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Species diversity and geographic distribution of *Gymnotus* (Pisces: Gymnotiformes) by nuclear $(GGAC)_n$ microsatellite analysis

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Abstract

Patterns of amplified DNA fragments flanked by $(GGAC)_n$ microsatellites, obtained by single primer amplification reaction (SPAR), from 198 *Gymnotus* specimens (Pisces: Gymnotiformes) sampled from 8 southeastern Brazilian river basins were analyzed. The species studied were *Gymnotus carapo*, *G. pantherinus*, *G. inaequilabiatus*, and *G. sylvius*. The indirectly obtained patterns reflected the distribution of simple sequence repeats in the nuclear genome of the specimens. Species-specific patterns of DNA amplification were found and were useful for the analysis of the geographic distribution of *Gymnotus* species. Monomorphic patterns were found in *G. carapo*, *G. pantherinus*, and *G. inaequilabiatus*. Three polymorphic patterns were found in *G. sylvius* populations. The SPAR technique could be a useful molecular tool in conservation programs involving communities of neotropical freshwater fish.

INTRODUCTION

The order Gymnotiformes is a monophyletic group of electrogenic freshwater fish. It comprises six families of which the family Gymnotidae is the most widely distributed in neotropical regions (Mago-Leccia, 1994). *Gymnotus* is the only genus of Gymnotidae and, until now, encompasses about 15 nominal species, although many undescribed species probably still exist (Nelson, 1994). In Brazil *Gymnotus* species are usually known as "sarapó" (pronounced "säräpô").

The species diversity of *Gymnotus* is well known in the Amazon basin where nine species are recognized (Albert *et al*., 1999), but few data are available about species diversity, distribution and population structure of this genus in other neotropical basins. In the southeastern Brazilian basins, four morphologically distinct *Gymnotus* species have been detected (Fernandes-Matioli *et al.*, 1998). Cytogenetic and electric organ discharge (EOD) analyses of these species have been carried out (Almeida-Toledo, 1978, Foresti *et al*., 1984, Foresti, 1987, Fernandes-Matioli, 1996; Fernandes-Matioli *et al*., 1997, 1998) but were inconclusive for species identification since some of these species present identical karyotypes and/or EOD patterns.

A very good specific molecular marker, obtained by the single primer amplification reaction (SPAR) technique, was described by Gupta *et al*. (1994). This technique is based on polymerase chain reaction (PCR) amplification of DNA markers using single primers of simple sequence repeats (SSR) or microsatellites.

The aim of the present work was the characterization of species and populations of *Gymnotus* from southeastern Brazilian basins using the SPAR technique to analyze species diversity and geographic distribution.

MATERIAL AND METHODS

Specimens

Four species, *Gymnotus carapo* (94 specimens), *G. pantherinus* (42 specimens), *G. sylvius* (58 specimens), and *G. inaequilabiatus* (four specimens) from eight southeastern Brazilian river basins were analyzed using the SPAR technique (Table I and Figure 1). The 58 *G. sylvius* specimens were also analyzed based on polymorphic *micro*11 patterns. All specimens were obtained from wild populations. Unless they were to be preserved for further analyses, the individuals were released at the collection site. Two other fish genera, *Eigenmannia* (Sternopygidae, Gymnotiformes) and *Pseudostegophilus* (Trichomycteridae, Siluriformes), from neotropical basins were included in the experiment to verify the efficacy of *micro*11. Tissue samples of specimens from these two species were kindly provided by Dr. D. Calcagnotto and Dr. M. de Pinna, respectively.

Total DNA

DNA was extracted from blood, fin and/or scales and was isolated by the standard phenol:chloroform protocol (Sambrook *et al.*, 1989).

Primers

Tetranucleotide repeat primers (16 bp) were employed. Pilot experiments were carried out using 36 combinations of the following oligonucleotides: $(GGGT)_4$, $(CACT)_4$, $(GGAT)_4$, $(AACC)_4$, $(GACA)_4$, $(GGAC)_4$, $(TAGG)_4$ and $(AAGC)_4$. Tetranucleotide primers were used because

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shorter or longer repeats produce inferior PCR products (Gupta *et al*., 1994). Other possible repeats were not used to avoid primer-dimers.

PCR conditions

One nanogram of DNA was amplified in a final volume of 30 µl containing 10 mM Tris-HCl, pH 8.4, 0.5% nonidet P-40, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM each of dNTP, 5 pmol primers, and 1.25 units of *Taq* DNA polymerase (Life Technologies). Amplifications were performed in a Perkin Elmer TC1 thermocycler for 35 cycles. The cycles consisted of 45 s at 94°C, 60 s at 53°C and 60 s at 72°C. All products were analyzed on 1.4% agarose gels stained with ethidium bromide. The molecular weight markers used were λ digests with *Hin*dIII and *Eco*R, pBR328 digests with *Bam*HI, *Bgl*I and *Hin*fI, and a 123-bp DNA ladder (Gibco BRL).

This work was conducted in three major steps. 1) We carried out PCR amplifications using different combinations of SSR primers in species previously identified by morphological criteria in a sample of 16 specimens. 2) After the characterization of a species-specific molecular marker we analyzed the geographic distribution of the 198 specimens sampled. 3) We analyzed the geographic distribution of the *micro*11 polymorphic patterns detected in *G. sylvius*.

RESULTS AND DISCUSSION

Thirty-six combinations of primers were tested in some specimens previously identified by morphological analysis (Britsk, H.A., personal communication; Fernandes-Matioli *et al*., 1998). The primer combinations $(TAGG)_4$ + $(GGAT)_4$ and $(GACA)_4$ + $(AAGC)_4$, and by SPAR, $(AAGC)_4$ and $(CACT)_4$, albeit presenting positive amplification, produced very faint bands and were not used. $(GACA)_4$ gave monomorphic interspecific patterns.

The most informative results were those obtained by SPAR using the primer $(GGAC)_4$ and this molecular marker was named *micro*11; species-specific amplification patterns were only observed with this marker (Figures 2). Amplification was obtained with *micro*11 in two other fish genera, *Eigenmannia* and *Pseudostegophilus* (Figure 2). The positive amplification showed that this marker may be useful for other fish groups.

The results obtained by the amplification of regions flanked by $(GGAC)_n$ microsatellite loci in the four species studied revealed great intraspecific conservation of the amplification patterns. The intraspecific conservation of this molecular marker in *Gymnotus* species may be used for species characterization and was recently included as a tool in the description of the species *G. sylvius* (Albert *et al*., 1999).

In the second step of our work we tested specimens from different populations. All samples were successfully

Table I - Samples of *Gymnotus* species analyzed.

Basin (number in Figure 1)	Species	Sample size
Paranapanema River (1)	*G. carapo*	34
	G. carapo	28
Tietê River (2)	*G. sylvius*	1
	G. inaequilabiatus	4
Ribeira de Iguape River (3)	*G. sylvius*	18
Pantanal do Miranda (4)	*G. carapo*	19
	G. sylvius	4
Tietê River (headwaters) (5)	*G. pantherinus*	20
Coastal rivers (6)	*G. pantherinus*	22
Paraíba do Sul River (7)	*G. carapo*	1
	G. sylvius	26
Mogi Guaçu River (8)	*G. carapo*	12
	G. sylvius	9

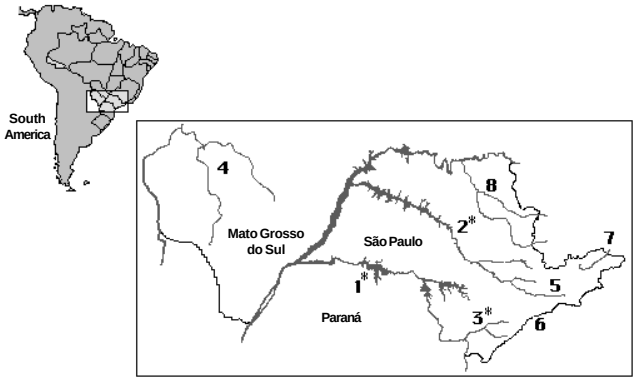

Figure 1 - Geographic localities of *Gymnotus* collection sites. 1, Paranapanema River (sample size: 34); 2, Tietê River (33); 3, Ribeira de Iguape River (18); 4, Pantanal do Miranda (23); 5, Tietê River (headwater) (20); 6, Coastal rivers (22); 7, Paraíba do Sul River (27); 8, Mogi Guaçu River (21). *, Sample previously studied by morphological analysis.

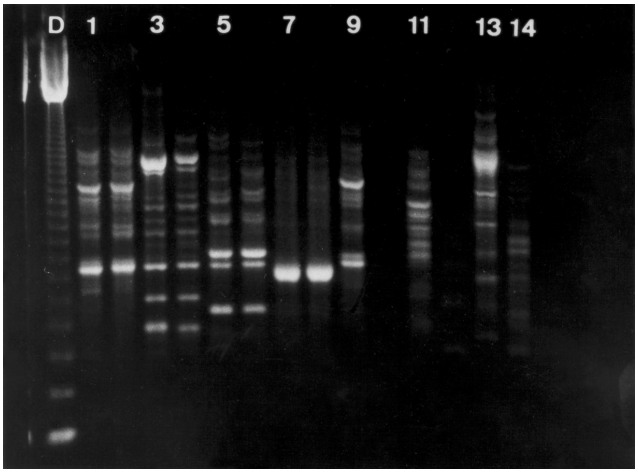

Figure 2 - Amplification products obtained with *micro*11 in two individuals of each *Gymnotus* species (lanes 1-8). D, 123-pb DNA ladder. Lanes 1,2, *Gymnotus carapo*; 3,4, *G. inaequilabiatus*; 5,6, *G. pantherinus*; 7,8, *G. sylvius*; 9 and 11, positive controls; 10, no amplification; 12, negative control; 13, *Eigenmannia*; 14, *Pseudostegophilus*.

analyzed. The use of the *micro*11 patterns in combination with morphological analysis allowed the identification of the geographic distribution of *Gymnotus* species in the southeastern Brazilian basins (Figure 3) and analysis using *micro*11 made it possible to quickly and easily identify a large number of specimens. All analyses were performed at least twice, with identical results. The species identification first obtained with *micro*11 in 64 specimens (20 *G. carapo*, 20 *G. pantherinus*, 20 *G. sylvius* and 4 *G. inaequilabiatus*) was subsequently confirmed by morphological analysis (Britsky, H., personal communication).

The total congruence between the morphological and molecular analyses is very useful because it would no longer be necessary to sacrifice numerous individuals or modify wild populations for analysis, permitting studies that do not interfere with biological conservation.

G. carapo was the most widely distributed of the species analyzed, being found in most of the small tributaries in southeastern Brazilian basins (Figure 3). *G. pantherinus* was only detected in the coastal river basins of the State of São Paulo (sample 6, Figure 1), and in the headwaters of the Tietê River (sample 5, Figure 1). *G. inaequilabiatus* was only found in tributaries of the Tietê River (sample 2, Figure 3). The populations of each of these three species presented a geographic distribution that was continuous in all the sampled basins. Populations of *G. sylvius*, on the other hand, were patchily assigned to five basins (samples 2, 3, 4, 7 and 8, Figure 3).

In some basins, *Gymnotus* populations of different species were found in sympatry, but in all cases one of the species was present at a significantly lower frequency. This was the case of the Paraíba do Sul River basin (sample 7, Figure 1), where 26 *G. sylvius* and one *G. carapo* were found sympatrically in a sample of 27 individuals (Figure 3). Sympatry involving *G. carapo* (N = 28) and *G. inaequilabiatus* (N = 4) was also observed in 32 specimens from the Tietê River basin (sample 2, Figure 3). However, there was no evidence of hybridization or introgression between the *Gymnotus* species found in sympatry. Furthermore, the species found in sympatry had very different chromosome numbers (Fernandes-Matioli *et al.*, 1998), a factor that usually precludes introgression or hybridization on a large scale.

Sympatry was also observed in some parts of the Mogi Guaçu River, Tietê River, and Pantanal do Miranda basins (samples 8, 2, 4, respectively, Figure 3). It is known that in these areas *Gymnotus* specimens are often sold as bait for fishing, so this species mixture might be attributable to human interference.

In the third step of the work, we analyzed the intraspecific *micro*11 pattern variation in 58 individuals of *G. sylvius* from different populations (see Table I). Three distinct patterns were found (A, B, C) (Figures 4b and 5). In the Paraíba do Sul River basin only the A pattern was found. The A and C patterns were found in the Ribeira do Iguape River basin, and all three patterns were found in the Mogi Guaçu River basin.

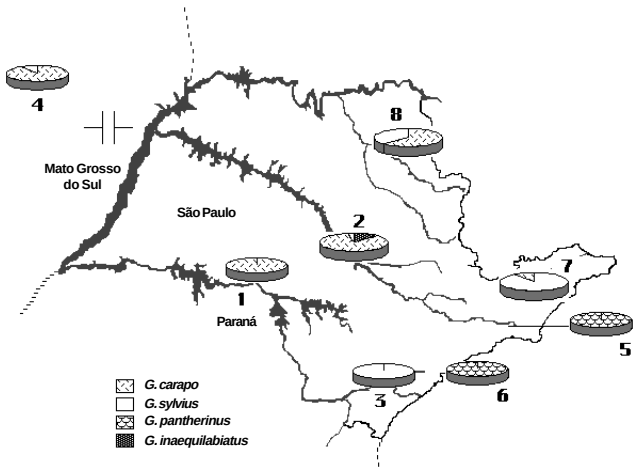

Figure 3 - Geographic distribution of four *Gymnotus* species. 1, Paranapanema River; 2, Tietê River; 3, Ribeira de Iguape River; 4, Pantanal do Miranda; 5, Tietê River (headwater); 6, Coastal rivers; 7, Paraíba do Sul River; 8, Mogi Guaçu River. The pie graphs indicate the ratio among the species observed at each collection site.

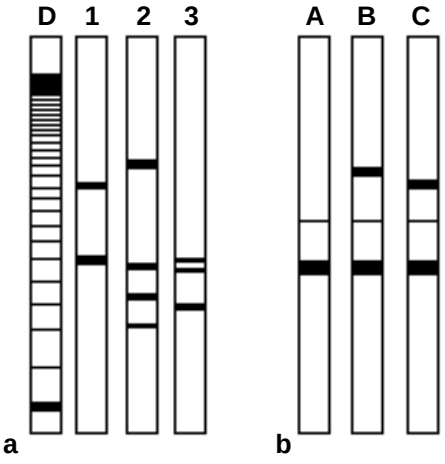

Figure 4 - Schematic drawing of *micro*11 patterns. a) Species-specific patterns: D, 123-bp DNA ladder; 1, *Gymnotus carapo*; 2, *G. inaequilabiatus*; 3, *G. pantherinus*. b) The three *micro*11 patterns obtained for *G. sylvius*.

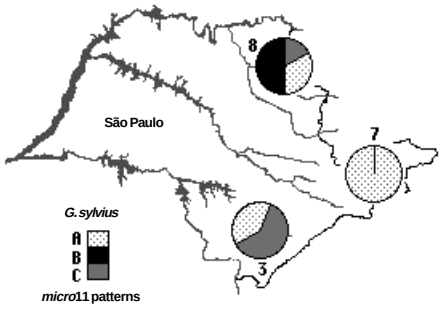

Figure 5 - Geographic distribution of *micro*11 patterns (see Figure 4) of three *Gymnotus sylvius* populations.

Since the data did not allow a classical analytical approach based on allele frequencies, a comparative study of intraspecific polymorphisms based on the *micro*11 marker was adopted. The variant patterns may have been due to spurious PCR products, making the variation entirely artefactual; however, taking into account the highly stringent conditions under which the experiments were performed, the *micro*11 intraspecific pattern variation could indeed be reflecting polymorphism within species. If so, the variation might be generated by inverted sequence blocks or might reside within some larger repeat structure, such as satellite sequences or ribosomal internal transcriber spacer regions. Alternatively, this variation might be associated with some kind of dispersed repeats, such as SINEs (short interspersed repeated sequences).

After testing different PCR conditions (e.g., annealing temperatures (50°, 51°, 52° and 53°C) and Mg^{2+} concentrations (2.0, 2.5, and 3.0 mM)), we concluded that *G. sylvius* has at least three polymorphic *micro*11 patterns (Figure 4b).

The presence of the A (N = 7) and C (N = 11) patterns in the Ribeira de Iguape River basin (sample 3; Figure 5) and only pattern A in the Paraíba do Sul River basin (sample 7; Figure 5) may indicate an ancestral polymorphism (Figure 5). The occurrence of a few *G. sylvius* specimens in the Tietê River basin (one specimen, sample 2; Figure 3) and in the Pantanal do Miranda basin (two individuals, sample 4; Figure 3) presenting the C and A patterns, respectively, was considered as evidence of species introduction because these patterns seem to be characteristic of other basins (see Ribeira de Iguape basin, sample 3, and Paraíba do Sul basin, sample 7; Figure 5). All the three *micro*11 patterns observed in *G. sylvius* were found in the Mogi Guaçu River basin (sample 8; Figure 5) (A, N = 7; B, N = 11, and C, N = 3, in a sample of 21 fish). The occurrence of pattern B only in the Mogi Guaçu River basin may indicate, in spite of the commercial profile of this site, that natural populations of *G. sylvius* might actually occur in this region since this was the only place where this pattern was detected. On the other hand, the presence of patterns A and C in the Mogi Guaçu River basin could be evidence of specimen introduction since they are characteristic of very distant populations.

Other studies involving *G. sylvius* (including the analysis of individuals that presented A, B, and C *micro*11 patterns) showed homogeneity in chromosome number (2n = 40, Fernandes-Matioli *et al*., 1998), and morphological and bioelectrical traits (Albert *et al*., 1999). According to these studies, the hypothesis of sibling species living in sympatry could be discarded.

The presence of only one *micro*11 pattern in the *G. sylvius* population from the Paraíba do Sul basin (sample 7; Figure 5) may be indicative of the loss of genetic variability due to genetic drift. This way, this population had probably originated from colonization events associated with the founder effect. In fact, this is very plausible because of the generally small size of the populations that migrate from one basin to another. This has already been observed in cichlid fish (Agnèse *et al*., 1997).

The present work outlines the first steps of an appropriate application of a novel molecular technique to improve the understanding of species diversity and geographic distribution of *Gymnotus* species, an approach which may be very useful in conservation programs involving communities of neotropical freshwater fish.

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RESUMO

No presente estudo foram analisados os padrões de amplificação de fragmentos de DNA nuclear flanqueados por microssatélites $(GGAC)_n$ obtidos a partir de 198 exemplares do gênero *Gymnotus* (Pisces: Gymnotiformes) amostrados em 8 bacias hidrográficas do sudeste brasileiro. As espécies analisadas foram *Gymnotus carapo*, *G. pantherinus*, *G. inaequilabiatus* e *G. sylvius*. Os padrões de amplificação foram obtidos através da técnica de SPAR (*single primer amplification reaction*) e refletem, indiretamente, a distribuição de seqüências repetitivas simples no genoma nuclear dos espécimens. Foram encontrados padrões de amplificação espécie-específicos, os quais foram utilizados como potentes ferramentas na análise da distribuição geográfica e diversidade de espécies de *Gymnotus*. Padrões monomórficos foram observados em *G. carapo*, *G. pantherinus* e *G. inaequilabiatus*. Três padrões polimórficos foram verificados em *G. sylvius*. Os resultados obtidos através da técnica SPAR indicam que esta é uma abordagem promissora como ferramenta molecular em programas de conservação de comunidades de peixes de água doce neotropicais.

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