

Genetic diversity of pacu and piapara broodstocks in restocking programs in the rivers Paraná and Paranapanema (Brazil)

Diversidade genética de estoques de Pacu e Piapara em programas de repovoamento nos rios Paraná e Paranapanema (Brasil)

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Abstract

The genetic diversity of *Piaractus mesopotamicus* (pacu) and *Leporinus elongatus* (piapara) broodstocks used in restocking programs in the rivers Paraná and Paranapanema is analyzed. One hundred and twenty specimens (two broodstocks of each species) from fish ponds in Palotina PR Brazil and in Salto Grande SP Brazil were assessed. Ten primers produced 96 fragments, comprising 68 (70.83%) and 94 (97.92%) polymorphic fragments for *P. mesopotamicus* and *L. elongatus* broodstocks, respectively. Differences ($p < 0.05$) in the frequency of 15 and 27 fragments were detected for each species, without exclusive fragments. Shannon Index (0.347 - 0.572) and the percentage of polymorphic fragments (57.3% - 94.8%) revealed high intra-population genetic variability for all broodstocks. Results of molecular variance analyses (AMOVA) showed that most variations do not lie between the broodstocks but within each broodstock (89%). Genetic (0.088 and 0.142) and identity (0.916 and 0.868) distance rates demonstrated similarity between the broodstocks of each species, corroborated by F_{st} (0.1023 and 0.1027) and N_m (4.18 and 4.33) rates, with a slight genetic difference due to genic flux. High intra-population genetic variability and similarity between the broodstocks of each species was also detected, proving a common ancestry.

Key words: Broodstocks. Genetic conservation. Genetic variability. *Leporinus elongatus*. *Piaractus mesopotamicus*.

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Resumo

O objetivo da pesquisa foi analisar a diversidade genética de estoques de Pacu (*Piaractus mesopotamicus*) e Piapara (*Leporinus elongatus*) utilizados em programas de repovoamento dos rios Paraná e Paranapanema. Foram analisados 120 exemplares (dois estoques de cada espécie) de pisciculturas das cidades de Palotina (Paraná) e da cidade de Salto Grande (São Paulo). Os 10 iniciadores produziram 96 fragmentos, dos quais 68 (70,83%) e 94 (97,92%) foram polimórficos para os estoques de *P. mesopotamicus* e *L. elongatus*, respectivamente. Foram observadas diferenças ($P < 0,05$) na frequência de 15 e 27 fragmentos para cada espécie, sem a presença de fragmentos exclusivos. Os valores do índice de Shannon (0,347 a 0,572) e da porcentagem de fragmentos polimórficos (57,3% a 94,8%) mostraram uma alta variabilidade genética intra-populacional para todos os estoques. Os resultados das análises de variância molecular (AMOVA) mostraram que a maior parte da variação está dentro de cada estoque (89%) e não entre os estoques. Os valores da distância (0,088 e 0,142) e identidade (0,916 e 0,868) genética demonstraram que existe similaridade entre os estoques de cada espécie, sendo corroborado pelos valores de F_{st} (0,1023 e 0,1027) e N_m (4,18 e 4,33) que mostraram uma moderada diferenciação genética com presença de fluxo gênico. Foi observada alta variabilidade genética intra-populacional e similaridade entre os estoques de cada espécie demonstrando uma origem em comum.

Palavras-chave: Conservação genética. *Leporinus elongatus*. Estoques de reprodutores. *Piaractus mesopotamicus*. Variabilidade genética.

Introduction

Brazil has a great diversity of native fish with relevant characteristics for breeding and with great commercial capacity (MARENGONI et al., 2006; BOSCOLO et al., 2011). Due to their excellent zootechnical traits for fish cultivation and to their high commercial and cultural value for riverine populations of the populations living near the rivers Paraná and Paranapanema, the *Piaractus mesopotamicus* (pacu) and the *Leporinus elongatus* (piapara) may be highlighted among migratory native fish species in Brazil. However, deforestation, deterioration of river waters, climatic changes and hydroelectric plants reduced natural broodstocks in Brazilian rivers (HATANAKA et al., 2006; LOPERA-BARRERO, 2009).

Although restocking programs are increasingly employed in Brazilian rivers as conservation strategies (SIROL; BRITTO, 2006), they must be foregrounded on a scientific base that would determine reproduction, genetic and biological methodologies without a decrease in genetic variability of broodstocks and consequently of

natural populations in the rivers. Genetic monitoring of the populations in restocking programs are highly relevant (LOPERA-BARRERO, 2009; POVH et al., 2008a), with special reference to dominant molecular markers (POVH et al., 2008b; GANAIE; ALI, 2016).

Due to dominant molecular markers, RAPD techniques are efficient to calculate precisely and inexpensively the genetic variability in simple population studies, including restocking programs of native populations. Reviews published during the last five years (ABD EL NABY et al., 2015; ALMEIDA et al., 2013; BEHRMANN et al., 2015; GANAIE; ALI, 2016; GOMES et al., 2013; HASAN; GOSWAMI, 2015; LOPERA-BARRERO et al., 2015; RAMOS et al., 2012; RIBEIRO et al., 2016) have shown that, in spite of the influence of several factors in technique, optimization protocols produced reliable results which have been accepted by the scientific community worldwide.

Current assay investigates the genetic diversity of *P. mesopotamicus* and *L. elongatus* broodstocks employed in restocking programs in the rivers Paraná and Paranapanema, Brazil.

Materials and Methods

One hundred and twenty samples of caudal fins (0.5 cm²) from four broodstocks (30 samples per broodstock) were collected. Two broodstocks of *P. mesopotamicus* (PacPAL) and *L. elongatus* (PiaPAL) were retrieved from a fish pond in Palotina PR Brazil (24° 12' S; 53° 50' W) and used for restocking programs in the river Paraná. The other two broodstocks *P. mesopotamicus* (PacSG) and *L. elongatus* (PiaSG) were retrieved from the Aquaculture and Hydrology Station of Duke Energy International in Salto Grande SP Brazil (22° 54' S; 50° 00' W) used for restocking programs in the river Paranapanema.

NaCl methodology, described by Lopera-Barrero et al. (2008), was employed for DNA extraction, quantified by spectrophotometer Shimadzu UV 1601, with wave length 260 nm and samples diluted for a concentration of 10 ng μL^{-1} . DNA integrity was verified by horizontal electrophoresis in agar gel 1% by buffer TBE 1X (500 mM Tris-HCl, 60

mM boric acid and 83 mM EDTA). Gel was stained with ethidium bromide (0.5 mg mL⁻¹) for 30 minutes and image obtained by EDAS (Kodak 1D Image Analysis 3.5).

Amplification followed procedures by Williams et al. (1990), modified. DNA was amplified in a 15 mL reaction volume using buffer 1X Tris-KCl, 2.5 mM MgCl₂, 0.46 μM primer, 0.2 mM of each dNTP, a Platinun Taq DNA Polimerase unit (Invitrogen®, USA) and 10 ng target DNA. DNA was denatured at 94°C for four minutes, followed by 40 cycles, every one consisting of one minute denaturation at 94°C; 90 seconds of primer annealing at 40°C and two minutes extension at 72°C. A final extension at 72°C was performed for seven minutes. RAPD reactions were amplified in a thermocycler Eppendorf Mastercycler Gradient and 30 different primers were tested with 10 bases for kits OPA, OPW and OPX (Operon Technologies Ltd. Valencia, USA), from which the most reproducible and best defined were selected (Table 1).

Table 1. Sequence of primers and nucleotides, percentage of puric bases (G+C), number of fragments (n) and base pairs of fragmente in *P. mesopotamicus* (A) and *L. elongatus* (B) broodstocks.

| Primers | Sequence | G+C | n (A) | Size (A) | n (B) | Size (B) |
|---------|---------------|-----|-------|----------|-------|----------|
| OPA02 | TGC CGA GCT G | 70 | 7 | 200-1100 | 7 | 350-1200 |
| OPA04 | AAT CGG GCT G | 60 | 6 | 500-1200 | 6 | 400-1300 |
| OPA16 | AGC CAG CGA A | 60 | 12 | 300-1700 | 14 | 200-1700 |
| OPW01 | CTC AGT GTC C | 60 | 10 | 300-1700 | 7 | 300-1500 |
| OPW02 | ACC CCG CCA A | 70 | 10 | 500-1800 | 10 | 400-1500 |
| OPW03 | GTC CGG AGT G | 70 | 11 | 300-1700 | 10 | 400-1500 |
| OPW04 | CAG AAG CGG A | 60 | 7 | 400-1500 | 8 | 500-1400 |
| OPW08 | GAC TGC CTC T | 60 | 9 | 300-1400 | 9 | 400-1500 |
| OPX01 | CTG GGC ACG A | 70 | 9 | 400-1500 | 10 | 300-1800 |
| OPX03 | TGG CGC AGT G | 70 | 8 | 400-1300 | 9 | 400-1700 |
| OPX04 | CCG CTA CCG A | 70 | 7 | 300-1300 | 6 | 400-1300 |
| Total | --- | --- | 96 | 200-1800 | 96 | 200-1800 |

Amplification products were separated in agar gel 1.5% and 15 mL of the amplified product and 2 mL of sample buffer (40% sucrose and 0.25%

bromophenol blue) in horizontal electrophorese were used. Electrophorese was conducted in a buffer TBE 0.5X (45 mM Tris-Borate and 1 mM

EDTA) for four hours at 70 volts. Quantification and amplification gels were visualized by UV radiation after exposure with ethidium bromide (0.5 mg/ mL⁻¹) for one hour. Image was photographed with Kodak EDAS (Kodak 1D Image Analysis 3.5, USA). Blank samples in gels (without DNA), double amplifications and electrophoresis, coupled to a strict selection of fragments enhanced quality and reproducibility of the amplified products

Fragment size obtained by amplification was estimated by comparing with standard Ladder 100 bp (Invitrogen®, USA). The presence or absence of fragments of identical molecular size constructed a similarity matrix based on Jaccard's similarity coefficient, with 1 as the presence of the fragment and 0 as its absence.

Genetic variability was determined by Shannon's genetic diversity index and by the percentage of polymorphic fragments calculated with PopGene 1.31 (YEH et al., 1999). TFPGA 1.3 (MILLER, 1997) calculated the distance and genetic identity (NEI, 1978) between broodstocks and the frequency of exact fragments (RAYMOND; ROUSSET, 1995). Arlequin 3.0 (EXCOFFIER et al., 2007) determined the genetic difference between broodstocks by *F_{st}* (WEIR; COCKERHAM, 1984) and for the analyses of molecular variance (AMOVA) (EXCOFFIER et al., 1992). Significance level of estimates was calculated by the randomized permutations method with 1000 and 10000 permutations. The same program was employed to determine the effective number of migrants (*N_m*). Wright (1978) proposal was employed for differentiation level by which rates between 0.00 and 0.05; 0.051 and 0.15; 0.151 and 0.25 and >0.25 respectively indicate small, medium, high and very high genetic differentiation. Statistical significance of *F_{st}* was calculated by test $X^2 [c^2 = 2n F_{st} (k-1); GL = (k-1) (s-1)]$, suggested by Workman and Niswander (1970), where *n* is the number of specimens in each group; *k* is the number of alleles; *s* is the number of groups.

Results and Discussion

The 10 primers produced 96 fragments varying between six (OPA04) and twelve (OPA16) (*P. mesopotamicus* broodstocks) and between six (OPA04 - OPX04) and 14 (OPA16) (*L. elongatus* broodstocks), featuring 68 (70.83%) and 94 (97.92%) polymorphic fragments for the two species respectively (Table 1). According to Telles et al. (2001), the number of fragments was sufficient to calculate reliable genetic variability.

There was a difference (*P*<0.05) in the frequency of fragments in *P. mesopotamicus* (15 fragments) and *L. elongatus* (27 fragments) broodstocks. Ten limiting fragments (fragments with frequency 1,000) were observed in the *P. mesopotamicus* broodstock in Palotina (PacPAL) but not reported in the broodstock in Salto Grande (PacSG). Low frequency fragments (less than 0.100) were not extant in the broodstocks (Table 2). On the other hand, *L. elongatus* broodstocks (Piapara Palotina: PiaPAL and Piapara Salto Grande: PiaSG) revealed limiting fragments in the two stocks (PiaPAL: 9; PiaSG: 3) and one low frequency fragment (primer OPA04 - 1300 bp) (Table 3). There were no exclusive fragments in the broodstocks, presupposing possible genetic similarity.

Shannon Index (SI) of genetic variability and percentage of polymorphic fragments (%PF) revealed high rates of intra-population genetic variability for all broodstocks (Table 4) even with fixed alleles (limiting) in several broodstocks. In fact, genetic variability was preserved in the fish ponds, probably due to the fact that a sample with sufficient genetic pool was provided at its formation and which prevented the founding effect. According to Lopera-Barrero (2009), the first thing to undertake in the implantation of pisciculture or restocking programs is the verification of broodstocks' genetic variability. If low genetic variability occurs, low survival rates and adaptation to the environment of released offspring will result.

Table 2. Primers (I), size (Tam) and frequency of fragments with significant rates by exact test ($P>0.05$) for *P. mesopotamicus* broodstock.

| I | Tam(A) | Frequency | | P ³ |
|-----|--------|---------------------|--------------------|----------------|
| | | PacPAL ¹ | PacSG ² | |
| A02 | 300 | 0.742 | 0.423 | 0.001 |
| A04 | 700 | 0.817 | 0.317 | 0.000 |
| A16 | 1500 | 0.817 | 0.367 | 0.000 |
| | 1700 | 1.000 | 0.293 | 0.000 |
| X01 | 500 | 1.000 | 0.247 | 0.000 |
| | 600 | 1.000 | 0.422 | 0.000 |
| | 800 | 0.517 | 0.225 | 0.001 |
| | 1200 | 1.000 | 0.293 | 0.000 |
| X03 | 500 | 1.000 | 0.394 | 0.000 |
| | 800 | 1.000 | 0.484 | 0.001 |
| | 1100 | 1.000 | 0.367 | 0.000 |
| X04 | 300 | 1.000 | 0.342 | 0.000 |
| | 400 | 0.635 | 0.247 | 0.000 |
| | 1200 | 1.000 | 0.293 | 0.000 |
| W01 | 300 | 1.000 | 0.367 | 0.000 |

¹PacPAL = pacu Palotina; ²PacSG = pacu Salto Grande; ³P: probability.

Table 3. Primers (I), size (Tam) and frequency of fragments with significant rates by exact test ($P>0.05$) for *L. elongatus* broodstocks.

| I | Tam (B) | Frequency | | P ³ | Continue ... |
|-----|---------|---------------------|--------------------|----------------|--------------|
| | | PiaPAL ¹ | PiaSG ² | | |
| A02 | 450 | 1.000 | 0.342 | 0.000 | |
| | 1200 | 0.553 | 0.293 | 0.004 | |
| A04 | 400 | 1.000 | 0.204 | 0.000 | |
| | 600 | 1.000 | 0.225 | 0.000 | |
| | 700 | 1.000 | 0.247 | 0.000 | |
| | 1000 | 1.000 | 0.270 | 0.000 | |
| | 1200 | 1.000 | 0.247 | 0.000 | |
| A16 | 1300 | 0.517 | 0.069 | 0.000 | |
| | 200 | 1.000 | 0.394 | 0.000 | |
| | 600 | 0.553 | 0.270 | 0.001 | |
| | 1100 | 1.000 | 0.342 | 0.000 | |
| X01 | 1700 | 0.553 | 0.247 | 0.002 | |
| | 300 | 0.553 | 0.247 | 0.001 | |
| X01 | 500 | 1.000 | 0.270 | 0.000 | |
| | 500 | 0.144 | 0.394 | 0.001 | |
| W01 | 600 | 0.184 | 0.484 | 0.004 | |
| W01 | 950 | 0.342 | 0.684 | 0.001 | |
| W03 | 700 | 0.247 | 0.452 | 0.006 | |
| | 1300 | 0.204 | 0.394 | 0.006 | |

| ... Continuation | | | | |
|------------------|------|-------|-------|-------|
| W04 | 500 | 0.183 | 0.452 | 0.001 |
| | 600 | 0.204 | 0.423 | 0.003 |
| | 700 | 0.163 | 0.394 | 0.001 |
| W08 | 700 | 0.144 | 0.394 | 0.001 |
| | 900 | 0.293 | 1.000 | 0.000 |
| | 1000 | 0.204 | 0.484 | 0.001 |
| | 1300 | 0.247 | 1.000 | 0.000 |
| | 1500 | 0.317 | 1.000 | 0.000 |

¹PiaPAL = piapara Palotina; ²PiaSG = piapara Salto Grande; ³P: probability.

Table 4. Number of specimens (N), Shannon Index (SI) and percentage of polymorphic fragments (PF) for *P. mesopotamicus* and *L. elongatus* broodstocks.

| Broodstocks | N | SI | %PF |
|---------------------|----|-------|------|
| PacPAL ² | 30 | 0.408 | 65.6 |
| PacSG ¹ | 30 | 0.347 | 57.3 |
| PiaPAL ⁴ | 30 | 0.572 | 94.8 |
| PiaSG ³ | 30 | 0.500 | 86.5 |

¹PacSG = pacu Salto Grande; ²PacPAL = pacu Palotina; ³PiaSG = piapara Salto Grande; ⁴PiaPAL = piapara Palotina.

High variability in broodstocks presupposes that offspring for restocking also have high variability, making feasible their release in the rivers Paraná and Paranapanema without jeopardizing the natural populations. In broodstock samplings from the same fish pond in Salto Grande for the species *P. mesopotamicus* and *L. elongatus*, Povh et al. (2008a) and Gomes et al. (2008) reported rates (SI = 0.289 and %FP = 56.36%; SI = 0.447 and %FP = 88.8%, respectively) similar to those in current research for PacSG and PiaSG. The above reveals good reproduction management of broodstocks and their viability in restocking programs. In another research on the genetic variability of *P. mesopotamicus* broodstocks in restocking programs in the river Paranapanema, Povh et al. (2009) reported high genetic variability (%PF = 71.4% - 75%; SI = 0.376 - 0.434) and thus adequate reproduction management. Almeida et al. (2013) assessed the genetic variability of broodstocks (natural population) and fingerlings in a *Salminus brasiliensis* restocking program and observed higher percentages of polymorphic fragments in natural populations than in fry

broodstocks and lower genetic diversity rates. The above corroborates the importance of adequate reproduction management for the variability of offspring.

Molecular variance (AMOVA) demonstrated that most variations lay within each broodstock and not between the broodstocks (89.77 for *P. mesopotamicus* and 89.73 for *L. elongatus*). Likewise, distance (0.088 and 0.142 for *P. mesopotamicus* and *L. elongatus*, respectively) and genetic identity (0.916 and 0.868 for *P. mesopotamicus* and *L. elongatus*, respectively) demonstrated similarity among the broodstocks of each species (Table 5). Results seem to show that broodstocks had a common ancestry. The hypothesis has been confirmed by the lack of exclusive fragments in the broodstocks of each species, Fst rate (0.1023 and 0.1027 for *P. mesopotamicus* and *L. elongatus*, respectively) and Nm rate (4.18 and 4,33 for *P. mesopotamicus* and *L. elongatus*, respectively) with a slight genetic difference (according to Wright's classification) with genetic flux (Table 6).

Table 5. Analysis of molecular variance (ANOVA), variation source (VS), sum of squares (SSQ), coefficient of variation (CV), percentage of variation (%V), distance (D) and genetic identity (I) for *P. mesopotamicus* and *L. elongatus* broodstocks.

| Groupings | VS | SSQ | CV | %V | D | I |
|--|-------|----------|----------|--------|-------|-------|
| PacPAL ¹ x PacSG ² | E.L | 45.900 | 1.18379 | 10.23* | 0.088 | 0.916 |
| | D.L | 602.400 | 10.38621 | 89.77 | | |
| | Total | 648.300 | 11.57000 | 100 | | |
| PiaPAL ³ x PiaSG ⁴ | E.L | 92.100 | 2.37770 | 10.27* | 0.142 | 0.868 |
| | D.L | 1204.600 | 20.76897 | 89.73 | | |
| | Total | 1296.700 | 23.14667 | 100 | | |

*P<0.05. E.L. = between broodstocks. D.L. = within broodstocks. ¹PacPAL = pacu Palotina; ²PacSG = pacu Salto Grande; ³PiaPAL = piapara Palotina; ⁴PiaSG = piapara Salto Grande.

Table 6. Fst, X² test for Fst, genetic difference according to Wright (1978) and number of emigrants (Nm) for different groupings analyzed in *P. mesopotamicus* and *L. elongatus* broodstocks.

| Groupings | Fst | Wright | X ² | Nm |
|--|---------|--------|----------------|------|
| PacPAL ¹ x PacSG ² | 0.1023* | Fair | 6.138 | 4.18 |
| PiaPAL ³ x PiaSG ⁴ | 0.1027* | Fair | 6.162 | 4.33 |

*P<0.05. ¹PacPAL = pacu Palotina; ²PacSG = pacu Salto Grande; ³PiaPAL = piapara Palotina; ⁴PiaSG = piapara Salto Grande.

Sporadic introductions of new broodstocks from natural populations or from broodstocks in captivity are normally used to increase the genetic variability of broodstocks in restocking programs (LOPERA-BARRERO et al., 2010). However, results in current analysis do not recommend an exchange of *P. mesopotamicus* and *L. elongatus* broodstocks between the fish ponds analyzed since their genetic similarity shuns the insertion of a new pool that improves conditions in restocking programs. On the contrary, no restrictions are recommended if exchange aims at replacing old broodstocks or those lost due to mortality.

High rates of intra-population genetic variability were reported within each broodstock, showing that the formation of broodstocks and the reproduction and genetic management were efficiently undertaken in the fish ponds studied. On the other hand, inter-population rates demonstrated similarity between broodstocks from each species and common ancestry. Results show the relevance

of genetic analyses in conservation programs with fish restocking.

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