Monitoring of genetic variability in *Colossoma macropomum* by using microsatellite markers

**Accepted 21st March, 2016**

**ABSTRACT**

Tambaqui fish (*Colossoma macropomum*) is a native species of great importance in Brazilian pisciculture. It is the species most farmed in captivity. However, many fish-farmers do not plan or manage the genetic variability of their stocks which may lead to the reduction of genetic variability. This research had as an objective to characterize the genetic variability and the genetic relationship of adults and juveniles Tambaqui fishes of captivity using 15 microsatellites loci. We found a hundred and three (103) alleles in the stock. The mean number of allele per locus was 6.3 (adults) and 7.5 (juveniles). The mean observed and expected heterozygosity in adults was 0.76 and 0.78, respectively, and in juveniles, it was 0.77 and 0.77, respectively. There were low endogamy rates with negative *F*IS values for the most loci, both in adults as in juveniles. We did not observe genetic structure in the stock (*F*ST=0.01). We observed private alleles in both generations. The dendrogram of genetic distances of shared alleles (*D*AS) suggested the occurrence of five groups. The groups 1 and 5 are the most divergent and thus, the more indicated for selection, aiming at the assisted reproduction. In conclusion, the genetic variability in the stock was elevated and the creation of a management plan towards to the genetic improvement of the stock seems to be promising.

**Key words:** Genetic monitoring, microsatellite, genetic diversity.

**INTRODUCTION**

The Amazonian tambaqui (*Colossoma macropomum*) is the most popular fish species in Brazil for freshwater aquaculture (Aguilar et al., 2013) and the central reasons for this domain is the fast growing in captivity, diverse feeding habits, rusticity and the good quality of the meat presented by such Amazon fish (Kubitza et al., 2007).

The importance of Tambaqui fish and the concerns related to the genetic management reflects in the great number of researches focusing on its genetic diversity, both in the natural environment as in pisciculture stocks (Calcagnotto, 1998; Calcagnotto and Toledo-Filho, 2000; Santos et al., 2009; Castro, 2008; Lopes et al., 2009; Jacometo et al., 2010; Farias et al., 2010). Some genetic studies performed with Tambaqui fish in pisciculture farms showed low genetic diversity (Calcagnotto and Toledo-Filho, 2000). Other studies showed that the reduction of genetic variability in pisciculture stocks occurs mainly due to inadequate reproductive management (Frost et al., 2006; Petersen et al., 2012), deficiency in the effective number of breeders (Aho et al., 2006) and unintentional breeders selection (Jacometo et al., 2010). The result of such actions may induce to endogamy, adaptability and progenies survival problems (Povh et al., 2008).

Microsatellites markers are repeats of sequences from two to six pairs set in tandem randomly distributed in genome. They are highly informative markers, once they allow the distinction of homozygotes from heterozygotes, they are multi-allelic and based in PCR. The broader population genetics study performed with native individuals of *C. macropomum* is the one of Santos (2010),...
Table 1. Estimative of genetic diversity in both generations, parental (G0) and juveniles (G1). (A) number of alleles/locus, (N) number of specimens, (P) number of exclusive alleles, (Ho) observed heterozygosity, (He) expected heterozygosity, (I) Shannon index, (Ht) total heterozygosity, (Fis) endogamy coefficient.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Loci</th>
<th>Adults (G0)</th>
<th>Juveniles (G1)</th>
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<tbody>
<tr>
<td></td>
<td>Cm1H8F</td>
<td>Cm1A11</td>
<td>Cm1D1</td>
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<tr>
<td>N</td>
<td>7</td>
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<tr>
<td>A</td>
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<td>1</td>
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<tr>
<td>I</td>
<td>1.73</td>
<td>2.11</td>
<td>2.14</td>
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<tr>
<td>Ho</td>
<td>0.57</td>
<td>1.00</td>
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<tr>
<td>He</td>
<td>0.79</td>
<td>0.87</td>
<td>0.88</td>
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<tr>
<td>Fis</td>
<td>0.34</td>
<td>-0.08</td>
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which sampled 21 sites distributed along Amazonas River, including some tributaries. In pisciculture, such markers have been used for genetic characterization of the stocks aiming at monitoring and controlling the genetic variability through generations (Rodriguez-Rodriguez et al., 2013).

In this study, 15 microsatellites loci were used to investigate the genetic variability levels and the genetic relationships of two generations of Tambaqui fish (G0 and G1), farmed in captivity. The obtained results provided basic information for selection and management strategies concerning the maintenance of the genetic variability of this stock.

MATERIALS AND METHODS

Samples from the caudal fin of seven adult specimens (Generation 0 to G0) and 60 juveniles (Generation 1 to G1) of C. macropomum were collected from the Experimental Farm of the Federal University of Amazonas, located at the BR-174, Km-38, in Manaus-AM, Brazil. The genomic DNA was extracted according to the protocol described by Murray and Thompson (1980). The polymorphism analysis for the microsatellites loci were made using 15 primers developed specifically for the species as described by Santos et al. (2009), Santana et al. (2012) and Hamoy et al. (2011). The genomic DNA was amplified in a final volume of 10 μl, containing: 1 μl buffer 10x Tris-KCl, 0.5 μl of MgCl₂ 1.5 mM, 1 mM of each primer R and F (the last one marked with HEX or FAM fluorescence), 0.25 μl of dNTP at 0.25 mM of each, 1U of TaqDNA Polymerase, 2.0 μl of DNA at 20 ng/μl.

The PCRs were performed in Thermocycler Veriti (Applied Biosystems) in the following conditions: a) Tambaqui Program 1: for the locus TB20 (annealing = 62°C), which consisted of an initial denaturation at 94°C for 1 min followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of hybridization of primer and 40 s of extension at 72°C, after a final extension at 72°C for 25 min; b) Tambaqui Program 2: for the loci Cm1H8F, Cm1A11, Cm1D1 and TB14 (annealing = 65°C), Cmc1r02, Cmc1r04, Cmc1r07, Cmc1r05 and Cmc1r03 (annealing = 60°C), TB21 and TB13 (annealing = 61°C) and TB1 (annealing = 45°C), consisted of 94°C for 1 min followed by 35 cycles, being 30 s of denaturation at 94°C, 30 s of hybridization of the primer and 1 min of extension at 72°C after a final extension at 72°C for 30 min.

The amplified products were observed in agarose gel 2% stained with ethidium bromide (0.05 mg/ml) for 15 min and photographed using a photo documentation system (EASY DOC 100 Bioagency). The products size was estimated by comparison with the marker of molecular weight of 1 kb Plus Ladder (Fermentas, Sinapse).

The PCR products were diluted in a proportion from 1/80 to 1/120 to genotyping reactions. A multiplex system (1 to 5) was performed organized according to size intervals of alleles and markings with different types of fluorescence to be distinguished in electrophoresis.
For analysis in ABI 3130 (Applied Biosystems) the products (in multiplex) were added to a mix with a marker of known molecular weight Gene Scan 500 and Formamidine HI-DI. We performed the genotyping according to the manufacturer’s instructions. The estimations of the alleles’ sizes of each locus were performed in GeneMapper v.4.0 software (Applied Biosystems). The data matrix with the sizes of alleles were submitted to the Micro-Checker software (Van et al. 2004) to ascertain the occurrence of technical artifacts such as the presence of null alleles, allele stutters and large drop-out.

Genetic diversity in the data set was determined by the number of alleles (A), the observed heterozygosity (Ho) and expected heterozygosity (He), the Shannon index (I), the total heterozygosity (Ht), number of exclusive alleles (P) and its frequency using the GenAlEx × 6.5 software (Peakall and Smouse 2006, 2012).

In order to estimate the deficiency or excess of heterozygotes and the endogamy coefficient (Fis) the software GENEPOP 3.3 was used (Raymond and Rouset, 1995), which also tested the adherence to the Hardy-Weinberg equilibrium (with 10,000 disremembering, 1,000 data sets and 10,000 interactions per set). An analysis of molecular variance AMOVA (exact test of Fisher at 5% of probability) was estimated in the Arlequin 3.5.1.2 software (Excoffier et al., 2005), as well as, the values of genetic differentiation FST (Weir and Cockerham, 1984) for the generations pairs. As a method for differentiation of the FST values, the Wright (1978) definition was used. To determine the genetic relationship between the adult and juvenile specimens the distance of shared alleles was estimated (DAs) (Chakraborty and Jin, 1993) and calculated with the Populations 1.2.28 software (Langella, 2002), grouped by the neighbor joining (NJ) method available in Mega version 3.1 software (Kumar et al., 2004).

RESULTS

Twelve out of 15 microsatellites loci optimized for the PCR conditions presented a satisfactory amplification. Three loci, Cm1C8, Cm1F4 and TB14, amplified unspecific products resulting in genotyping errors and thus, were not considered in the analysis. The 12 microsatellite loci produced a total of 103 alleles; 76 of them in adults (G0) and 91 in juveniles (G1), with a mean of 6.3 (adults) and 7.5 (juveniles) alleles per locus. The number of alleles per locus (A) varied from 4 to 9 in adults and from 3 to 11 in juveniles (Table 1). The allelic frequencies varied between the two generations and 12 private alleles in adults (G0) and 27 private alleles in juveniles (G1) was observed (Figure 1). The mean observed heterozygosity for both generations was statistically insignificant (Table 1). In adults (G0), the observed heterozygosity varied from 0.14 for the locus TB20 to 1.00 for the locus Cm1A11. In juveniles (G1), the observed heterozygosity varied from 0.60 for the locus TB13 to 0.88 for the locus Cm1D1. For the juveniles (G1), the expected heterozygosity had greater amplitude varying from 0.49 to 0.87 for the locus TB13 and TB20, respectively.

Concerning the inbreeding estimations the coefficient (Fi), a variation in the twelve loci was observed, but such variation was not significantly different from zero (p < 0.05) for most of them. The greatest inbreeding indexes were observed in the loci TB20 (Fi = 0.84 and 0.29) in adults (G0) and in juveniles (G1), respectively, and in the loci Cm1H8F (Fi = 0.17) and TB20 (Fi = 0.29) in juveniles (G1). The loci TB13 (Fi = -0.22) and Cmacr103 (Fi = -0.27) indicated an excess of heterozygotes in juveniles.

Fisher exact test revealed a deviation in Hardy-Weinberg Equilibrium (HWE) for the locus TB20 in adults (G0) and equilibrium deviation in eight loci (Cm1H8F, Cm1A11, Cm1D1, Cmacr102, Cmacr107, Cmacr105, TB20, TB21) in juveniles (G1) (Table 1). The genetic distances of shared alleles (DAs) varied from 0.25 to 1.0 and the frequencies distribution showed that 46.7% of the sample concentrated in an interval from 0.71 to 0.80. These values indicated that approximately 47% of the analyzed stock has a high value of genetic divergence, thus, the stock is genetically variable (Figure 2).

In relation to the dendrogram, the establishment of five groups was observed (Figure 3). The groups 1 and 5 showed the greatest genetic divergence and, thus, they are the most indicated for the selection of couples aiming at the assisted reproduction.

DISCUSSION

The genetic variation detected by the 12 microsatellite loci confirmed the high content of information in this type of marker for studies of genetic variability monitoring of fishes kept in piscicultures (Rodriguez-Rodriguez et al., 2013) or in native stocks (Santos, 2010; Hamoy et al., 2012, Santana et al., 2012). The explanation for the observed differences of allelic frequencies in the generations G0 and G1 and for the presence of alleles with low frequency may be due to the selection pressure on the adults previously. Eighteen out of the 25 matrices kept in captivity in the farm died probably due to inadequate management. Romana-Eguia et al. (2005) also observed alleles with low frequency when analyzing the effect of mass selection in genetic improvement of Nile tilapia. Jacometo et al. (2010), using RAPD markers to analyze the genetic variability of three stocks of C. macropomum from Rondônia, Sergipe and Mato Grosso, reported a bottleneck effect produced by unintentional selection observed during the reproductive management. According to these authors, high levels of genetic variability were observed for the three stocks, and, although the absence of genetic structure at least three exclusive fragments were observed.

The other genetic parameters observed in the present
Figure 1. Private alleles frequencies in the parental generation (G0) in blue and juveniles generation (G1) in red. Microsatellites loci: Cm1H8F, Cmacr102, Cm1A11, Cmacr104, Cm1D1, Cmacr107.

Figure 2. Graphic of genetic relationships of the specimens.
Figure 3. Dendrogram of genetic distances of shared alleles (DAS). The letter (A) beside some numbers means specimens adults.
research, such as observed heterozygosity (Ho), expected heterozygosity (He) and number of alleles per locus (A), were similar to those found by Santos et al. (2009), Hamoy et al. (2011) and Santana et al. (2012). Based on these parameters, we concluded that the degree of genetic diversity for the most of the loci analyzed considering both the adults (G0) and the juvenile (G1) specimens was high, indicating a considerable genetic variability in the studied stock (Table 1). We did not expect this result once the stocks kept in captivity tend to lose variability because of the selection effect (Lopera Barreiro et al., 2010).

On the other hand, high levels of genetic variability were already reported for natural and captivity populations of Tambaqui fish (Santos et al., 2007; Jacometo et al., 2010). Santos et al. (2007) analyzed the control region of mtDNA from wild samples of Tambaqui fish from the Upper Solimões, Medium and Low Amazonas rivers, and concluded that the Tambaqui fishes occupying the main stream of these rivers formed a great panmictic population. Although, the authors reported high levels of genetic variability and absence of structure, they called attention to the clear and drastic reduction of the population size in the census of the species.

Similar to results here observed of high genetic variability, the study of Aguiar et al. (2013) that analyzed the control region of mtDNA from wild life kept in captivity besides tambating hybrids verified a total of 67 haplotypes of which 57 were exclusives and 16 shared. Seven of these haplotypes were observed in both wild and captivity specimens and one haplotype was observed in the four analyzed populations.

According to the authors, this result was unexpected due to the results obtained by Gomes et al. (2012) that related just two haplotypes (H1 and H2) for 93 specimens of tambaqui from 10 different farms. Aguiar et al. (2013) paid attention to the fact that different approaches for the breeding and management within the same geographic region can lead to contrasting implications for the genetic diversity of tambaqui. In the east of Pará State, the management practices lead to a decrease of genetic variability (Gomes et al., 2012) whereas, in the west the stocks that kept the wild representation led to the production of genetically diversified stocks (Aguiar et al., 2013).

Our results showed that the tambaqui fish stock kept in the experimental farm from the Federal University of Amazonas has good levels of genetic variability. The selection of more divergent specimens is highly recommended for keeping this variability and the establishment of a genetic improvement program aiming at the increase in production seems to be promising.

ACKNOWLEDGEMENT

The authors are grateful to FAPEAM for their financial support.

REFERENCES


