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Genetic characteristics of Tambaqui broodstocks in the state of Rondônia, Brazil: implications on production and conservation

Caracterização genética de estoques de Tambaqui do Estado de Rondônia (Brasil): implicações na produção e na conservação

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Abstract

In recent years, the genetic monitoring of broodstocks in fish farming has been greatly highlighted due to its importance in the management and improvement of their production and conservation. Current study evaluates the genetic diversity of four Tambaqui broodstocks (*Colossoma macropomum*) in the state of Rondônia (Brazil) and discusses activities towards the species´s correct production management and conservation. Nine primers were employed to analyze 94 specimens from four fish farms in the municipalities of Ji-Paraná (JP), Ouro Preto do Oeste (OP), Presidente Médici (PM) and Rolim de Moura (RM). Differences in the frequency of 38 fragments, with an exclusive fragment in JP and OP, were reported. High polymorphism (52.40 to 64.60%) and Shannon Index (0.313 to 0.382) rates were observed. The analysis of molecular variance (AMOVA) demonstrated that most variation is within each stock. The identity and genetic distance between the groups ranged between 0.927 and 0.954 and between 0.047 and 0.076 respectively, with shortest distance between the OPxPM and JPxRM groups. Genetic differentiation ranged from moderate to high (Fst = 0.081 to 0.179) and the number of migrants per generation was moderate (Nm = 3.83 to 6.24). As a rule, stocks showed high genetic variability and moderate / high differentiation and genetic distance between them. The results allowed direct conservation programs and increase the productivity of this species in fish farming through breeding programs.

Key words: Broodstocks. *Colossoma macropomum.* Fish. Genetic diversity. Genetic variability.

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Resumo

Nos últimos anos, o monitoramento genético de estoques na piscicultura tem recebido grande destaque devido a sua importância no gerenciamento e melhora da sua produção e conservação. Dessa forma, o objetivo deste estudo foi avaliar de forma inédita a diversidade genética de quatro estoques de reprodutores Tambaqui (*Colossoma macropomum*) do estado de Rondônia (Brasil) e através dela, discutir ações que contribuam com o correto manejo produtivo e conservação dessa espécie. Utilizaram-se nove iniciadores para analisar 94 indivíduos de quatro pisciculturas dos municípios Ji-Paraná (JP), Ouro Preto do Oeste (OP), Presidente Medici (PM) e Rolim de Moura (RM). Foram encontradas diferenças nas frequências de 38 fragmentos, com um fragmento exclusivo em JP e OP. Altos valores de polimorfismo (52,40 a 64,60%) e índice de Shannon (0,313 a 0,382) foram observados. A análise de variância molecular (AMOVA) demostrou que a maior parte da variação está dentro de cada estoque. A identidade e distância genética entre os agrupamentos variou de 0,927 a 0,954 e 0,047 a 0,076, respectivamente, com menor distância entre os agrupamentos OPxPM e JPxRM. A diferenciação genética variou de moderada a alta (Fst = 0,081 a 0,179) e o número de migrantes por geração foi moderado (Nm = 3,83 a 6,24). De forma geral, os estoques apresentaram alta variabilidade genética e moderada/alta diferenciação e distancia genética entre eles. Os resultados permitirão direcionar programas de conservação e aumentar a produtividade dessa espécie na piscicultura através de programas de melhoramento genético.

Palavras-chave: *Colossoma macropomum.* Estoque de reprodutores. Diversidade genética. Peixes. Variabilidade genética.

Introduction

During the last years world aquaculture has had great growth and achievements. The 2010 world production was estimated to be over 59 million tons, whereas the 2012 production reached more than 66.6 million tons (FAO, 2014). The 2012 fish production in Brazil totaled 392,493 tons, with the northern region achieving almost 73,000 ton during the same year. The state of Rondônia, with 25,141 tons of fish production, ranks first in the region (IBGE, 2013).

Colossoma macropomum (Characiformes: Characidae), popularly known as Tambaqui, is a species native to the rivers Amazon and Orinoco (ARAUJO-LIMA; GOULDING, 1997; JACOMETO et al., 2010). In Brazil, the Tambaqui ranked second in production, with 88,718 tons in 2012, or 22.6% of all Brazilian fish production, with tilapia (43.1%) ranking first. The state of Rondônia ranks second in the production of Tambaqui (15,650 tons), whereas the state of Mato Grosso ranks first with a production of 18,880 tons (IBGE, 2013). High production, easy reproduction and high commercial value were the factors that boosted production (ARAUJO-LIMA; GOULDING, 1997). In fact, the fish is specially appreciated by river populations (MENEZES et al., 2008).

The characterization of genetic variability is relevant for production management (JACOMETO et al., 2010), establishment and maintenance of improvement (OLIVEIRA et al., 2012) and conservation programs (LOPERA-BARRERO, 2009) as an integral part in the formation, maintenance and correct use of broodstocks. Several studies have revealed that the formation of broodstocks with low genetic variability may cause difficulties related to endogamy cycles, adaptation capacity and survival of offspring (LOPERA-BARRERO et al., 2010a; RODRIGUEZ-RODRIGUEZ et al., 2010; LOPERA-BARRERO et al., 2014) and may affect the genetic potential of the species (MOREIRA et al., 2007). Low genetic diversity is negatively correlated with adaptation potential to changes in environmental conditions (RASHID et al., 2012; LYNCH et al., 1995; LOEW, 2000). Management and conservation strategies may be successfully planned when correct information exists on the genetic composition of the species (MUNEER et al., 2011).

Research for the evaluation of genetic diversity, currently possible with molecular markers, is important for the conservation of the species (PANARARI-ANTUNES et al., 2011). Due to its high capacity to detect polymorphism and its easy and low-cost implementation (LIU; CORDES, 2004), dominant markers have been successfully employed in the characterization of genetic broodstocks and natural fish populations (GOMES et al., 2013; JACOMETO et al., 2010; HALFEN et al., 2012; FERREIRA et al., 2015; HAN et al., 2015).

Due to the importance of the Tambaqui in Brazilian broodstocks and the lack of genetic studies in the region under analysis, current analysis evaluates the genetic diversity of four *C. macropomum* broodstocks in the state of Rondônia (Brazil) and discusses activities that would contribute towards the correct production and conservation management of the species.

Materials and Methods

Biological material from the caudal fin were retrieved from four fishponds in the state of Rondônia, Brazil, precisely from the municipalities of Ji-Parana - JP (10° 52′ 51″ S; 61° 56′ 31″ W, with 25 samples); Ouro Preto do Oeste - OP (10° 42' 42'' S; 62° 15' 18'' W, with 25 samples); Presidente Médici - PM (11º 10' 31" S; 61º 54' 05" W, with 24 samples) and Rolim de Moura - RM (11° 43′ 48″ S; 61° 46′ 47″ W, with 20 samples).

DNA extraction was performed according to methodology by Lopera-Barrero et al. (2008a), namely, 550 µL of lysis buffer (50 mmol L^{-1} Tris-HCl, 50 mmol L^{-1} EDTA, 100 mmol L^{-1} NaCl, 1% SDS) and 200 µg mL^{-1} proteinase K were added in microtube with the fins. Samples were incubated in a warm bath at 50ºC for 12 h. DNA was precipitated with 600 µL of NaCl solution (5 mol L^{-1}) and centrifuged for 10 min at 12,000 rpm. DNA was transferred to different microtubes and precipitated with 700 µL of iced absolute ethyl alcohol and incubated for two hours at -20ºC; they were then washed with 700 µL ethyl alcohol 70%, suspended in 80 µL of TE buffer solution (10 mmol L^{-1} Tris pH 8.1 mmol L^{-1} EDTA) and 30 µg mL^{-1} RNase were mixed. Samples were incubated for 40 min in a warm bath at 37ºC and stored at -20ºC.

DNA was quantified in spectrophotometer Shimadzu UV 1601, wavelength 260 nm, and diluted for a concentration of 10 ng μL^{-1}. DNA integrity was verified in horizontal electrophoresis in agar gel 1% at 70 volts for 60 min, in a buffer solution TBE 1X (500 mmol L^{-1} Tris-HC1, 60 mmol L^{-1} boric acid, 83 mmol L^{-1} EDTA). DNA was marked by ethidium bromide (0.5 µg mL^{-1}) for 30 min and image registered by photographic system EDAS (Kodak 1D Image Analysis 3.5).

Amplification conditions were based on procedures by Williams et al. (1990), with modifications. DNA was amplified in a 15 µL reaction volume by buffer 1X Tris-KCl, 2 mmol L^{-1} of MgCl2, 0.46 µmol L^{-1} initiator, 0.2 mmol L^{-1} of each dNTP, a Platinum unit Taq DNA Polymerase (Invitrogen®, Carlsbad, USA), and 10 ng DNA. DNA had an initial denaturation at 94ºC for 4 min, followed by 40 cycles, with 1 min of denaturation at 94ºC; 30 seconds of annealing of primer at 40ºC, and 2 min extension at 72ºC. Further, a final extension at 72ºC for 7 min was performed. Reactions were amplified in an Eppendorf Mastercycler Gradient. Thirty primers of kits OPA, OPW and OPX (Operon Technologies Ltd., Valencia, USA) were tested, whilst nine with the best definition and reproducibility were selected: OPA02, OPA16, OPW01, OPW02, OPW03, OPW04, OPW08, OPW19 and OPX04 (Table 1).

Amplified products were separated in agar gel 1.5%, while 15 µL of the amplified product and 2 µL of the sample buffer (40% sucrose and 0.25% bromophenol blue) were used in horizontal electrophoresis. A negative sample (without DNA) was added to verify contamination

and amplification issues. Electrophoresis was performed in buffer solution TBE 0.5X (45 mmol L^{-1} Tris-Borato and 1 mmol L^{-1} EDTA) for 4 h, at 70 V. Each quantified and amplified gel was visualized with UV, and then exposed with ethidium bromide (0.5 µg mL^{-1}) for 1 h. Image was photographed with Kodak EDAS (Kodak 1D Image Analysis 3.5, New York, USA).

Table 1. Primer sequence, percentage of puric bases (G+C), number of fragments and base pairs of fragments in *Colossoma macropomus* broodstocks.

Primer	Sequence (5'→3')	% G + C	Fragments	Base pairs
OPA02	TGC CGA GCT G	70	14	320 – 2100
OPA16	AGC CAG CGA A	60	12	280 – 2500
OPW01	CTC AGT GTC C'	60	10	330 – 1300
OPW02	ACC CCG CCA A'	60	08	500 – 1400
OPW03	GTC CGG AGT G	70	06	550 – 1200
OPW04	CAG AAG CGG A	70	05	250 – 750
OPW08	GAC TGC CTC T	60	12	450 – 1900
OPW19	CA AAG CGC TC	60	08	300 – 1250
OPX04	CCG CTA CCG A	70	07	380 – 930
Total	---	---	82	250 – 2500

Fragment size was estimated by comparing DNA Ladder standard 100 bp (Invitrogen®, Carlsbad, USA). The presence or absence of fragments of identical molecular sizes was used to build a similarity matrix based on Jaccard´s similarity coefficient with 1 as the presence of the fragment and 0 as absence.

Shannon genetic diversity index and polymorphic fragment percentage were calculated by PopGene 1.31 (YEH et al., 1999). TFPGA 1.3 (MILLER, 1997) was employed to determine the frequency of fragments by exact test (RAYMOND; ROUSSET, 1995).

Arlequin 3.0 (EXCOFFIER et al., 2005) determined the genetic differentiation between broodstocks by F_{st} estimates (WEIR; COCKERHAM, 1984) and by molecular variance AMOVA (EXCOFFIER et al., 1992). The estimates´ significance level was verified by randomized permutation with 1000 and 10 000 permutations. Broodstocks were grouped two by two, in all possible combinations, to analyze their molecular variance: JP x OP; JP x PM; JP x RM; OP x PM; OP x RM and PM x RM. The program was also employed to determine the number of migrants per generation (Nm).

Greatness of genetic differentiation between broodstocks followed definition by Wright (1978), where F_{st} rates between 0.00 and 0.05; 0.051 and 0.15; 0.151 and 0.25 and > 0.25 respectively show small, moderate, 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)], proposed by Workman and Niswander (1970) where n is the number of specimens in the two groups; k is the number of alleles; s is the number of groups.

Results and Discussion

Variability and genetic structure

The nine selected primers produced 82 fragments sized between 250 bp and 2500 bp. Total number of fragments varied between five (OPW04)

and 14 (OPA02). Biggest fragment (2500 bp) was obtained by the amplification of primer OPA16, whilst the smallest one (250 bp) was obtained by OPW04 (Table 1). According to Telles et al. (2001), the number of fragments obtained is more important than the primers used. This suggests that 50 fragments are sufficient to obtain reliable genetic variability rates, or rather, the number of fragments obtained in current analysis revealed a reliable estimate of genetic variability of broodstocks, Similar results were obtained by Lopera-Barrero et al. (2010b) who observed 90 fragments with nine primers when they characterized the genetic variability of *Brycon orbignyanus* stocks. Similarly, Lopes et al. (2009) reported 100 fragments with 10 primers when calculating the genetic variability of *C. macropomum* stocks.

A frequency difference ($p<0.05$) in 38 out of the 82 fragments was reported. Low frequency fragments (less than 0.100) in OP (OPW19: 700 bp) and PM (OPW01: 430 bp and 1300 bp; OPW19: 700 bp) were registered. On the other hand, 41 excluded fragments were reported in the four broodstock groups (JP: 9; OP: 8; PM: 11; RM: 13) (Table 2). Excluded fragments may have been triggered by loss of genetic variability due to management conditions in captivity or to founding effect (genetic variability by which the stocks were formed). Trend is mainly verified in OP and PM stocks where low frequency fragments were reported. In spite of these conditions, limiting fragments (fragments with frequency 1000) were also reported in the four stocks (JP: 3; OP: 9; PM: 3; RM: 3) and an exclusive fragment in JP (OPW04: 280 bp) and OP (OPW19: 800 bp). The above revealed high genetic variability within each stock. If the stock is formed by a few specimens (founding effect) or if there is a reduced number of specimens (bottleneck effect), genetic variability may decrease and cause low or even the absence of fragments (JACOMETO et al., 2010). However, it may not be stated that these two effects affected frequencies since there is no information either on reproduction management within each fish culture or on the origin of these specimens.

Genetic variability rates, calculated by Shannon´s genetic variability index (SI) and by the percentage of polymorphic fragments (PF) showed that there was high intra-population variability, and thus corroborating limiting fragments. In comparative terms, there was a greater variability rate in JP, followed by PM, RM and OP (Table 3). Similar results were reported by Lopera-Barrero et al. (2008b) in *Brycon orbignyanus* broodstocks where an offspring with six excluded fragments was reported, or rather, loss of genetic variability when compared with broodstock, but with high intra-population genetic variability. Povh et al. (2008) also reported four excluded fragments in *Piaractus mesopotamicus* broodstocks, or rather, a lower genetic variability when compared with natural population. However, the intra-population genetic characterization was high.

Genetic variability in current study shows that, in spite of captivity conditions and artificial selection to which stocks are normally exposed, the correct reproduction managements were made. According to Moreira et al. (2007) and Povh et al. (2009), inadequate managements in a fish culture may cause loss of genetic variability and endogamy mainly due to the mating of kin specimens. Lopes et al. (2009) also analyzed broodstocks from towns in the state of Rondônia and reported high intra-population genetic variability (Boa Esperança: IS = 0.469 and %FP = 77%; Vale Verde: IS = 0.440 and %FP = 75%), attributed to adequate reproduction management.

Table 2. Characterization of base pairs and frequency of fragments with significant rates by exact test (p<0.05) for *Colossoma macropomum* broodstocks.

OPA02					
bp	JP	OP	PM	RM	P
2100	0.236	1.000	0.646	0.134	0.001
1900	---	1.000	1.000	0.408	0.000
1650	0.110	---	0.110	0.329	0.000
1500	0.209	---	0.460	---	0.000
750	0.388	0.717	---	0.684	0.000
700	---	---	0.592	0.329	0.000
580	0.264	0.717	---	0.684	0.000
530	---	1.000	0.500	1.000	0.000
OPA16					
bp	JP	OP	PM	RM	P
1600	0.248	0.622	0.264	---	0.000
850	0.792	0.564	1.000	0.452	0.000
400	0.341	0.127	---	0.163	0.000
280	0.277	1.000	1.000	0.225	0.000
OPW01					
1300	0.323	0.354	0.087	---	0.000
800	0.796	0.388	0.323	---	0.000
490	0.796	0.592	0.183	0.580	0.000
430	0.423	---	0.087	0.126	0.000
390	0.544	1.000	---	---	0.000
330	0.543	0.323	0.264	0.657	0.000
OPW02					
1400	---	0.434	0.209	---	0.000
950	0.460	---	---	1.000	0.000
850	0.646	0.553	0.544	0.258	0.000
600	1.000	1.000	0.500	---	0.000
OPW03					
800	0.510	---	---	1.000	0.000
720	---	0.800	---	0.452	0.000
650	1.000	1.000	0.544	---	0.000
OPW04					
280	0.106	---	---	---	0.000
OPW08					
1750	0.460	1.000	---	0.553	0.002
600	0.711	0.510	0.134	0.684	0.000
530	---	0.175	0.158	0.452	0.002
OPW19					
1050	---	0.800	0.460	0.452	0.000
800	----	0.128	---	---	0.000
700	0.128	0.083	0.021	0.452	0.000
OPX04					
930	---	0.337	0.646	0.684	0.000
750	0.434	1.000	0.646	0.194	0.000
600	1.000	---	0.592	---	0.000
530	0.151	0.434	---	---	0.002
380	0.400	0.400	0.711	---	0.001

p = probability <0.05.

AMOVA results show that the greatest variation between groupings was reported within and not among stocks. This fact suggests that possibly some specimens of each stock are very similar and evidence their common ancestry (Table 4). In fact, F_{st} revealed consanguinity among groups with moderate (JP x OP; JP x RM; OP x PM) and high (JP x PM; OP x RM; PM x RM) genetic differentiation between stocks, according to classification by Wright (1978), with genic flow (from 3.83 to 6.24).

Table 3. Number of specimens (N), Shannon Index (SI) and percentage of polymorphic fragments (PF) for *Colossoma macropomum* broodstocks.

Stocks	N	SI	PF
JP	25	0.382	64.6
OP	25	0.313	52.4
PM	24	0.333	58.5
RM	20	0.321	53.7

JP: Ji-Paraná; OP: Ouro Preto do Oeste; PM: Presidente Médici; RM: Rolim de Moura.

Table 4. Analysis of molecular variance (AMOVA), variation source (FV), sum of squares (SMQ), coefficient of variation (CV) and variation percentage (%V) for different groupings in *Colossoma macropomum* broodstocks.

Groupings	FV	SMQ	CV	%V
JP x OP	E.L	21.440	0.61137	9.03*
	D.L	295.480	6.15583	90.97
	Total	316.920	6.76720	100
JP x PM	E.L	42.086	1.40498	15.47*
	D.L	360.873	7.67816	84.53
	Total	402.959	9.08314	100
JP x RM	E.L	20.993	0.64274	8.74*
	D.L	288.540	6.71023	91.26
	Total	309.533	7.35297	100
OP x PM	E.L	18.578	0.52020	8.18*
	D.L	274.382	5.83791	91.82
	Total	292.959	6.35811	100
OP x RM	E.L	25.824	0.96303	17.88*
	D.L	190.220	4.42372	82.12
	Total	216.044	5.38675	100
PM x RM	E.L	34.225	1.29144	17.60*
	D.L	254.025	6.04821	82.40
	Total	288.250	7.33965	100

*P<0.05. E.L. = between stocks. D.L. = within stocks. JP: Ji-Paraná; OP: Ouro Preto do Oeste; PM: Presidente Médici; RM: Rolim de Moura.

Greatest genetic differentiation was reported between stocks OP x RM (Table 5). Although information on the precise origin of stocks is lacking, results presuppose that the four *C. macropomum* broodstocks groups were formed from natural populations, stocks or fingerlings of the same origin, forming a single gene pool.

Table 5. F_{st}, X^2 test for F_{st}, genetic differentiation according to Wright (1978) and number of migrants (Nm) for the different groupings analyzed for *Colossoma macropomum* broodstocks.

Groupings	F_{ST}	Wright	X^2	Nm
JP x OP	0.090*	Moderate	9.000	5.24
JP x PM	0.155*	High	15.190	4.97
JP x RM	0.087*	Moderate	7.830	6.24
OP x PM	0.081*	Moderate	7.938	6.21
OP x RM	0.179*	High	16.110	3.83
PM x RM	0.176*	High	15.488	4.39

*P<0.05. JP: Ji-Paraná; OP: Ouro Preto do Oeste; PM: Presidente Médici; RM: Rolim de Moura.

Productive and conservation management

High genetic variability in current study demonstrates correct reproduction management in the fish cultures under analysis. However, further studies should be undertaken on the offspring of these specimens to discover whether high variability will be maintained in the next generations. Decrease in genetic variability through deficiencies during the formation of stocks and through inadequate reproduction management may enhance endogamy cycles and low adaptability and survival of offspring (FROST et al., 2006; ORTEGA-VILLAÍZAN ROMO et al., 2006; GARDNER et al., 2010). In any conservation program (LOPERA-BARRERO, 2009), improvement program (OLIVEIRA et al., 2012) or fish production procedures, genetic monitoring of broodstocks is required to determine whether these specimens effectively contributed with their specific genetic potential.

Management procedures should be undertaken to avoid consanguineous mating that would cause endogamy processes. This will rarely occur if good reproduction management (adequate broodstock selection, mating, stock formation, introduction of new broodstocks and others) is posited (BARROSO et al., 2005; MACHADO-SCHIAFFINO et al., 2007). The introduction of new broodstock from different natural populations or stocks is usually employed by breeders to increase the genetic variability of broodstocks (LOPERA-BARRERO et al. 2008b), even though the method is not needed in the broodstocks analyzed since they already had high genetic variability.

Recent studies with dominant molecular markers to evaluate the genetic variability of *C. macropomum* (LOPES et al., 2009; JACOMETO et al., 2010) or of other species (ALMEIDA et al., 2013; GOMES et al., 2013) have provided satisfactory results through a highly important and efficacious technique for the genetic characterization of specimens, populations and stocks.

Results revealed stocks with high genetic variability, with highest rates in JP and PM. Analysis of groupings showed moderate and high genetic differentiation among the stocks, with high genetic variation between OP x RM stocks. The assessment of the genetic variability of stocks is an important tool for reproduction management in fish culture since it directs the activities of important management for production and conservation and new possibilities in the use of genetic improvement programs.

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