Original Article

GENETIC DIVERSITY OF *Piaractus mesopotamicus* (Characiformes: Characidae) BROODSTOCKS USING IN THE RESTOCKING PROGRAM OF TIETÊ RIVER, BRAZIL

DIVERSIDADE GENÉTICA DE ESTOQUES DE Piaractus mesopotamicus *(*Characiformes, Characidae*) UTILIZADOS NO PROGRAMA DE REPOVOAMENTO DO RIO TIETÊ, BRASIL*

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ABSTRACT: The restocking programs are being used more frequently as methods of fish conservation. However, the reduction in the genetic diversity can affect survival of juveniles used in this programs and cause effects on the wild populations. The aim of this study was assess the genetic diversity in three broodstocks (BA, BB and BC) of pacu *Piaractus mesopotamicus* of a Hydropower plant in São Paulo - Brazil, using in restocking program of Tietê River. Nine RAPD primers were amplified used extracted DNA from 89 fin-clipping samples. Sixty-nine fragments were polymorphic, 15 had frequencies with significant differences ($P<0.05$), seven were excluded, and six were fixed fragments. High values for polymorphic fragments (47.83% to 71.01%) and Shannon index (0.270 to 0.424) were observed. Most of the genetic variation was found within the groups through the AMOVA analysis, which was confirmed by the results of the identity and genetic distance. Ancestry levels (F_{ST}) among the groups values indicated little and moderate genetic differentiation. The estimate of number of migrants by generation (Nm) indicated levels of gene flow. Moderate genetic divergence between groups (0.214 to 0.259) was observed. The results indicate high (BA and BB) and moderate (BC) variability within broodstocks and genetic differentiation among them. The fish stocks analyzed represent a genetic base that will allow the fish technicians to release juveniles without genetic risks to wild populations present in the river. These genetics procedures can be used as models for other migratory species.

KEYWORDS: Fish. Genetic conservation. Pacu. RAPD.

INTRODUCTION

The Tietê, an important Brazilian river of the State of São Paulo, runs through the metropolitan area of São Paulo City (1,136 km), up to the Paraná River, at the border with Mato Grosso do Sul. Along the way, are installed several dams.

The massive acceleration in plans for hydropower development in the Brazil has led to growing concern over the potential environmental and over the impact on the basin's fisheries. The dams favor local and regional economic development, but they also bring serious and irreversible alterations in the natural hydrologic regime of rivers and the ichthyofauna (AGOSTINHO et al., 2008). According to Agostinho et al. (2003), these dams have broken the migratory path, interfered with the life cycle of numerous aquatic organisms, and produced major changes in ecosystems. The loss of connectivity and habitat availability as well changes in the discharge regime of the river cause shifts in migratory fish populations located up and downstream of a dam (GUBIANI et al., 2007).

Recently, there has been in Tietê River a decline in the population of various indigenous migratory fishes like the pacu (*Piaractus mesopotamicus*). *P. mesopotamicus* is classified in the order Characiformes, family Characidae, sub-family Serrasalminae (Holmberg, 1887). Is endemic in the Neotropical region and widely distributed along the Paraná, Paraguay and Uruguay basins (URBINATI; GONÇALVES, 2005). While the causes of the decline are unknown, water pollution, habitat loss and overfishing may have contributed. Thus, restocking program for this species is being conducted actuality in the Tietê River.

Although they have been made several decades in Brazil, inquiries about efficiency of restocking programs must be usual because losses in the genetic variability (AGOSTINHO et al., 2005). Many programs have not been successful and a major reason for this is improper broodstock management in the hatcheries in the short or long

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term (BORREL et al., 2007, LOPERA-BARRERO, 2009). When this happens, inbreeding depression occur leading to a decrease in performance for traits such as growth rate, survival, and viral susceptibility (PEREZ-ENRIQUEZ et al., 2009).

In this context, the genetic variation of broodstocks using in this programs must be monitored to increase the preservation of wild fishing resources in restocking programs. With this objective, the RAPD molecular marker (Random Amplified Polymorphic DNA) has been applied to estimate the genetic diversity in broodstocks (LOPERA-BARRERO et al., 2008a; POVH et al., 2009; JACOMETO et al., 2010).

The aim of this study, with RAPD marker, was to evaluate the genetic diversity in three *P. mesopotamicus* broodstocks, using in restocking program of Tietê River.

MATERIAL AND METHODS

The experiment was realized between May 2010 and February 2011. We collected 89 fin-clipping samples from three captive broodstock of *Piaractus mesopotamicus* reared in the AES Hydropower plant (21°29'98''N and 49°78'40"W), São Paulo, Brazil. These stocks, maintained in captivity six years ago, are important because they have been enhancing the fish restocking program in the São Paulo Rivers. The origin of stocks:

- Broodstock A and B: unknown origin. However, it is known that has the first generation (F1) of individuals collected in wild populations.
- Broodstock C: unknown origin. However, it is known that has the first generation of individuals in captivity (F0).

The DNA was isolated from fin-clipping with 0.5 cm^2, and the extraction was based on the methods described by Lopera-Barrero et al. (2008b). The samples were treated with 550 µL lise buffer (50 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, and 1% SDS), 7 µL proteinase K (200 µg/mL) per sample, and incubated overnight at 50ºC. Then, 600 µL 5 M NaCl were added to each sample before being centrifuged for 10 min at 12000 rpm. The aqueous layer was removing carefully to new microtubes where the DNA was precipitated with 700 µL of freezing ethanol and incubated later at -20°C for 2 h. The DNA samples were centrifuged again, washed with 700 µL 70% ethanol, re-suspended in TE buffer (10 mM Tris and 1 mM EDTA), and treated with 6 µL RNAse (30 µg/mL) at 38ºC for 40 min. The DNA was quantified in the Shimadzu spectrophotometer with absorbance at 260 nm. The samples were diluted to the concentration of 10 ng/µL. The DNA quality was checked using agarose gel electrophoresis buffered with TBE 1X (500 mM Tris-HC1, 60 mM boric acid and 83 mM EDTA) at 70 volts for 1 h. Thereafter, the gels were photographed using the L-PIX Image Transiluminator software 1.3 (Loccus Biotechnology – Molecular Image).

The genomic DNA was amplified in the reaction volume of 15 µl using the buffer Tris-KCl 1X (Tris-HCl 20 mM pH 8.4 and KCl 50 mM), 2.5 mM $MgCl_2$, 0.46 mM primer (oligonucleotides), 0.2 mM from every dNTPs, one unit of Platinum Taq DNA Polymerase and 10 ng of DNA. The RAPD reactions were amplified in the "Eppendorf Mastercycler® Gradient" thermocycler, programmed to 40 cycles, initial step of denaturation at 94ºC for 4 min and a final step of extension at 72ºC for 5 min. Every cycle consisted of 1 min at 94ºC, 90 s at 40ºC and 2 min at 72ºC.

We evaluated 60 primers from the Operon Kit (Operon Technologies Inc. in Alameda, California, USA). The different stocks were evaluated by five selected primers with a good standard to the amplification. The product from the amplification was separated in agarose gel at 1.4%. We used 15 µl of the amplified product and 2 µl of the sampling buffer (40% sucrose and 0.25% bromophenol blue) in horizontal electrophoresis. The electrophoresis was carried out at 70 V for 4 h (3 V/cm) using the TBE 1X buffer. Every reaction had a negative control (N) where all the previous components but DNA were added to the solution. A bath with ethidium bromide at 0.5 µg/ml for 30 min was used to reveal the gel. Thereafter, the gels were photographed using the L-PIX Image Transiluminator software 1.3.

Size of the fragments was determined by comparison with a 100 pb DNA Ladder (Invitrogen®, USA). The presence or absence of identical molecular-sized fragments was used to construct a similarity matrix based on the calculation of the Jaccard similarity coefficient, codifying 1 if a fragment was present and 0 if absent.

Based on this matrix, the genetic divergence was estimated by the Mantel test, using the Monte Carlo method, by Mantel-Struct program (MILLER, 1999). The percentage of polymorphic fragments and the Shannon index were obtained from the POPGENE software version 1.31 (YEH et al., 1999). The software TFPGA 1.3 (MILLER, 1997) was used to estimate the identity and genetic distance among the groups. The frequency of fragments was estimate by the exact test.

The software ARLEQUIN 3.0 (EXCOFFIER et al., 2005) was used to determine the genetic differentiation using the estimates of the F_{ST}, the number of migrants per generation (Nm) and the molecular analysis of variance – AMOVA. The molecular variance between the groups was evaluated after combining all of them in the following groups: BAxBB, BAxBC and BBxBC. The significance of these tests was determined by the random permutation method using from 1,000 to 10,000 permutations. The significance of the F_{ST} was tested by the X^2 [$c^2 = 2n\ F_{st}$ (k-1); GL = (k-1) (s-1)] after (WORKMAN; NISWANDER, 1970) in which ***n*** is the number of individuals in two groups, ***k*** is the number of alleles and ***s*** is the number of groups. The magnitude of the genetic differentiation between these groups was based on the Wright definition (WRIGHT, 1978) as having little differentiation when F_{ST} is ranging between 0 and 0.05, moderate when F_{ST} is ranging between 0.051 and 0.15, high when the F_{ST} is ranging between 0.151 and 0.25 and very high when the F_{ST} is above 0.25.

RESULTS AND DISCUSSION

The RAPD analysis was appropriate to evaluate the genetic diversity within and between *P. lineatus* stocks. The main problems towards the dominant performance of this technique (BENTER et al., 1995) were overcome through the amplification tests and standardization of samples carried out in conjunction with the negative controls in the different amplifications. The isolation of high quality DNA is essential for many molecular biology applications using polymerase chain reaction (CHAKRABORTY et al., 2008; LOPERA-BARRERO et al., 2008b). In the present study, an optimum quantity of DNA was found in all the extracts of the fish with various primers.

Sixty-nine amplified fragments were polymorphic (100%). The number of clear and reproducible fragments generated per primer ranged from five (primer OPW01) to 11 (primer OPW02). The biggest fragment (2072bp) was obtained from the primer OPA10 and the smaller (250bp) from the primers OPA10 and OPW02 (Table 1).

Table 1. Nucleotide sequence in the primers, percentage of G+C bases, number of fragments (NF), number of polymorphic fragments (NPF), and size of the amplified fragments from the *P. mesopotamicus* broodstocks.

Primers	Sequence (3’ → 5’)	% (G+C)	NF	NPF	Size (bp)
OPA05	AGG GGT CTT G	60	08	08	300-1300
OPA10	GTG ATC GCA G	60	09	09	250-2072
OPA16	AGC CAG CGA A	60	07	07	300-1100
OPW01	CTC AGT GTC C	60	05	05	280-750
OPW02	ACC CCG CCA A	70	11	11	250-2050
OPW03	GTC CGG AGT G	70	07	07	380-1130
OPW16	CAG CCT ACC A	60	09	09	280-1100
OPX01	CTG GGC ACG A	70	07	07	480-1350
OPX03	TGG CGC AGT G	70	06	06	300-950
Total	-	-	69	69	250-2072

Fifteen in 69 fragments had significant differences in their frequencies (P<0.05). Six fixed fragments (frequency of 1.000) in BA were observed, demonstrated high genetic variability in this broodstock as compared to BB and BC. Exclusive fragments were not found. Seven were excluded fragments - frequency of 0.000 (BB = 3 and BC = 4) (Table 2). The presence of excluded fragments within each stock can be explained by the bottleneck effect or founder effect (JACOMETO et al., 2010). The bottleneck effect is the reduction of an existing population to a small number of individuals. The founder effect is the formation of stocks with a few individuals who normally do not represent the genetics of natural populations. These effects may cause to loss of genetic variability and allele (MOREIRA et al., 2007; JACOMETO et al., 2010). Fragments with low frequency not found in the three broodstocks. Thus, despite the presence of excluded fragments, there is a high genetic variability within BB and BC.

The percentage of polymorphic fragments - %FP (BA = 71.01%; BB = 65.22%; BC = 47.83%) and the Shannon index – SI (BA = 0.424; BB = 0.387; BC = 0.270) were higher (BA and BB) and moderate (BC) values. By comparing the broodstocks was observed higher genetic variability in BA (agreeing with the fixed fragments) and lowest genetic variability in BC. These results showed that BA and BB were formed from a large number of individuals with high genetic variation that allowed the maintenance of variability. This

result is expected due to the source of the stocks (wild populations). Similar results were observed by Povh et al. (2009) in three broodstocks using in restocking program of Paranapanema River (%FP: BA = 75%, BB = 72.30%, BC = 71.40%; SI: BA = 0.434, BB = 0.424, BC = 0.376) demonstrated high genetic variability. High values of %FP (74.15% to 92.90%) and SI (0.381 to 0.522) also were observed by Lopera-Barrero et al. (2009) in four *P. mesopotamicus* broodstocks used in restocking programs.

Table 2. Characterization, size and frequency of fragments with significant values using the exact test on the *P. mesopotamicus* broodstocks.

Primer	Size (bp)	BA	BB	BC	p*
OPA05	550	1.000	0.357	0.423	0.000
	500	0.811	0.357	0.342	0.000
	300	1.000	0.814	0.484	0.002
OPA10	2072	1.000	0.247	0.509	0.001
	1750	1.000	0.553	---	0.001
	1000	0.234	0.452	0.191	0.000
OPW02	1200	1.000	---	0.520	0.000
	900	1.000	0.509	0.520	0.000
OPW03	850	0.811	0.452	0.742	0.004
OPW16	1000	0.811	---	0.517	0.000
	950	0.814	0.342	---	0.000
	830	0.737	0.270	0.537	0.001
OPX01	1350	0.629	---	0.357	0.000
OPX03	500	0.169	0.394	---	0.002
	400	0.190	0.293	---	0.001

BA = broodstock A; BB: broodstock B; BC: broodstock C. *P<0.05.

On the other hand, in BC was observed moderate genetic variability, due inappropriate reproduction management. The gene pool of stocks in aquaculture is sometimes characterized by reduced diversity (FOPP-BAYAT et al., 2010). Small effective number of contributing parents (N_e) (BORREL et al., 2007), inappropriate selection of parents, founder effect, misuse of the reproduction period and the breeding system used in mating (POVH et al., 2010) are some constraints capable of inducing significant losses in the genetic variability. This loss of genetic variability in broodstocks used in restocking programs may cause less survival of juveniles and, consequently, the wild populations will be permanently affected. Lopes et al. (2008) observed a decrease of genetic variability in *P. lineatus* offspring used in restocking programs (broodstock: %FP = 85.71, offspring: %FP = 79.37), possibly due to inadequate reproductive management.

According to the analysis of molecular variance (AMOVA), most of the genetic variation occurs within every group of *P. lineatus* unlike between them. These results were confirmed by the estimates of the identity and the genetic distance (Table 3).

Table 3. Molecular analyses of variance (AMOVA), genetic distance and genetic identity for the different groups of *P. mesopotamicus*.

Groups	Source of variation	Sum of squares	Variance components	% of variation	Genetic distance	Genetic identity
BA x BB	B.G	13.550	0.2369	3.55*	0.021	0.979
	W.G	373.667	6.4425	96.45		
	Total	387.217	6.6794	100		
BB x BC	B.G	27.614	0.7686	13.45*	0.063	0.939
	W.G	282.013	4.9476	86.55		
	Total	309.627	5.7162	100		
BB x BC	B.G	13.668	0.2878	5.26*	0.050	0.950
	W.G	295.298	5.1807	94.74		
	Total	308.966	5.4685	100		

BA = broodstock A; BB: broodstock B; BC: broodstock C. B.G = between group; W.G = within group *P<0.05.

The AMOVA analysis, genetic distance and identity showed similarity between BA, BB and BC, which reflects a common origin or individual exchange between the broodstocks. The similarity between BA and BB is surprising, since it was thought that the two groups have different origin. On the other hand, the similarity between BC and BA / BB demonstrates the existence of a common origin. According to the genetic distance values is more closely related to BB. Founder effect or not intentional exchange between them during reproductive management may have contributed to this similarity. Unfortunately, the management decisions that were made to reproductive season are unknown.

The F_{ST} and genetic divergence showed high ancestry and divergence between groups, suggest a little and moderate genetic differentiation. Corroborating this hypothesis, the Nm was low in all the groups suggesting a low gene flow (Table 4). BA and BB revealed little genetic differentiation confirmed the genetic distance values. Due to management information absence, can not be said to have a common origin or individual exchange between the broodstocks. The F_{ST} (0.052), Nm (5.72) and genetic divergence - GDIV (0.214) values suggest that BB is the parents origin of the juvenile stock, corroborated the genetic distance (0.050). However, it is not possible to dismiss the paternity of BA, which also shows similarity values (F_{ST}: 0.135, Nm: 4.55, GDIV: 0.232, GDIS: 0.063).

Table 4. F_{ST}, X^2 test for the F_{ST}, genetic differentiation according to Wright (1978), number of migrants (Nm) and genetic divergence in the different groups of *P. mesopotamicus*.

Groups	Number of fish	F_{ST}	Wright	X^2	Nm	Genetic divergence
BA x BB	60	0.035*	Little	4.200	13.97	0.259
BA x BC	59	0.135*	Moderate	15.930	4.55	0.232
BB x BC	59	0.052*	Moderate	6.136	5.72	0.214

BA = broodstock A; BB: broodstock B; BC: broodstock C. *P<0.05.

Thus, based of these results, it is suggested that the reproductive and genetic management of four *P. mesopotamicus* stocks analyzed in this study should be performed as genetically similar stocks. This suggestion should be applied especially when stocks are used in restocking or breeding programs. The main farmers worries are arise from environmental aspects of management since immediate improvement can be obtained, ignoring the genetic aspects (BORREL et al., 2007). According Altukhov (2006), the disproportional elimination of some genotypes and the underuse or irregular reproduction of other genotypes create negative consequences resulting in low population survival and reductions in productivity. Good broodstock management practices, such as using large N_e, minimizing variance of family size, using single pair mating and maintaining genetic integrity of stocks, should be practiced by farmers (NA-NAKORN; MOEIKUM, 2009). Given their importance, more information should be required on genetic variation which has important implication in conservation programs, development fish culture technology (MU et al., 2011) and establishment of hatcheries and restocking programs (LOPERA-BARRERO et al., 2008a).

Enhancement of fish populations by restocking is one of the most controversial approaches to fisheries management (FRASER, 2008). These programs can, at least in some cases, benefit fisheries by increasing the catch of desirable fish and the capital value of the fishery in the short term (BEREJIKIAN et al., 2008). However, risks associated with its failure of stimulating the population size (ARAKI; SCHMID, 2010), increase the ecological competition between wild and captive-bred individuals (ELDRIDGE; NAISH, 2007), impact in other species in the ecosystem (ARAKI et al., 2009) and reduce the genetic diversity and population viability of wild stocks (KITADA et al., 2009) are questionable.

Understanding genetic patterns is increasingly important for developing effective fishery conservation strategies, management, and remediation efforts (AN et al., 2011). Thus, the correct election of the individuals to be used in the formation of broodstock (BORREL et al., 2007) and their genetic evaluation can offer important bases for formulating reproductive management strategies (SØNSTEBØ et al., 2007). These strategies will permit safe exchange of broodstocks among fish farms stations to break up cycles of endogamy that are common in controlled environments (MOREIRA et al., 2007).

On the other hand, introduction of nonlocal stocks may result in loss of the genetic integrity of wild populations, for example, disease resistance, hardiness and misregulation of gene expression

(NA-NAKORN; MOEIKUM, 2009; NORMANDEAU et al., 2009). Potential alteration of the genetic composition of the wild population may have significant consequences in terms of reduction to response to changing environments (BOURRET et al., 2011). Thus, for genetic diversity conservation of wild populations of *P. mesopotamicus*, all the fish released during restocking program must represent these populations genetically (LOPERA-BARRERO, 2009).

In summary, the current results indicated high (BA and BB) and moderate (BC) genetic variability within the stocks and little and moderate genetic differentiation among them. The fish stocks analyzed represent a large genetic base that will allow the fish technicians to release juveniles without genetic risks to wild populations. Genetic monitoring using the molecular markers as the RAPD on the wild populations, broodstocks and progenies that have participation in restocking programs is fundamental to avoid declines in the genetic variability, prevent its effects on the wild populations, and allow for insights into the preservation, management and reproduction of the *P. mesopotamicus*.

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RESUMO: Os programas de repovoamento estão sendo usados com mais frequência como métodos de conservação de peixes. No entanto, a redução na diversidade genética pode afetar a sobrevivência dos juvenis usados nesses programas e causar efeitos sobre as populações selvagens. O objetivo deste estudo foi avaliar a diversidade genética de três estoques de reprodutores (BA, BB e BC) de pacu *Piaractus mesopotamicus* de uma hidrelétrica em São Paulo - Brasil, do programa de repovoamento do rio Tietê. Nove iniciadores RAPD amplificaram-se usando DNA extraído de 89 amostras de nadadeira. Sessenta e nove fragmentos foram polimórficos, 15 tiveram frequências com diferenças significativas ($P<0.05$), sete foram excluídos e seis foram fragmentos limitantes. Observaram-se altos valores de fragmentos polimórficos (47,83% a 71,01%) e de índice de Shannon. A maior variação genética observou-se dentro dos grupos através da análise de AMOVA, sendo confirmado com os resultados de identidade e distância genética. Os níveis de F_{ST} entre os grupos indicaram pequena e moderada diferenciação genética. O número de migrantes por geração (Nm) indicou níveis de fluxo gênico. Observou-se moderada divergência genética entre grupos (0,214 a 0,259). Os resultados indicaram alta (BA e BB) e moderada (BC) variabilidade dentro dos estoques e diferenciação genética entre eles. Os estoques de peixes analisados representam uma base genética que permitirá aos produtores liberar juvenis sem riscos genéticos para as populações naturais presentes no rio. Esses procedimentos genéticos podem ser utilizados como modelos para outras espécies migradoras.

PALAVRAS-CHAVE: Conservação genética. Pacu. Peixe. RAPD.

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