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Research Article

RAPD markers indicate the occurrence of structured populations in a migratory freshwater fish species

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Abstract

Many factors have contributed to the destruction of fish habitats. Hydroelectric dams, water pollution and other environmental changes have resulted in the eradication of natural stocks. The aim of this study was to detect the genetic variation in *Prochilodus marggravii* from three collection sites in the area of influence of the Três Marias dam (MG) on the São Francisco river (Brazil), using the RAPD technique. The results obtained revealed that the fish in the downstream region nearest the dam have a higher similarity coefficient than those from the other sampling sites that may be related to differences in environmental characteristics in these regions. Additionaly, significant differences in the band frequencies were observed from one collection site to another. These both findings suggest the occurrence of a structured population and have important implications for the conservation of the genetic variability of distinct natural *P. marggravii* stocks.

Key words: Prochilodus, RAPD, genetic structure, conservation genetic, fish.

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Introduction

Prochilodontidae fish are widely distributed throughout South America and have been reported in almost all South American hydrographic basins (Mago-Leccia, 1972).

Thirteen species of the *Prochilodus* genus are recognized (Castro, 1990) and three species, *P. marggravii*, *P. affinis*, *and P. vimboides*, live in the São Francisco basin (Britski, 1988), which comprises an area equivalent to 7.4% of Brazil's territory (Paiva, 1982; 1983). The two former species are migratory and are considered very important species for inland fishing in this river basin (Paiva, 1983). However, a dam built in 1960 on the main channel of the São Francisco river in the municipality of Três Marias (MG) prevents migratory fish from lower areas reaching most of the river's upper tributaries. There is no information on the effect of the construction of a new dam on the populational genetic structure of Neotropical migratory fish fauna. It is known that migratory fish require favorable conditions, determined by endogenous and exogenous factors, which guide migration and spawning. Free fish migration across a dam is often imparied and genetic variation and allelic frequency of migrating populations can be changed. Moreover, the environmental conditions prevailing in this region's hydrographic system have undergone profound changes both upstream and downstream from the dam (Sato and Osório, 1986). Drastic environmental conditions, caused mainly by reduced water flow, lower water temperature and oxygen rates just downstream from the dam, may be influencing some of the biological features of the living organisms in this area (Sato *et al*., 1995). Histological studies of gonads in *P. marggravii* have revealed reproductive disturbances in the specimens caught in the downstream area nearest the dam when compared to individuals from more distant regions (Sato *et al*., 1995).

Little is known about the population genetics of *Prochilodus*. Previous enzyme electrophoresis, used to quantify the genetic variations of *P. lineatus* collected from three sites in the Paraná river basin, detected no genetic divergences among the three samplings, suggesting a unique genetic pool of *P. lineatus* spread along the studied area (Revaldaves *et al*., 1997).

Another useful methodology to assess genetic variations in fish populations is the RAPD (Random Amplified Polymorphic DNA) technique, which is used in fishery management and conservation genetics of wild populations. Based on the amplification of genomic DNA by PCR (Polymerase Chain Reaction) with arbitrary nucleotide sequence primers, RAPD can detect high levels of DNA polymorphisms and can produce fine genetic markers

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(Williams *et al*., 1990; Welsh and McClelland, 1990). Nevertheless, RAPD analysis has some limitations that must be considered. It shows dominant inheritance and marker/marker homozygotes cannot be distinguished from marker/null heterozygotes. In addition, it is unable to assign bands to specific loci unless a previous pedigree analysis is performed. In applying this method, it is assumed that populations are under the Hardy-Weinberg equilibrium, that polymorphic bands segregate in the Mendelian way, and that marker alleles from different loci do not co-migrate to the same position in the gel (D'Amato and Corach, 1996).

The potential use of RAPD in genetic mapping and population genetics has been widely documented for a large variety of organisms, including fish (Postlethwait *et al*., 1994; Foo *et al*., 1995; Bielawski and Pumo, 1997; Caccone *et al*., 1997; Dergam *et al*., 1998; Nadig *et al*., 1998; Liu *et al*., 1999).

In order to assess the genetic variation of *P. marggravii* in the area of influence of the Três Marias dam on the São Francisco river, RAPD reactions were performed on samples from three collection sites. The results promise to be useful for the fishery management, aquaculture and stock conservation of this species.

Material and Methods

Sampling sites

Adult individuals of *Prochilodus marggravii* were caught in November 1996, January 1997 and March 1997 at three sampling sites along the São Francisco river (Três Marias region). The first site (region A) comprises the proximate area below the dam (with inhospitable conditions caused by the dam's construction). The second location lies between an area approximately 10 km below the dam (Cascalheira) and the confluence of the Abaeté and São Francisco rivers (region B). The third area (region C) consists of a stretch of about 20 km below this confluence in the São Francisco river (Figure 1). Fifty-six individuals of *P. marggravii* were analyzed: 19 from region A, 20 from region B and 17 from region C.

DNA extraction, PCR and electrophoresis

Genomic DNA from liver tissue was sampled. Standard proteinase-K digestion was followed by phenol:chloroform extraction (Sambrook *et al*., 1989). A set of 10 decamer oligonucleotides (Operon Technologies) was used in this study as single primers in the Polymerase Chain Reaction (PCR) (Table 1). The amplification conditions, slightly modified, were based on Williams *et al*. (1990), and several tests were carried out to verify the best amplification conditions (DNA and $MgCl_2$ concentrations). PCR was performed in 50 mM KCl, 10 mM Tris pH 8.3, 2 mM $MgCl_2$, 100 µM each of dNTPs (Amersham Pharmacia Biotech), 5 pmol of 10-base primer, 0.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech), and 25 ng of genomic DNA in a final volume of 25 µL. Amplifications were made in an MJ Research thermal cycler, model PT100, programmed for an initial denaturation step, 4 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. The program was concluded with an additional 3 min at 72 °C step before cooling to 4 °C. One negative control (absence of template DNA) was included for each set of amplifications. The reaction products

Figure 1 - Map of the Três Marias region, showing the sampling sites. A: downstream from the dam, B: region between Cascalheira and the Abaeté river, and C: downstream from the confluence of the Abaeté and São Francisco rivers.

Table 1 - Primer sequences and amplification product characteristics. Y = Yes, N = No.

Primer	Sequence (5' → 3')	Polymorphic
OPP-4	GTGTCTCAGG	N
OPP-6	GTGGGCTGAC	Y
OPP-7	GTCCATGCCA	Y
OPP-8	ACATCGCCCA	Y
OPP-9	GTGGTCCGCA	Y
OPP-10	TCCCGCCTCA	Y
OPP-11	AACGCGTCGG	Y
OPP-12	AAGGGCGAGT	Y
OPP-15	GGAAGCCAAC	Y
OPP-18	GGCTTGGCCT	N

were separated by electrophoresis on a 1.4% agarose gel stained with ethidium bromide and 8% polyacrylamide gel stained with 0.17% silver nitrate. In each case, electrophoretic runs were standardized for a comparative analysis of the band patterns obtained.

Data analysis

Individual RAPD patterns were compared within and between sampling sites. Only reproducible well-marked amplified fragments were scored. For each genotype, the presence and absence of fragments were recorded as 1 or 0, respectively. A pairwised comparison of banding patterns was evaluated by Jaccard's similarity coefficient (Jaccard, 1901), using the NTSYS-PC analysis software (Rohlf, 1993). Statistical analyses were performed in order to test the significance of similarity coefficients for each primer among the sampling sites, using the nonparametric Kruskal-Wallis test of the Biostat 2.0 software program (Ayres *et al*., 2000), which compares three or more size independent samples. Dunn's post-test of the Biostat 2.0 software program (Ayres *et al*., 2000) was also used to identify which sites show significant differences from each other. Fisher's Exact Test was performed using the Biostat 2.0 program (Ayres *et al*., 2000) to ascertain the existence of significant differences in band frequencies among the collections sites.

Results

Ten primers were tested prior to the analysis, with the amplification products ranging from 300 to 3000 bp. A low variable pattern was observed among *P. marggravii* individuals when the OPP-4 and OPP-18 primers were used, while the remaining primers produced polymorphic patterns (Table 1). No characteristic and/or diagnostic bands were found for any sampling site. All the primers examined produced reproducible RAPD fragment patterns. Figure 2 shows an amplification pattern obtained with the OPP-12 primer. Four of the 10 tested primers were chosen (OPP-7, OPP-10, OPP-12, OPP-15), based on profile sharpness and on adequate variation levels for statistical tests. A total of 31 loci were analyzed and the results of Fisher's Exact Test revealed five loci with significantly different frequencies among the sampling sites (Table 2).

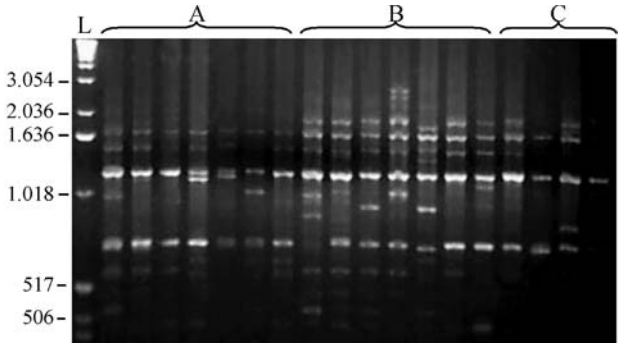

Figure 2 - RAPD patterns from *Prochilodus marggravii* using primer OPP-12 from three sampling sites in the São Francisco river, section A: individuals collected from region A, B: individuals from region B, and C: individuals from region C. Molecular weight marker ladder 1 kb is denoted in lane L.

The similarity coefficients of Jaccard (1901) within and between sampling sites are given in Table 3, which shows that the lowest value of the similarity coefficients within samples was obtained for individuals from region C (0.442), whereas regions A and B presented Jaccard's coefficient values of 0.636 and 0.604, respectively. The Kruskal-Wallis test revealed that the mean similarity coefficients of Jaccard for each primer among the sampling sites were statistically significant ($KW = 7.538$; $p = 0.011$). Pairwise comparisons (Dunn's Multiple Comparisons test) demonstrated that the differences observed between regions A and B and between B and C were not significant ($p > 0.05$), but a statistically significant difference was found between regions A and C ($p < 0.05$), indicating a considerable genetic discrepancy between these regions. Jaccard's coefficient used between sampling sites showed a higher value between A and B (Table 3).

Discussion

RAPD can be an efficient tool to differentiate geographically and genetically isolated populations, and has been used to verify the existence of locally adapted populations within a species that may have arisen either through genetic selection under different environmental conditions or as a result of genetic drift (Fuchs *et al*., 1998).

Since homozygotes cannot be distinguished from heterozygotes by the RAPD technique, the absence of amplification of a band in two genotypes does not necessarily represent genetic similarity between them. Jaccard's similarity coefficient, which excludes negative co-occurrences, has been widely employed for statistical analysis of RAPD markers (Duarte *et al*., 1999). In the present study, our statistical analyses showed considerable genetic variation among individuals of *P. marggravii* from collection sites A and C.

Populational genetic differentiation can be driven by ecological, evolutionary and historical factors. In *Barbus neumayeri*, genetic differentiation among sampling sites that presented different oxygen rates could represent the effects of selective pressure (Chapman *et al*., 1999). The well-developed homing instinct of salmonid fish seems to be a decisive factor leading to strong population subdivisions (Ryman, 1983). An evolutionary unit can be identified for each tributary, with particular genetic traits possibly related to local adaptation and/or to inbreeding. In *Oncorhynchus nerka*, genetic differences were found between two populations inhabiting regions with distinct en-

Table 2 - Characterization of amplification products, band size in base pairs (bp) and their relative frequencies in the A (fA), B (fB) and C (fC) sampling sites; and *P*-values of Fisher's Exact Test found for sites A-B; B-C and A-C.

Primer	PCR products	bp	band frequencies			p-value		
			fA	fB	fC	A-B	B-C	A-C
OPP-7	band 1	1050	0.8235	0.7500	0.4615	0.6880	0.1426	0.0056*
	band 2	1020	0.0588	0.1875	0.2308	0.3353	1.0000	0.2903
	band 3	950	0.8823	0.8750	0.8750	1.0000	0.3640	0.3598
	band 4	900	0.1765	0.2500	0.2308	0.6880	1.0000	1.0000
	band 5	720	0.1176	0	0	0.4848	-	0.4920
	band 6	700	0.7059	0.9375	0.7692	0.1748	0.2994	1.0000
OPP-10	band 7	1900	0.9000	0.9412	0.8333	1.0000	0.5534	0.6196
	band 8	1800	0.0500	0.0582	0	1.0000	1.0000	1.0000
	band 9	1600	0.0500	0.0582	0	1.0000	1.0000	1.0000
	band 10	1400	0.0500	0	0.2500	1.0000	0.0602	0.1361
	band 11	1100	0.1000	0.1176	0.0833	1.0000	1.0000	1.0000
	band 12	1000	0.9000	0.9412	0.6667	1.0000	0.1296	0.1651
	band 13	800	0.2000	0.2353	0.4167	1.0000	0.4223	0.2400
OPP-12	band 14	1650	0.9000	1.0000	0.6923	0.4879	0.0227*	0.1824
	band 15	1400	0.9000	0.9444	0.5385	1.0000	0.0124*	0.0351*
	band 16	1200	1.0000	1.0000	0.9231	-	0.4194	0.3939
	band 17	1180	0.0500	0.1667	0.0769	0.3282	0.6207	1.0000
	band 18	1150	0.1000	0	0	0.4879	-	0.5076
	band 19	1100	0.1000	0.0556	0	1.0000	1.0000	0.5076
	band 20	1016	0.0500	0.0556	0.1538	1.0000	0.5575	0.5473
	band 21	800	0.1500	0.1111	0	1.0000	0.6207	0.6253
	band 22	750	0.2000	0.1667	0.0769	1.0000	1.0000	1.0000
	band 23	720	0.1000	0.1111	0.1538	0.4737	0.5575	0.1477
	band 24	700	0	0.0556	0.1538	0.0035*	1.0000	0.0002*
	band 25	680	0.6000	1.0000	0.6154	0.0934	1.0000	0.2018
	band 26	600	0.3000	0.0556	0.0769	1.0000	0.3662	0.3662
OPP-15	band 27	1100	0.2105	0.2222	0.0714	1.0000	0.6285	1.0000
	band 28	950	0.8947	0.8421	0.7857	1.0000	0.4587	0.7187
	band 29	900	0.6842	0.7368	0.5714	1.0000	0.4587	0.7157
	band 30	850	0.8421	0.9474	0.7857	0.6039	1.0000	0.2882
	band 31	800	0.3158	0.2632	0.7143	0.7311	0.0135*	0.0036*

*****indicates significant difference; – indicates test could not be performed.

Table 3 - Jaccard's similarity coefficients within and between sampling sites (A, B and C) for each primer (OPP-7, OPP-10, OPP-12, OPP-15); SD = Standard Deviation.

	Within sampling sites			Between sampling sites		
Primer	A	B	C	A-B	A-C	B-C
OPP-7	0.564	0.610	0.459	0.565	0.504	0.518
OPP-10	0.663	0.662	0.466	0.658	0.554	0.543
OPP-12	0.739	0.548	0.418	0.631	0.472	0.539
OPP-15	0.579	0.597	0.424	0.599	0.540	0.539
Mean ± SD	0.636 ± 0.081	0.604 ± 0.047	0.442 ± 0.024	0.613 ± 0.040	0.518 ± 0.037	0.535 ± 0.011

vironmental conditions (Hendry *et al*., 2000). Furthermore, some river or lake systems contain metapopulations composed of distinct breeding units (Carvalho, 1993; Hansen and Loeschcke, 1994).

Although further studies are required to confirm the repeatability of the patterns observed, at least three hypotheses can be put forward to explain the possible genetic structure observed in the *P. marggravii* in the Três Marias region.

First, the fish downstream from the confluence of the Abaeté and São Francisco rivers (region C) comprise a panmictic population (panmictic population model). During the reproduction period, one fraction migrates towards the dam; the higher similarity coefficient observed in individuals downstream from the dam (region A) is the result of genetic drift (Figure 3a).

Second, the findings reported on here possibly reflect the homing behavior of *P. marggravii* individuals, similarly to wild trout populations (Ryman, 1983). In this case, animals from region C comprise a metapopulation with distinct reproductive populations, which historically complete their migration to different parts of this hydrographic system (Figure 3b). Genetic differences are determined by different evolutionary pathways of breeding populations in each tributary, while the remaining genetic similarities represent the putative genetic pool of a common ancestor stock.

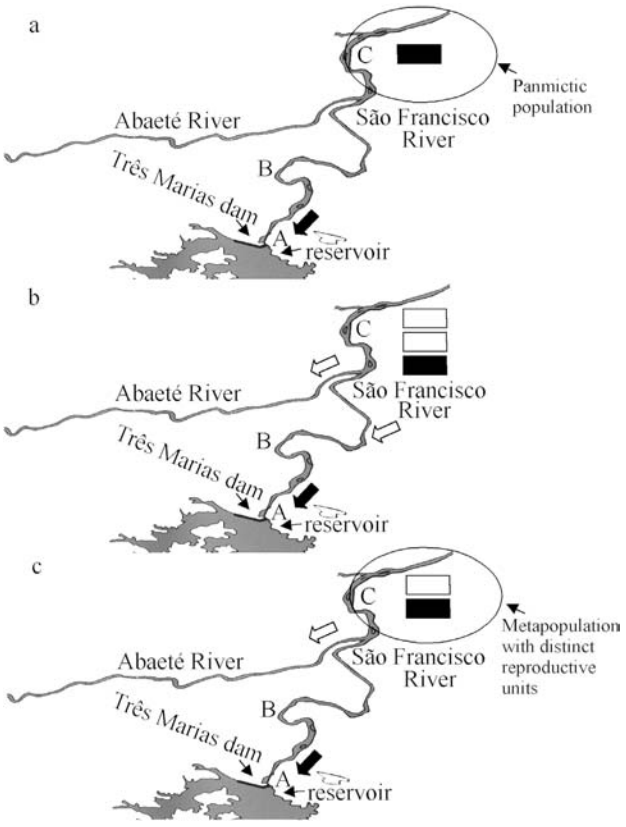

Figure 3 - Genetic structure models for *Prochilodus marggravii* from the Três Marias region (São Francisco river). a: Panmictic model; b: Homing model; c: Structured population model. A: region A, B: region B, and C: region C. White and black squares indicate distinct reprodutive units; arrows indicate the migration routes.

In a third hypothesis only a minor fraction of this metapopulation completes its migration towards the dam (region A) during the reproductive period, while the majority possibly migrate to locations with environmental conditions more favorable for reproduction (structured population model). Individuals from different populations present co-migrational behavior during the reproductive period (Figure 3c).

Competition for greater resources is very common in nature. Considering that the area downstream from the dam lacks the optimal conditions for reproduction, the most vigorous individuals are expected to take over the best habitats, establishing territories and leaving the less favorable habitats for the weaker individuals during fish migration (Krebs and Davies, 1996). Previous studies have demonstrated that specimens collected from region C, which offers more favorable reproductive conditions, are larger than the fish in regions A and B. At its confluence with the São Francisco, the Abaeté river supplies the former with higher water temperature, flow and oxygen rates than the conditions downstream from the dam (Sato *et al*., 1995). It is well known that hydroelectric dams create new obstacles for the natural dispersion of migrant fish, directly affecting their survival and reproduction by changing thermal and hydrodynamic conditions (Agostinho *et al*., 1992).

Findings similar to those observed here in *P. marggravii* have also been reported for other migratory fish species studied in the same area. In *Brycon lundii*, the presence of a characteristic band in 100% of specimens from regions A and B, albeit in only 27.3% of individuals from region C, also suggested the occurrence of structured populations (Wasko and Galetti, 2002), reinforcing the argument that the model of structured populations is probably the one best suited to illustrate the distribution of *P. marggravii* in the Três Marias region on the São Francisco river.

These ideas highlight an important aspect of the genetic conservation of this species. The practice of releasing cultured fish into the wild is widespread, but without a careful genetic analysis, it may damage the goals of preservation, leading to the homogenization of populations and decreasing species diversity (Taggart and Ferguson, 1986; Fritzner *et al*., 2001). Furthermore, it is important to recognize that different scenarios require different measures. Hence, the aim of conservation programs should be to develop an integrated strategy that conserves as much genetic diversity within the species as possible, and ensures the presence of utilizable fish resources (Hansen and Loeschcke, 1994).

The findings reported in this study nevertheless reveal important implications for the fishery management, aquaculture, and stock conservation of *P. marggravii* in the Três Marias region of the São Francisco river basin. The scenario, however, is not complete and further samplings from additional sites, mainly along large tributaries such as the Abaeté river, taken during different seasons of the year, should be analyzed.

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