hours; the amplified fragments were visualized under ultraviolet light and the gel was photographed for analysis. The size of the fragments was estimated by comparison with 100 bp ladder marker (Invitrogen ™). The fragments of ISSR were treated as dominant markers and judged as binary characters: present (1) or absent (0), so a binary matrix was produced and from it were estimated the indices of genetic diversity within and among populations. The accuracy of band assignment was studied by repeating in a minimum of five replicates; the bands were bright enough that presence/absence scoring was not confounded by simple intensity differences and the bands were distinct enough in size from the surrounding bands.

The pairwise genetic distance matrix between individuals was obtained by the Jaccard similarity index, and used to construct the Neighbor-Joining dendrogram with the program FreeTree and Mega 3.1. The scatter plot of principal coordinates was constructed

using the programs DistPCoA and Statistica 7.1. Genetic differentiation was examined by applying the Mantel test, with 10,000 permutations for the Jaccard similarity matrix using the Mantel-Struct 1.0 program. The analysis of molecular variance, expected heterozygosity and the value of genetic differentiation ($F_{ST}$) were obtained using the program Arlequin 3.5.1.2. The linear regression between $F_{ST}$ and geographic distance was carried out using Statistica 7.1 using the data in Table 1.

## RESULTS

Of the 65 loci scored 58 were polymorphic, with 0.797 intrapopulation variation and 0.203 interpopulation variation. The polymorphic *vs.* total number of loci per each primer was: 15/17 for the $(GGAC)_3A$ primer; 15/17 for the $(GGAC)_3C$ primer, 14/16 for the $(GGAC)_3T$ primer and 14/15 for the $(GGAC)_4$ primer. Primer $(GGAC)_3A$ produced a fragment of 250 bp uniquely from the population of Marmeleiro-PR and another fragment of 210 bp uniquely from populations of SP. The amplified fragments ranged from 200 to about 1,500 bp (Fig. 2A).

The dendrogram analysis identified two main groups whose compositions show that there is a clear relationship between genetic distance and geographical distance, and the population of Marmeleiro-PR proved to be differentiated from the other populations analyzed (Fig. 2B). The scatter plot of principal coordinates built with the two major eigenvectors (0.245 and 0.122 of variation, respectively), also obtained with the Jaccard similarity index, corroborates the dendrogram, since it shows that the population of Marmeleiro-PR is separated from the SP populations (Fig. 2C).

Genetic differentiation among populations of *P. cuvieri* quantified by the Mantel test showed no significant correlation only between the populations of Eng. Schmidt-SP and Talhado- SP (the smallest geographic distance), showing that these populations are not genetically differentiated ($p = 0.473$, $r = -0.011$ and $Z_{10,000} < Z$). Values of genetic dissimilarity, which is the genetic variability within and among populations, were also obtained through this test (Table 1). The intrapopulational genetic dissimilarity was almost the same for all populations, being 0.469 in the population of Marmeleiro-PR; 0.449 in Nova Itapirema-SP; 0.454 in Eng. Schmidt-SP and 0.446 in Talhado-SP. The expected heterozygosity was almost the same for all populations; 0.513 ± 0.101 in Marmeleiro-PR; 0.488 ± 0.101 in Nova Itapirema-SP; 0.457 ± 0.091 in Eng. Schmidt-SP and 0.506 ± 0.101 in Talhado-SP. The index of interpopulation genetic differentiation ($F_{ST}$) was high between populations of the two different regions PR/SP ($F_{ST} \geq 0.25$), moderate among the population of Nova Itapirema and the other two SP populations ($0.25 > F_{ST} \geq 0.05$), and not different among the populations of Schmidt and Talhado ($F_{ST} < 0.05$). These results corroborate the genetic difference among Marmeleiro-PR population and SP populations, and indicate that genetic dissimilarity is related to geographical distance, as the correlation between $F_{ST}$ and geographic distance ($p = 0.002$, $r = 0.966$) (Table 1, Fig 3).

## DISCUSSION

Although the studied populations of *P. cuvieri* are present in small preserved fragments of Atlantic Forest and Cerrado, the genetic variability present is high (around 0.45), and 0.797 of all variation is intrapopulation. A similar result was found by Telles et al. (2006) in a study with RAPD marker in 214 individuals from 18 populations of *P. cuvieri*, where most of the genetic variation was found among individuals of the same fragment (0.898). When the authors analyzed only the 6 local populations with more than 12 individuals (12 to 23 individuals), the result obtained was qualitatively similar, and indicated that unbalanced sample sizes and the relatively small sample size in some populations did not qualitatively affect the evaluation of overall population genetic structure (using AMOVA and Bayesian estimate of $\theta^B$). In relation to the expected heterozygosity ($H_e$), our study showed high values ranging from 0.46 to 0.51. Similar results were obtained by Conte et al. (2011) using microsatellite markers in 85 individuals from 5 populations (14 to 21 individuals/populations), with high observed heterozygosities ($H_o$) ranging from 0.31 to 0.59 (and $H_e$ ranging from 0.30 to 0.58). As already shown in other studies, forest fragments can contribute to the maintenance of species diversity of frogs (Silva and Rossa-

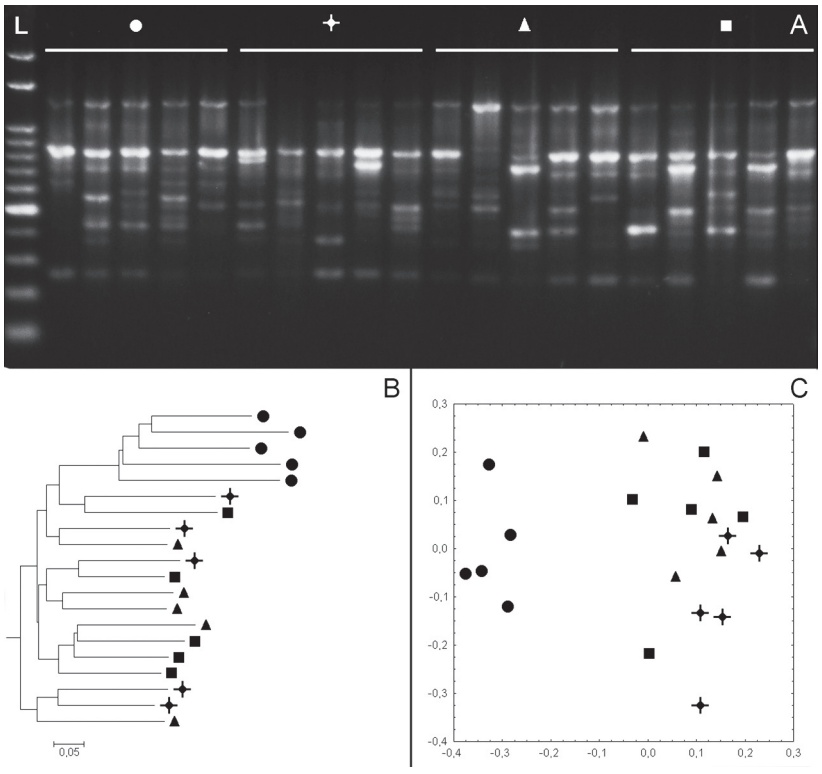


**Figure 2:** Analysis of P. cuvieri populations using ISSR markers: (A) *agarose gel electrophoresis* of PCR products obtained from the amplification of DNA samples using the primer (GGAC)3T; (B) Neighbor-joining dendrogram based on Jaccard similarity index with 10,000 bootstrap resamplings. (C) Scatter plot of main coordinates based on Jaccard similarity index. (L) 100 bp ladder, (●) Marmeleiro-PR, (✦) Nova Itapirema-SP, (▲) Eng. Schmidt-SP and (■) Talhado-SP.

**TABLE 1**

Pairwise comparisons of *Physalaemus cuvieri* populations.

| Pairwise comparisons | Distance (Km) | $F_{ST}$ | p | r | Z | $Z_{10\,000}$ | Genetic dissimilarity |
|---|---|---|---|---|---|---|---|
| Marmeleiro-PR x Nova Itapirema-SP | 663.88 | 0.359 | 0.007 | 0.861 | 15.849 | 13.900 | 0.634 |
| Marmeleiro-PR x Eng. Schmidt-SP | 699.08 | 0.292 | 0.009 | 0.759 | 14.854 | 13.383 | 0.594 |
| Marmeleiro-PR x Talhado-SP | 717.33 | 0.288 | 0.007 | 0.687 | 14.679 | 13.228 | 0.587 |
| Nova Itapirema-SP x Eng. Schmidt-SP | 35.40 | 0.072 | 0.114 | 0.185 | 11.871 | 11.611 | 0.475 |
| Nova Itapirema-SP x Talhado-SP | 53.42 | 0.058 | 0.183 | 0.145 | 11.668 | 11.446 | 0.467 |
| Eng. Schmidt-SP x Talhado-SP | 21.06 | -0.005 | 0.473 | -0.011 | 11.208 | 11.220 | 0.448 |

Approximate distance (km), interpopulation genetic differentiation index ($F_{ST}$), significance level (p), correlation of two matrices (r), robustness of the relation original data (Z) and after 10,000 permutations ($Z_{10\,000}$), genetic dissimilarity using Jaccard Coefficient.

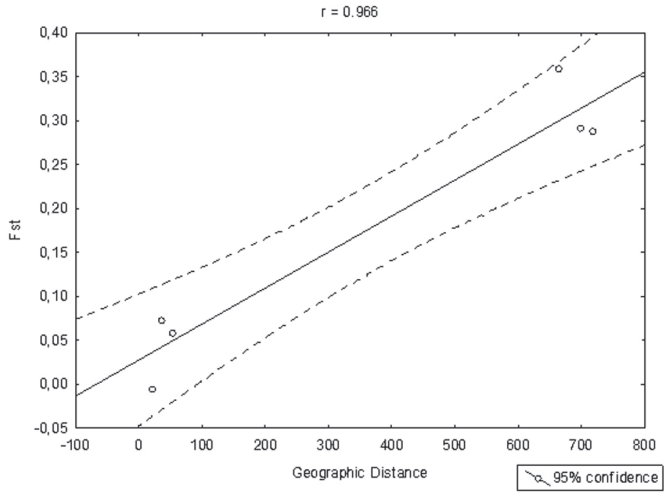


**Figure 3:** Linear regression between FST and geographic distance among P. cuvieri populations.

Feres, 2007; Silva and Rossa-Feres, 2011), and the present study supports that the genetic diversity can also be maintained, and thus the populations would be potentially more protected from the extinction factor due to environmental changes.

Gene flow is geographically restricted to nearby populations, indicated by $F_{ST}$ that increases in direct proportion to the distance. In our study, this correlation was observed, where the highest $F_{ST}$ occurred between populations of the two different regions PR/SP (distance ≥ 663.8 Km; Table I, Fig. 3). Similar results were obtained in *P. cuvieri* by Conte et al. (2011) using microsatellite markers, with high $G_{ST}$ values ($G_{ST} \geq 0.20$) observed between populations isolated by long distances (ranging from 381 Km to 2936 Km). Telles et al. (2006), in RAPD studies with *P. cuvieri* at a much smaller spatial scale, indicate that genetic similarity among local populations tends to be slightly larger than expected by chance alone. Lampert et al. (2003), working with populations of *Physalaemus pustulosus* separated by 260 meters on average, that is, a very small range of spatial distance, showed strong positive correlation between genetic and geographic distances. Although genetic diversity can be produced by mutation, genetic drift and selection, the differentiation between populations tends to increase with the absence of gene flow, and these data can contribute to the identification of evolutionarily significant units (ESUs) (Varvio et al., 1986). The preservation of ESUs maximizes the potential for future evolutionary success of species or population groups of species (Hey et al., 2003). The use of ESUs as targets for conservation actions is increasing, so it is very important that preliminary studies identify quickly, accurately and correctly an ESU. In the present study it was possible to determine by the ISSR marker that the level of interpopulation genetic differentiation is high among the population of Marmeleiro-PR and other SP populations ($F_{ST} > 0.288$), suggesting that this population has a genetic composition quite different from the others.

The frequent occupation of many areas of agriculture and livestock has reduced many characteristic biomes of Brazil to small fragments, surrounded by a landscape highly modified and degraded with an edge effect greatly increased, which is reflected in changes of abiotic factors resulting from the abrupt transition between two adjacent ecosystems that affect population dynamics (Murcia, 1995; Rambaldi and Oliveira, 2003). Decreases in the potential for dispersal of species, the number of species and population size can also be observed, resulting in reduced genetic variability (Fahrig, 2003). According to Ohmer and Bishop (2011), studies from the last two decades indicate climate change and habitat loss as the factors most related to the worldwide decline of amphibians. Nevertheless, the small fragments are often the last remnants of a biota and play an important role in the preservation of these species (Silva et al., 2011).

As recommended by Fraser and Bernatchez (2001) and Aleixo (2009), the degree of vulnerability of an ESU should be assessed at the planning stage of conservation, to prevent a species listed as threatened from losing one of its ESUs before conservation action. The ISSR proved to be an efficient and useful molecular marker for the preliminary diagnosis of genetic diversity in populations of amphibians; it has a low cost, high repeatability and produces quick results, since it does not require prior knowledge of sequence to be amplified. Moreover, for the preliminary diagnosis of genetic diversity, the limited sample size should be compensated by a higher number of loci per sample. Thus it can be used as a tool for future conservation projects, helping in making decisions about the preservation of fragments, and identification of ESUs before they are threatened.

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