Impacts of stocking on the genetic diversity of *Colossoma macropomum* in central Amazon, Brazil

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Genet. Mol. Res. 15 (2): gmr.15027700
Received September 22, 2015
Accepted December 8, 2015
Published April 7, 2016
DOI http://dx.doi.org/10.4238/gmr.15027700

ABSTRACT. Tambaqui (*Colossoma macropomum*) is the main fish species farmed on a commercial scale in northern Brazil. In view of the current scenario of Brazilian aquaculture, studies on the genetic improvement and reproductive management of captive tambaqui are crucial in identifying the genetic variability of broodstocks and devising management practices. Genetic diversity of three tambaqui broodstocks in western Amazon was evaluated using molecular markers. Fin samples were collected from 89 fish; 38 from Balbina, 30 from a hatchery in Rio Preto da Eva, and 21 from the experimental farm of the Federal University of Amazonas (UFAM). Ten primers were used for the analysis of diversity and genetic structure. Of the 152 bands produced, 146 were polymorphic. The proportion of polymorphic loci showed little variation among the three stocks. The lowest and highest rates were found in the Rio Preto da Eva (80.92%) and Balbina (85.53%) stocks, respectively. Heterozygosity (H) and Shannon (I) indices were similar among the stocks; the lowest values were found in Balbina (H = 0.279 and I = 0.419), and the highest in UFAM (H = 0.294 and I = 0.439). Following analysis of the genetic structure and relationship, the sample was

divided into two groups, with the Balbina stock clearly deviating from the others. The results suggest that, to increase genetic variability, molecular information may be used instead of replacement of wild breeders. The groups characterized here can be used in genetic improvement programs with other tambaqui broodstocks from different areas of South America.

Key words: Genetic variability; Amazonian fish; Genetic structure; Broodstock; Genetic management

INTRODUCTION

Aquaculture is a promising activity for promoting the sustainable development of the Amazon, because it combines rational management of water resources and forests, maintaining its economic feasibility for years. This is possible because of the techniques developed to farm native species with high commercial value, such as tambaqui (*Colossoma macropomum*). This omnivorous fish shows good growth and feed conversion rates with artificial feed, and its hardiness allows it to survive in low water quality conditions, particularly at high temperatures and low oxygen concentrations (Freitas et al., 2014). For these reasons, tambaqui is the main species farmed on a commercial scale in Northern Brazil.

The success of fish farming is associated with the management and maintenance of genetic variability in broodstocks, which is supported by planned cross-matings that promote continuous improvement in productivity. The first tambaqui broodstocks in Brazil were reared in the 1980s from a few wild fish; however, inbreeding reduced stock variability over the years, compromising fish vigor and lowering their resistance to management practices and diseases (Calcagnotto and Toledo-Filho, 2000). After a number of years, the genetic quality of new tambaqui broodstocks in the states of Mato Grosso and Rondônia was assessed using molecular markers (Lopes et al., 2009). A national genetic improvement program was then established, to monitor genetic variability and cross-mating within and between different stocks. Breeders from different areas and novel genotypes were eventually introduced into the program (Ponzoni, 2006; Gjedrem and Baranski, 2009).

Currently, the application of molecular markers is an essential research field in aquaculture. They are used in different improvement programs for genetic characterization of broodstocks, hybrid identification, diversity estimates, determination of crossbreeding rate, and construction of genetic maps (Gasques et al., 2014). The inter simple sequence repeat (ISSR) markers are suitable for that purpose, because it is easy to use and multilocus genetic analysis can be performed rapidly (Ng and Tan 2015). In native fish, ISSR has been used to study intra- and interspecific genetic diversity (Fonteles et al., 2011; Gasques et al., 2014). It has also been applied to differentiate tambaqui from pacú (*Piaractus mesopotamicus*), pirapitinga (*Piaractus brachypomus*), and their hybrids (Fonteles et al., 2011).

Given the current conditions of tambaqui farming, research related to its genetic improvement and reproductive management in captivity is a priority when identifying the degree of variability in broodstocks and guiding their management. The present study used ISSR molecular markers to evaluate the genetic diversity of tambaquis from three different fish farms in the state of Amazonas, to prepare an experimental broodstock that can be used in genetic improvement programs with tambaqui stocks from other areas.

MATERIAL AND METHODS

Sampling

Tambaqui breeders were obtained from three fry production units; 38 fish from the Balbina Center of Technology, Training and Production in Aquaculture (CTTPA-Balbina), 30 from a hatchery in Rio Preto da Eva, and 21 from the experimental farm of the Federal University of Amazonas (UFAM) (Table 1). The 89 fish were microchipped before being stocked in the facilities of Embrapa Western Amazon, Manaus, Amazonas state. Caudal fins were clipped and the samples were preserved in liquid nitrogen for DNA extraction.

Table 1. Characteristics of tambaqui, *Colossoma macropomum*, stocks.

Stock	Localization	Hydrographic origin	Population	N
Balbina	Presidente Figueiredo	several rivers	Founding	38
Rio Preto da Eva	AM010, 113 km	Rio Negro, AM	-	30
UFAM	BR174, 38 km, Manaus	Rio Negro, AM	Wild x Wild	21

ISSR Markers

DNA was extracted from the caudal fin samples, according to the protocol modified by Lopera-Barrero et al. (2008). The amount and quality of the DNA was determined using a NanoDrop spectrophotometer and 0.8% agarose gel electrophoresis, respectively. The samples were diluted to 50 ng/mL to optimize DNA amplification.

The PCR reactions were performed in 20-µL aliquots of the diluted sample, using 50 ng DNA, 1X buffer with 2.0 mM $MgCl_2$, 0.5 mM dNTPs, 0.5U Taq DNA polymerase (Biotika), and 0.25 pM primers. Initial amplification conditions were: denaturation at 94°C for 3 min, followed by 40 cycles of 30 s at 92°C. To ISSR primer selection, ninety-four primers (UBC Primer Set#9) were used annealing at 45°C for 1 min, elongation at 72°C for 2 min, and final elongation at 72°C for 10 min. Ten of the 94 ISSR primers were selected based on polymorphism and annealing temperature gradient tests.

The same PCR protocol was adopted to study the different broodstocks, with an annealing temperature varying from 48° to 63°C, depending on the primer (Table 2). The amplified fragments were separated on a 1.5% agarose gel and a binary matrix, assigning code (1) for band presence and (0) for absence in the 10 primers used.

Table 2. ISSR primers selected for the genetic diversity analysis in three tambaqui (*Colossoma macropomum*) stocks in central Amazonia, Brazil.

Primer	Repetition	Ta (ºC)	N	P (%)
UBC 808	$(AG)_8C$	56°C	13	100
UBC 811	$(GA)_8C$	62°C	9	100
UBC 834	$(AG)_8YT$	60°C	15	100
UBC 841	$(GA)_8YC$	63°C	15	100
UBC 842	$(GA)_8YG$	62°C	18	88.88
UBC 866	$(CTC)_6$	60°C	8	100
UBC 876	$(GATA)_2(GACA)_2$	45°C	19	100
UBC 880	$(GGAGA)_3$	50°C	17	88.23
UBC 899	CAT $(GGT)_3$ CAT TGT TCC A	48°C	20	100
UBC 900	ACTT $(CC)_2$ CAG GTT AAC ACA	48°C	18	94.40

Ta = annealing temperature; N = Number of bands; P (%) = Rate of polymorphic bands.

Data analysis

The stocks were first evaluated with POPGENE 1.32 software, using descriptive statistics for population genetics (Yeh et al., 1997). Heterozygosity was determined by gene diversity, according to Nei (1973). GENALEX 6.41 (Peakall and Smouse, 2006) was used to perform principal coordinates analysis (PCoA), based on the genetic distance between the pairs of individuals and analysis of molecular variance (AMOVA). The relationship between stocks was determined using the software STRUCTURE (Pritchard et al., 2000) and Bayesian structural inference (Falush et al., 2007). Twenty independent simulations and 200,000 iterations were performed for each number of groups (K; 1 to 8). The most probable K was selected based on DK values, according to Evanno et al. (2005). The individuals with ancestry (Q) higher than 0.80 were allocated as members of the groups formed, whereas the others (Q < 0.80) were considered admixtures.

RESULTS

ISSR markers

The 10 ISSR primers selected in the polymorphism test produced 152 bands, 146 of which were polymorphic. A lower number of bands were obtained using the UBC 866 primer and a higher number using the UBC 899 primer. Most primers were 100% polymorphic, except for the UBC 842, 880 and 900 primers, which showed monomorphic bands and polymorphism rates ranging from 88.23 to 94.40%. The high polymorphism rate indicated that the ISSR markers selected were highly informative regarding genetic variability in the stocks (Table 2).

Genetic diversity

The variation between the three *C. macropomum* broodstocks was evaluated by direct observation of the polymorphisms and estimates based on allele frequency of 146 polymorphic ISSR loci. The two most contrasting stocks in terms of proportion of polymorphic loci (P) and number of alleles (N_A) were Balbina (P = 85.53% and N_A = 1.855) and Rio Preto da Eva (P = 80.92% and Na = 1.809). Based on allele frequency, the most marked difference in genetic heterozygosity (H) and Shannon index (I) was between Balbina (H = 0.279 and I = 0.419) and UFAM (H = 0.293 and I = 0.439). The effective number of alleles varied only in the Balbina stock (Table 3).

Table 3. Summary of genetic diversity analysis of three tambaqui broodstocks in central Amazonia, Brazil.

Stock	N	P (%)	N_A	N_E	uH	I
Balbina	38	85.53	1.855	1.479	0.283	0.419
Rio Preto da Eva	30	80.92	1.809	1.500	0.293	0.429
UFAM	21	83.55	1.836	1.501	0.301	0.439
Total	89	96.05	1.960	1.576	0.336	0.502

N = number of individuals sampled per stock; P (%) = proportion of polymorphic loci; N_A = number of alleles observed; N_E = effective number of alleles; uH = unbiased expected heterozygosity; I = Shannon index.

Genetic structure

The PCoA and genetic structure results of tambaqui broodstocks allowed identification of groups with close genetic relationships as a function of ISSR marking of the 89 breeders analyzed.

In the PCoA, the sum of proportions of the first two main coordinates represented 57.96% of the total ISSR variation in all the stocks. The number of groups determined by the maximum delta produced by STRUCTURE was K = 2, which means that the 89 genotypes, defined by the relative proportions of Q, derived from two genetically distinct groups.

The two analyses were complimentary in the distribution of the 89 individuals and identification of stocks in the two inferred genetic groups. Group 1 was composed of Balbina fish, except for three admixture individuals. Group 2 was composed of 20 breeders from the Rio Preto da Eva stock and eight from the UFAM stock, exhibiting a close relationship between them. A total of 26 admixture individuals were identified in the groups (Q < 80%; data not shown) (Figure 1).

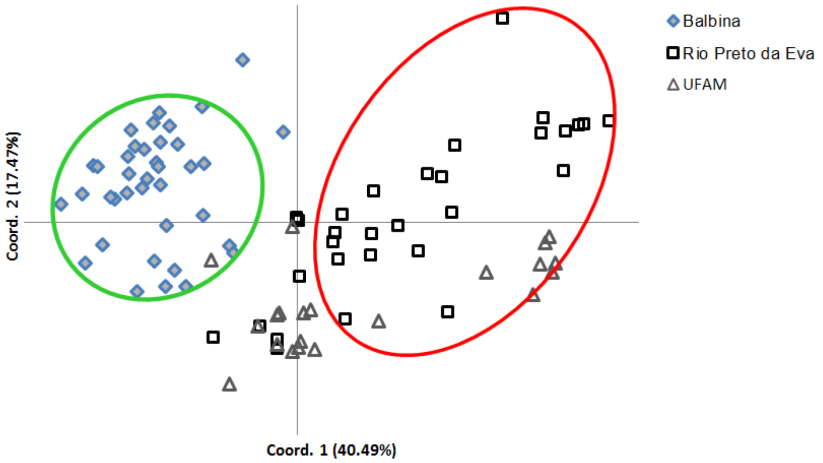

Figure 1. Principal coordinates analysis (PCoA) and structure analysis of 89 tambaquis sampled from three broodstocks. Circles correspond to the two (K = 2) groups inferred by STRUCTURE.

The AMOVA was performed considering the distribution of genetic variability in the original stocks and in the Bayesian groups. The first AMOVA showed that the genetic variability of the ISSR markers was more concentrated within the broodstocks (78%) than between them (22%) (Table 4). The AMOVA based on the inferred groups (K = 2, N = 63, Q > 80%) showed intra- and intergroup genetic variation of 71 and 29%, respectively (Table 4). The genetic differentiation was estimated between the predefined groups (broodstocks) and the inferred groups (K = 2), represented by PhiPT values of 0.220 and 0.290, respectively. Both results suggest an interchange of genetic material among the broodstocks, especial those of more recent origin (Rio Preto da Eva and UFAM).

Table 4. Analyses of molecular variance of three tambaqui broodstocks and groups inferred by STRUCTURE.

Source of variation	d.f.	SS	MS	Est. Var	% Var
Among stocks	2	407.856	203.928	6.303	22
Within stocks	89	1896.121	22.048	22.048	78
PhiPT = 0.222, P < 0.01					
Among groups	1	297.685	297.685	8.769	29
Within groups	62	1329.893	21.450	21.450	71
PhiPT = 0.290, P < 0.01					

d.f. = degrees of freedom; SS = sum of squared observations; MS = mean of squared observations; Est. var. = estimated variance; % Var. = percentage of total variance; P value estimates are based on 999 permutations; PhiPT = proportion of total genetic variance between individuals within populations.

DISCUSSION

ISSR Markers

ISSR has been one of the primary markers used for a rapid analysis of the diversity and genetic structure of populations, owing to the number of polymorphic loci produced in microsatellite regions. For species with high economic value in aquaculture, ISSR has been used alone or in conjunction with mtDNA, to access intra- and interspecific phylogenetic relationships, and variation between stocks or their genetic structure (Bignotto et al., 2009; Xiaoxiao et al., 2011). In the present study, the 10 ISSR primers selected showed optimal efficiency, producing polymorphic patterns above 88.2% for the three broodstocks studied. Using the same class of markers and number of primers, Jacometo et al. (2010) obtained a lower proportion of random amplified polymorphic DNA (RAPD) polymorphisms for tambaquis, ranging from 72.92 to 83.33%.

Genetic diversity

In general, estimates on genetic diversity in natural tambaqui populations are high, irrespective of the marker used. Other studies on wild populations sampled in different areas of the Amazonian Basin show high genetic diversity, assessed by mitochondrial DNA (Santos et al., 2007) or microsatellites (Santos et al., 2009; Santana et al., 2012). On the other hand, comparative studies on wild and farmed specimens show conflicting results. Isozyme markers identified loss of genetic variability in the wild population (Santos et al., 2012), whereas mitochondrial DNA sequences revealed similar levels of genetic diversity between wild and farmed samples (Aguiar et al., 2013).

In the present study, the Shannon index ranged from 0.419 (Balbina) to 0.439 (UFAM), showing similar diversity to that of other tambaqui stocks. In previous studies using RAPD, the Shannon index for broodstocks in Rondônia state ranged from 0.44 to 0.46 (Lopes et al., 2009), and in four stocks from different regions of Brazil it ranged from 0.39 to 0.45 (Jacometo et al., 2010). According to Nei's (1973) measure of genetic diversity, average heterozygosity of the three broodstocks was 0.336, with the lowest estimate found for Balbina stock (0.283). Similarly, estimates of genetic diversity were close to those described for tambaqui broodstocks analyzed using RAPD markers (Jacometo et al., 2010). Variations in estimated genetic diversity of the samples may be attributed to the history and level of genetic management of the stocks, although this has been scarcely documented.

The relationship between genetic diversity and breeder origin has been discussed by Calcagnotto and Toledo-Filho (2000). They suggested that the low number of individuals used to establish the first tambaqui broodstocks in the early 1980s accounts for the low genetic diversity found in current stocks. In this respect, the Balbina stock can be included as a pioneer in Amazonian aquaculture and represents a mixture of native and non-native individuals. The UFAM and Rio Preto da Eva stocks are more recent and are formed primarily by breeders from natural populations of the Rio Negro. Cropped stocks can lose genetic diversity due to founder effects, artificial selection, genetic drift, inadequate cross-mating strategies, or unbalanced sex ratios (Machado-Schiaffino et al., 2007; Wang et al., 2012).

Genetic structure

Bayesian clustering is more parsimonious in ascertaining population structure compared

to traditional methods (Warnock et al., 2010). The genetic relationship between individuals of the three stocks was structured into two different groups, which were supported by the combined results of the PCoA and structure analysis. With a few exceptions, group 1 was associated with the Balbina stock, whereas the graphic distribution of the individuals in group 2 was independent of sampling source. Records on the formation of stocks are scarce, but the results highlight the genetic distance between the oldest broodstock (Balbina) and the two most recent stocks composed of fish captured in the Rio Negro. Only three admixture individuals were detected in the Balbina stock, whereas most individuals in the other stocks were admixture, according to the Bayesian clustering. The structure pattern reveals that the loss of genetic variability in the Balbina stock was sufficient to make the group very homogeneous and different from the other stocks. Populations in captivity may undergo genetic divergence by genetic drift, founder effects, and inbreeding (Alarcon et al., 2004; Pampoulie et al., 2006).

The AMOVA revealed high genetic variability within the broodstocks and significant genetic differentiation ($P < 0.01$) in both analyses. The results partially agree with those reported by Jacometo et al. (2010), who found high genetic variability in tambaqui broodstocks from Northern Brazil, but with low intergroup differentiation and genetic distance. Endogamy is one of the main causes of reduced genetic variability and differentiation among broodstock individuals and populations (Taniguchi, 2003).

Tambaquis have been farmed in Brazil for more than 30 years. During this time, new stocks were created in producing areas in the state of Amazonas. Genetic isolation of the original Balbina stock and the recent introduction of new individuals (wild and selected) explain the genetic structure determined as a function of ISSR. Intraspecific differentiation of stocks involves a microevolutionary process (Waldman, 1999). According to Hutchings and Fraser (2008), the main agents of evolutionary changes induced by aquaculture include inadvertent selection, founder effects, genetic drift, inbreeding, multi-generational hybridization, and outbreeding depression.

Impact of farmed stocks on genetic diversity

The first years of tambaqui fry production in Brazil were satisfactory, but over the generations, the resistance to management, and the tolerance to environmental fluctuations, such as the thermal amplitude in certain seasons, decreased, whereas susceptibility to diseases increased and growth became limited (Calcagnotto and Toledo-Filho, 2000). In recent decades, the expansion of aquaculture in the western Amazon encouraged the commercial production of fry, reducing the dependence of CTTPA stock on Balbina. The use of fewer effective breeders can create genetically distinct groups that can change the genetic profile in relation to the original population in the long run (Caroffino et al., 2008). Thus, genetic monitoring of tambaqui broodstocks must be continuous.

The findings of the present study indicate the need for regular breeder replacement, preferentially with wild specimens and using molecular information to amplify genetic variability within the stocks, especially those from the Balbina reservoir. The main application of the results depends on the contribution of future cross-mating planning, which may enhance genetic variability of the stocks and provide genetic gains if features that meet aquaculture objectives are selected. Therefore, the implementation and maintenance of genetic improvement programs that combine research groups and the private sector can guide the management of genetic variability in tambaqui stocks, increasing the profits of tambaqui farming.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by Embrapa (Brazilian Agricultural Research Corporation: Projects AQUABRASIL and #02.12.01.020.00.03.003), FINEP (Project DARPA/SEPA/SEPROR), the National Council of Technological and Scientific Development (CNPq #471655/2010-4), and the Amazon Research Foundation (FAPEAM/PAPAC #062.00890.2014).

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