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Genetic diversity of Tambaqui (Teleostei - Characidae) broodstocks from Northern region of Brazil using microsatellite markers

Diversidade genética de estoque de Tambaqui (Teleostei - Characidae) da região Norte do Brasil usando marcadores microssatélites

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Highlights:
Molecular study in fish farmed Tambaqui.
Moderate genetic differentiation between stocks.
Positive F_{IS} (inbreeding coefficient) in three stocks.

Abstract

The Amazonian fish Tambaqui (*Colossoma macropomum*) is the most common native species in Brazil. This species has the highest production rate in the Northern region, especially in the State of Rondônia. The genetic evaluation of Tambaqui is an extremely important to increase productivity in fish farms or improve the adaptability in restocking natural populations. The objective of this study was to evaluate the genetic diversity of three Tambaqui broodstocks in Rondônia, Brazil. Six microsatellite markers were used to analyze a total of 89 breeders collected from three fish farms located in Ji-Paraná (JP), Ouro Preto do Oeste (OP) and Presidente Médici (PM). A total of 37 alleles between 140 and 310 bp were found, including the presence of exclusive and low frequency alleles in the three broodstocks. The average values of observed heterozygosity ranged from 0.404 (PM) to 0.499 (JP). The F_{IS} coefficient values were positive for the three broodstocks, demonstrating a deficit of heterozygotes. The Molecular Variance Analysis (AMOVA) showed greater variation within the stocks than between them. The genetic differentiation was moderate and significant between the stocks, with higher differentiation between JP x PM and lower between OP x PM. The Bayesian analysis designated an optimal value of K = 3 groupings. Although there is moderate genetic diversity between broodstocks, the high F_{IS} indicates a possible decline of diversity in the next generations, and therefore, the incorporation of new breeders is suggested to increase the genetic diversity in the three stocks.

Key words: *Colossoma macropomum.* Conservation. Genetic variability. Microsatellites. SSR.

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Resumo

O peixe amazônico Tambaqui (*Colossoma macropomum*) é a espécie nativa mais produzida no Brasil. Esta espécie **é** a mais criada na região Norte, principalmente no Estado de Rondônia. A avaliação genética de Tambaqui é extremamente importante para aumentar a produtividade nas pisciculturas ou melhorar a adaptabilidade no repovoamento de populações naturais. O objetivo deste estudo foi avaliar a diversidade genética de três estoques de Tambaqui em Rondônia, Brasil. Seis marcadores microssatélites foram utilizados para analisar um total de 89 reprodutores coletados em três pisciculturas localizadas em Ji-Paraná (JP), Ouro Preto do Oeste (OP) e Presidente Médici (PM). Foram encontrados 37 alelos entre 140 e 310 pb, incluindo a presença de alelos exclusivos e de baixa frequência nas três ninhadas. Os valores médios de heterozigosidade observada variaram de 0,404 (PM) a 0,499 (JP). Os valores do coeficiente de F_{IS} foram positivos para as três ninhadas, demonstrando déficit de heterozigotos. A Análise de Variância Molecular (AMOVA) mostrou maior variação dentro dos estoques do que entre eles. A diferenciação genética foi moderada e significativa entre os estoques, com maior diferenciação entre JP x PM e menor entre OP x PM. A análise bayesiana designou um valor ótimo de K = 3 agrupamentos. Embora exista uma diversidade genética moderada entre os filhotes, o alto SIF indica um possível declínio da diversidade nas próximas gerações e, portanto, sugere-se a incorporação de novos criadores para aumentar a diversidade genética nos três estoques.

Palavra-chave: *Colossoma macropomum*. Conservação. Microssatélites. SSR. Variabilidade genética.

Introduction

World aquaculture fish production in 2018 reached 82.1 million tons, which an increase of 2.6 million tons over that of the year 2016 (Food and Agriculture Organization [FAO], 2020). Following the same trend, in Brazil, in 2019 the production was 758 thousand tons, representing an increase of 4.9% in relation to the previous year (Peixe BR, 2020). The production of native fish in Brazil represented about 38% (287,930 tons) of the total production, with emphasis on the species of Tambaqui (*Colossoma macropomum*) (Peixe BR, 2020). This species has the highest production rate in the Northern region of Brazil and is the most produced native species in the country, with the State of Rondônia being its main producer with 69 thousand tons (Peixe BR, 2020).

Colossoma macropomum is a species belonging to the order Characiformes, family Characidae, and is naturally present on the banks of the Amazon and Orinoco rivers. It is a very important fish due to its ease of cultivation, high productivity, easy reproduction and high commercial value (Araujo-Lima & Goulding, 1997; Costa, Freitas, Gomes, Carneiro, & Martins, 2016). Its production is currently widespread, spanning several fish farms in the Southeast, Central West and Northeast regions of Brazil. Despite its commercial and cultural importance, its populations have been reported to be declining in the basins where it naturally occurs, with extractives being one of the main causes (Moraes et al., 2017).

Due to population reduction in their natural habitats, *C. macropomum* breeding units should be considered for the generation of fingerlings for both genetic improvement and conservation purposes (Lopera-Barrero et al., 2015; Ribeiro et al., 2016; Santos, Santana, Sá Leitão, Paula Silva, & Almeida Val, 2016). However, it is necessary that the females and males breeders have high genetic variability to transfer to their progeny, since inbreeding depression can lead to a decrease in zootechnical characteristics of interest (Rodriguez-Rodriguez et al., 2013) fundamental to the success of genetic improvement programs, and adaptability, in the case of restocking natural populations.

One of the most common methods for this purpose is the analysis of genetic diversity and population structure through microsatellite markers (SSR- Simple sequence repeats), which is one of the most widespread tools used for the characterization

of fish stocks (Lopera-Barrero et al., 2016; Santos et al., 2016; Souza et al., 2018a). Since they have a codominant inheritance pattern, show a high number of polymorphisms and generate a large amounts of informative data (Abdul-Muneer, 2014), SSRs allow the characterization of several genetic parameters that are useful for the management of fish stocks.

Microsatellite markers in the literature has been developed for the characterization of fish stocks. Tambaqui is the most produced native species in Brazil with a highest production rate at the northern region, so its genetic evaluation is extremely important to increase productivity in fish farming. Priorly, the literature does not take into consideration ancestral studies for the subsequent reproduction and maintenance of genetic diversity in fish populations (Brabo et al., 2016; Moraes et al., 2017; Peixe BR, 2020). The objective of this paper is to evaluate the genetic diversity of three broodstocks of *C. macropomum* from the State of Rondônia. In this context, one contribution of this work is to consider previous genetic studies in order to complement the genetic data of these breeding stocks.

Materials and Methods

The study was approved by the Committee of Ethics in the use of animals of the State University of Londrina (CEUA_UEL nº18610.2016.00). Caudal fin samples (approximately 0.5 cm²) were randomly collected of males and females from three fish farms in the State of Rondônia, Brazil: 27 samples from Ji-Paraná - JP (10° 53' 07"S, 61° 56' 06"W), 30 samples from Ouro Preto do Oeste - OP (10° 44' 53"S, 62° 12' 57''W), and 32 samples from Presidente Médici - PM (11° 10' 31"S, 61° 54' 05"W). These three fish farms consist of *C. macropomum* broodstock and have the purpose of distributing fingerlings to other fish farms. On the other hand, JP, PM and OP were previously analyzed by Ribeiro et al. (2016) implementing the RAPD technique (dominant molecular marker), proposing a new study using the co-dominant marker (SSR) to determine and validate the results.

DNA extraction was accomplished using a NaCl protocol performed according to the methodology described by (Lopera-Barrero et al., 2008). The DNA was quantified using a SLIPQ 026-Quantificador L-Quant spectrophotometer (Loccus Biotecnology, Ribeirão Preto, Brazil). Samples were diluted to 30 ng/μL. DNA integrity was checked using 1% agarose gel electrophoresis, run at 100 volts for 60 minutes. The gel was visualized in a transilluminator with ultraviolet light and was photographed with a Kodak EDAS camera (1D Image Analysis 3.5 Kodak, USA).

PCR was performed in a final volume of 15 μl, with 1X Tris-KCl buffer, 2.0 mM of $MgCl_2$, 0.8 μM of the primers (Forward and Reverse), 0.2 mM of dNTP, one Platinium Taq DNA Polymerase unit (Invitrogen®, Carlsbad, USA) and 60 μg of DNA. For amplification, initially, DNA was denatured at 94°C for four minutes, subsequently followed by 30 cycles of 60 seconds of denaturation at 94°C, 60 seconds of annealing and 60 seconds of extension at 72°C. Cycling was followed by 10 minutes of a final extension at 72°C. The amplified primers were: Cm1A8 (EU685306), Cm1A11 (EU685307), Cm1C8 (EU685308), Cm1D1 (EU685309), Cm1E3 (EU685310) and Cm1H8 (EU685315) (Santos, Hrbek, & Farias, 2009). The recommended annealing temperatures for the primers as described by Santos et al. (2009) were used. PCR was performed on a Veriti® thermal cycler (Applied Biosystems®, Austin, TX, U.S.A.).

The amplified products were separated on 10% polyacrylamide gel (acrylamide: bis-acrylamide - 29: 1), with electrophoresis conducted in TBE 0.5X buffer at 180 volts and 250 MA for eight hours. Subsequently, gel staining was performed with silver nitrate for visualization of the microsatellite alleles. The gels were photographed and analyzed using Adobe Photoshop CC (64 Bit) software.

Photographs of the gels were aligned and allele sizes were calculated using a 100 bp DNA ladder (Invitrogen®, Carlsbad, USA).

The number of alleles, expected (He) and observed heterozygosity (Ho), Hardy-Weinberg equilibrium test (HWE), analysis of molecular variance - AMOVA and genetic differentiation (F_{ST}) were estimated by the Arlequin 3.0 program (Excoffier, Laval, & Schneider, 2005). Allele frequency, allelic richness (Ar) and the inbreeding coefficient (F_{IS}) were calculated using FSTAT 2.9.3.2 (Goudet, 2002). The effective number of alleles (Ae) was calculated for each locus using GenAlex version 6.5 software (Peakall & Smouse, 2012). Gene flow (Nm) between stocks was calculated using the GENEPOP 4.0.6 program (Rousset, 2008). For differentiation of Fst values, the definition proposed by Wright (1978) was used, were values from 0.00 to 0.05, 0.051 to 0.15, 0.151 to 0.25 and > 0.25 indicate small, moderate, high and very high genetic differentiation. A dendrogram based on the genetic distance defined by Nei (1978) was constructed with a UPGMA analysis using MEGA version 5.0 software (Tamura et al., 2011). The STRUCTURE v.2.3.3 software (Pritchard, Stephens, & Donnelly, 2000) was used to verify the existence of possible groupings (K) of genetically similar populations, following the mixed model of clusters with a length period of 250,000 followed by 1,000,000 repetitions of MCMC (Markov chain Monte Carlo), the software assumed mixed ancestry between the correlated populations with allelic frequencies. Estimates of K (number of clusters) were obtained from simulations performed with K ranging from one to five (K = 1-5), reproducing 20 runs for each hypothetical value of K. The number of clusters was determined using the method proposed by Evanno, Regnaut and Goudet (2005) implemented on the Structure Harvester website (Earl & vonHoldt, 2012).

Results and Discussion

The six microsatellite loci generated 37 alleles. The total number of alleles per locus ranged from five (Cm1C8, Cm1E3 and Cm1H8) to eight (Cm1A8), with sizes between 140 bp (Cm1A8) to 310 bp (Cm1E3). The number of alleles identified in loci Cm1A11, Cm1C8 and Cm1H8 was similar to that observed in two broodstocks investigated by Moraes et al. (2017). However, the six loci presented lower numbers of alleles than those found by Santos et al. (2009) in a wild population collected on the São Miguel Island, Pará, who obtained number of alleles between 7 (Cm1A8) and 21 alleles (Cm1H8) for the same loci implemented in this study. On the other hand, J. D. P. Aguiar et al. (2018) found an average number of alleles between 2.83 and 8.58 in broodstocks of *C. macropomum* in different regions of Brazil. The differences in the number of alleles observed can be attributed to the structure of these populations in different regions and may additionally be due to the reproductive management adopted in the fish farm (JP, OP and PM), which can lead to the disappearance of alleles in cases of intentional selection during mating.

Exclusive alleles and low frequency alleles (alleles with frequency <0.100) were observed in all stocks. JP was the stock that presented the highest number of alleles for both parameters: six exclusive alleles and 11 low frequency alleles (Table 1). The low allele frequencies, which tend to increase intrapopulation genetic differentiation, can be caused due to confinement, genetic bottlenecks or founder effect, which can be provoked by selection during the reproductive processes within each stock. In addition, the high number of exclusive alleles characterized an interpopulation genetic distancing in the stocks, mainly in the JP stock. The presence of these alleles suggests a restricted gene flow and presence of genetic drift (Azevedo et al., 2013), possibly related to the limited number of breeders in the stocks and their physical isolation.

Table 1
Number (N), allele size (bp), exclusive alleles and low frequency alleles observed in *Colossoma macropomum* stocks

	Cm1A8	Cm1A11	Cm1C8	Cm1D1	Cm1E3	Cm1H8
N	8	7	5	7	5	5
pb	140-215	200-260	225-260	160-230	280-310	256-282
Exclusive alleles (Frequency)						
JP	200(0.111); 215(0.148)	-	231(0.038)	-	305(0.093); 310(0.014)	282(0.019)
OP	180(0.033)	200(0.017)	-	-	-	-
PM	174(0.016)	-	260(0.097)	160(0.281)	-	-
Low frequency alleles (Frequency)						
JP	140(0.074); 150(0.074)	230(0.038); 237(0.96); 260(0.077)	225(0.038); 231(0.019)	200(0.074)	310(0.050)	275(0.053); 282(0.079)
OP	180(0.033)	200(0.017); 232(0.033); 237(0.067); 260(0.017)	-	182(0.067); 200(0.067); 230(0.017)	300(0.043)	262(0.067)
PM	174(0.016)	-	260(0.097)	-	300(0.093)	275(0.097)

JP: Ji-Paraná, OP: Ouro Preto, PM: Presidente Médici
<0.100 low frequency alleles.

When analyzed individually, significant deviation ($p < 0.05$) from the Hardy-Weinberg equilibrium was found for most loci (Cm1A8, Cm1C8, Cm1D1, Cm1E3 and Cm1H8 in JP; Cm1A8, Cm1D1, Cm1E3 and Cm1H8 in OP; and Cm1E3 and Cm1H8 in PM). According to Waples (2015), deviation from the Hardy-Weinberg equilibrium occurs due to forces such as selection, genetic drift, mutation or migration, all of which tend to change allele frequencies over time. In the case of finite and small populations, as found in fish farms, it is likely that a non-random selection of breeders and genetic drift are the main forces acting on the allele frequencies, corroborating the presence of exclusive and low frequency alleles found in the three broodstocks. The low values of effective alleles (Ae) in relation to allelic richness (Ar) reinforce the existence of several alleles segregating at low frequency (Table 2).

The average values of expected heterozygosity (He) were higher than those of the observed heterozygosity (Ho) for all stocks, which resulted in a significant ($P<0.05$) positive mean F_{IS} (Table 2), demonstrating a heterozygote deficit. In a study with *C. macropomum* broodstocks in Brazil, Santos et al. (2016) found He ranging from 0.48 to 0.63 and Ho from 0.26 to 0.52, which demonstrated a heterozygote deficit shown by the F_{IS}. Moraes et al. (2017) also found values of this coefficient from 0.229 to 0.348 in two broodstocks in the Northern region. It is expected that in stocks kept in captivity, there will be a decrease in genetic diversity as a consequence of intentional selection and of the mating among parental (Jacometo et al., 2010; Lopera-Barrero et al., 2015; Santos et al., 2016). Also, founder effect, caused by a small number of individuals in the base population, can be a driving factor in the loss of genetic diversity in fish farms for altering allele frequencies through genetic drift (Santos et al., 2016; Moraes et al., 2017). Therefore, it is possible that the broodstocks analyzed in the present study have reduced genetic diversity due

to the presence of this founder effect, which can be supported by the deficiency of heterozygotes in most loci, and consequently the deviation from HW equilibrium, and the significant positive F_{IS}, indicating the occurrence of inbreeding. Reproductive management aiming to maximize the use of breeders in mating, in order to avoid reproductive dominance of certain animals, whether male or female, can be strategies used to minimize the effects of drift and assist in the conservation of the genetic diversity of the broodstock (Souza et al., 2018b).

Table 2
Number of alleles per locus (Na), allelic richness (Ar), effective alleles (Ae), observed (Ho) and expected heterozygosity (He), Hardy-Weinberg equilibrium test (HWE) and inbreeding coefficient (F_{IS}) in the Colossoma macropomum stocks

Stocks	Locus	Na	Ra	Ae	Ho	He	F_{IS}
JP	Cm1A8	6	6	4.528	1.000	0.793*	-0.266
	Cm1A11	6	6	3.808	0.615	0.751	0.185
	Cm1C8	4	4	2.220	0.230	0.560*	0.593
	Cm1D1	5	5	3.710	0.740	0.744*	0.005
	Cm1E3	4	4	3.265	0.200	0.711*	0.724
	Cm 1H8	4	4	2.481	0.210	0.613*	0.663
	Mean	5	5	3.335	0.499	0.695	0.287*
OP	Cm1A8	4	4	2.675	1.000	0.636*	-0.586
	Cm1A11	7	6	3.614	0.733	0.735	0.003
	Cm1C8	3	3	2.675	0.533	0.636	0.165
	Cm1D1	6	6	2.699	0.466	0.640*	0.274
	Cm1E3	3	3	2.159	0.000	0.548*	1.000
	Cm 1H8	4	4	2.620	0.233	0.628*	0.633
	Mean	5	4	2.740	0.494	0.637	0.228*
PM	Cm1A8	3	3	2.022	0.500	0.513	0.026
	Cm1A11	2	2	1.992	0.500	0.505	0.012
	Cm1C8	3	3	2.385	0.451	0.590	0.238
	Cm1D1	3	3	2.589	0.562	0.623	0.099
	Cm1E3	3	3	2.378	0.185	0.590*	0.690
	Cm 1H8	4	4	3.070	0.225	0.685*	0.674
	Mean	3	3	2.406	0.404	0.584	0.313*

He * significance found in the Hardy-Weinberg equilibrium test at the 0.05 level.
JP: Ji-Paraná; OP: Ouro Preto do Oeste; PM: Presidente Médici
F_{IS}*: $P < 0.05$.

The AMOVA showed greater variation are within stocks than the between them ($p < 0.05$). Genetic differentiation (F_{ST}), according to the classification defined by Wright (1978), indicates a significant moderate differentiation between the three stocks (Table 3). Low gene flow (Nm) was identified between the stocks, which was smaller between JP x PM (0.256), followed by OP x PM (0.316), and larger between JP x OP (0.341). The dendrogram showed the formation of two groupings: one

comprised of the OP and PM populations and the other containing the JP individuals (Figure 1). Bayesian analysis performed with Structure software revealed K = 3 groupings (Figure 2), and the cluster arrangements corroborated the groupings produced in the dendrogram, these figures (Figure 1 and Figure 2) are new results obtained based in the co-dominant technique applied in these population.

Table 3
Analysis of molecular variance (AMOVA) and genetic differentiation (F_{ST}), using the classification defined by Wright (Wr), of *Colossoma macropomum* stocks

Variation Source	Sum of squares	Variance Components	Percentage of variation	F_{ST}	Wr
Between the stocks	20.086	0.147	10.48*	0.105	Moderate
Between individuals Within the stocks	112.819	0.520	3.69		
Within the individuals	107.500	1.208	85.82		

* P < 0.05.

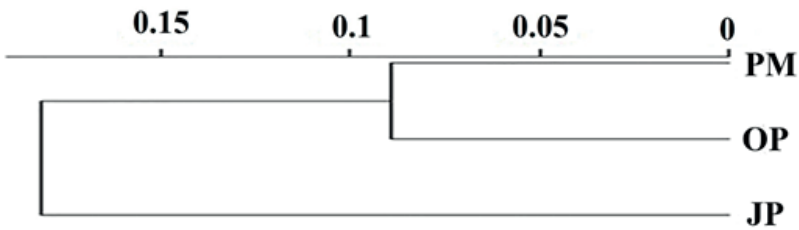

Figure 1. Dendrogram results for the three broodstock of *Colossoma macropomum* based on 6 microsatellite markers. JP: Ji-Paraná; OP: Ouro Preto do Oeste; PM: Presidente Médici.

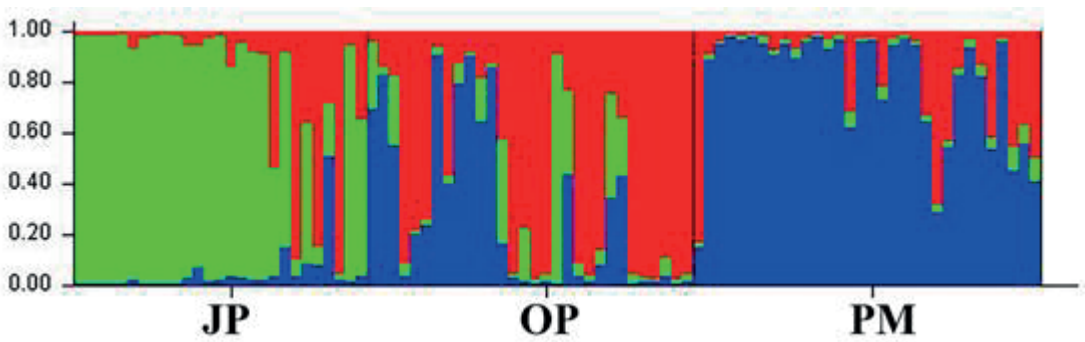

Figure 2. Bayesian cluster analysis (K = 3) of the 6 microsatellite loci in the three broodstock of *Colossoma macropomum*, run in the Structure program. JP: Ji-Paraná; OP: Ouro Preto do Oeste; PM: Presidente Médici.

The characterization of the genetic diversity in captive stocks should be a priority for the development of strategies to reduce genetic erosion and minimize the risk of inbreeding depression (J. Aguiar et al., 2013). Our results indicated the presence of moderate genetic diversity in the studied broodstocks; however, they warn of a possible decline in the diversity of the upcoming generations. For this reason, incorporation of new breeders is recommended (Souza et al., 2018a) in order to neutralize the effects of genetic drift and to improve genetic diversity indexes. As emphasized by Santos et al. (2016), an apparent solution would be to increase the gene flow between stocks and natural populations or that between other *C. macropomum* stocks, being careful to base the incorporation of gene flow on genetic analysis. This could increase the genetic variability of isolated broodstocks, as in the present case, through the incorporation of new genetic material. Furthermore, knowing that the selection of breeders with better phenotypic characteristics is common and sometimes unavoidable for reproductive units, constant genetic monitoring could help in the implementation of measures that could keep the rate of inbreeding under control.

Future studies should be conducted in order to evaluate whether the present inbreeding is affecting zootechnical parameters such as growth rate and fertility in these stocks. The data generated will help in making decisions regarding the management of fish farm activities, since elucidation of the genetic relationships between breeders will allow for better control of mating with the incorporation of new breeders.

Conclusion

Moderate genetic diversity is present within the studied stocks; however, a high heterozygote deficit evidenced by the F_{IS} coefficients was found. Moderate genetic differentiation (F_{ST}) was been found between broodstocks. The incorporation of new breeders based on genetic analysis is recommended in order to increase gene flow within broodstocks and to reduce the rate of inbreeding.

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