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Genetic diversity of *Salminus brasiliensis* (Characiformes: Characidae) collected in the passage ladder of the Canoas I hydropower plant in the Paranapanema River, Brazil

Diversidade genética de *Salminus brasiliensis* (Characiformes: Characidae) coletados na escada de transposição da Hidrelétrica de Canoas I, rio Paranapanema, Brasil

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Abstract

The aim of this study was to evaluate the genetic diversity of *Salminus brasiliensis* collected three times in the passage ladder of the hydropower plant Canoas I, in the Paranapanema River (Brazil). Fish samples were collected on 14 (CI14), 18 (CI18) and 25 (CI25) February 2008. Eight primers using RAPD technique were evaluated. Seventy-nine in 105 fragments amplified using these primers were polymorphic fragments (75.2%), 32 had frequencies with significant differences ($P<0.05$), 10 had low frequencies, 25 were excluded, and four were fixed fragments. One exclusive fragment was found in the CI14 sample. High values for polymorphic fragments and genetic diversity index of Shannon were observed for the CI14 and CI18. Low ancestry levels among the groups were indicated by the F_{ST} values that indicated high genetic differentiation. In all the three groups, the estimates of the number of migrants by generation (Nm) indicated low levels of gene flow. Most of the genetic variation was found within the groups through the AMOVA analysis, which was confirmed by the results of the identity and genetic distance. The results indicate high variability within the groups and genetic differentiation among them.

Key words: Dourado, fish transposition, genetic variability, RAPD, restocking programs

Resumo

O objetivo do presente estudo foi avaliar a diversidade genética de *Salminus brasiliensis* coletados em três períodos diferentes na escada de transposição da hidrelétrica de Canoas I no rio Paranapanema

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(Brasil). Os peixes foram coletados no dia 14 (CI14), 18 (CI18) e 25 (CI25) do mês de fevereiro de 2008. Foram avaliados oito iniciadores com a técnica de RAPD. Dos 105 fragmentos amplificados pelos iniciadores, 79 foram polimórficos (75,2%), 32 tiveram diferenças significativas ($p<0,05$) na frequência, 10 fragmentos tiveram baixa frequência, 25 fragmentos foram excluídos e quatro fragmentos foram fixados. Um fragmento exclusivo foi encontrado em CI14. Observaram-se altos valores de porcentagem de fragmentos polimórficos e índice de diversidade genética de Shannon em CI14 e CI18. O valor de F_{st} mostrou que existe uma baixa ancestralidade entre os agrupamentos sugerindo uma alta diferenciação genética entre os grupos amostrados. O valor de número de migrantes por geração (Nm) calculado foi baixo nos três grupos, sendo considerado como baixa presença de fluxo gênico. De acordo com a AMOVA, a maior parte da variação genética está dentro de cada grupo e não entre os grupos, sendo confirmado com as estimativas da identidade e da distância genética. Os resultados deste estudo indicam que existe uma alta variabilidade genética e diferenciação genética entre os grupos amostrados.
Palavras-chave: Dourado, programas de repovoamento, RAPD, transposição de peixes, variabilidade genética

Introduction

The Paranapanema River with a basin of 100,800 km² is part of the border line between the states of Paraná and São Paulo, and one of the most important left-bank tributary into the Parana River (BRITTO et al., 2003).

Recently, there has been in the Paranapanema River a decline in the population of various wild fishes like the dourado (*Salminus brasiliensis*). The main factors responsible for this decline have been the intense deforestation in the river banks, excessive fishing of juveniles, drainage of nearby lagoons, the regulatory hydrological regimen, the water pollution, and the introduction of exotic fishes (AGOSTINHO; THOMAZ; GOMES, 2005; POVH et al., 2008, LOPERA-BARRERO, 2009, VIANA et al., 2010). Moreover, the Paranapanema River has 10 hydropower plants as the Canoas I that changed the original river flow into a sequence of water reservoirs (LEUZZI et al., 2004). These dams have broken the migratory path, interfered with the life cycle of numerous aquatic organisms, and produced major changes in ecosystems (AGOSTINHO; GOMES; SUZUKI, 2003).

The hydropower plant of Canoas I was built in 1998 (LOPES et al., 2007) and the passage ladder was built within the above scenario to permit an alternative route to the natural flow of the migratory species. However, these ladders have been criticized for their behavior unidirectional (BRITTO; SIROL, 2006), selectivity of the fish (AGOSTINHO et al., 2007a), and their inefficiency in allowing downstream return by adults, eggs, and larvae (AGOSTINHO et al., 2007b). Therefore, even with a fish ladders, a dam can restrict the gene flow between fish populations located up- and downstream of the barrier, likely leading to interpopulational structuring (ESGUÍCERO; ARCIFA, 2010).

Another decision that has made possible to preserve the fish fauna is the regional development of restocking programs (POVH et al., 2010). Although they have been releasing fish for about three decades in Brazil, risks associated with its failure of stimulating the population size (ARAKI; SCHMID, 2010), increase the ecological competition between wild and captive-bred individuals (ELDRIDGE; NAISH, 2007), impact in other species in the ecosystem (ARAKI; COOPER; BLOVIN, 2009) and reduce the genetic diversity and population viability of wild stocks (AGOSTINHO; THOMAZ; GOMES, 2005; KITADA et al., 2009) are questionable.

In this context, the genetic variation of wild fish populations must be monitored and the correct election of the individuals to be used in the formation of broodstock is a crucial step (BORREL et al., 2007) to increase the preservation of wild fishing resources in restocking programs. Thus, the

RAPD (Random Amplified Polymorphic DNA) has been applied to estimate the genetic diversity of various wild fish populations (PAMPONET et al., 2008; LOPERA-BARRERO et al., 2010a), the stock levels in captivity (POVH et al., 2009) and the fish lines (LUPCHINSKI JUNIOR et al., 2008).

The objective of the present study was to evaluate, using the RAPD technique, the genetic diversity of *Salminus brasiliensis* collected in the passage ladder of the hydropower plant Canoas I in the Paranapanema River, in Brazil.

Material and Methods

Sampling characteristics

Fish samples collected in the passage ladder of the hydropower plant Canoas I (22º 56' SL, 50º 31' WL) on 14 February 2008 were named as CI14, on 18 February they were named as CI18 and on 25 February they were named as CI25. The sampling number collected every time was 81, 54 and seven fish, respectively. Thereafter, a total of 142 samples from their caudal fins were collected. Every caudal sample weighed between 300 and 400 mg. These samples were stored in micro tubes with ethyl alcohol before the DNA analysis. The specimens were maintained at the Estação de Hidrologia e Aquicultura at the Duke Energy International (Salto Grande – SP) to produce offspring to supply the restocking program in the Paranapanema River.

DNA extraction and quantification

Forty caudal fin samples taken from fish collected in the CI14 and CI18 periods, and seven collected in the CI25 were analyzed. The analysis was carried out in the Laboratory of Molecular Biology in the Núcleo de Pesquisa Peixegen/DZO at the Universidade Estadual de Maringá (UEM-PR). The DNA protocol followed the recommendation of Lopera-Barrero et al. (2008). In the micro tubes, was added 550 µL of lyses buffer (50 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl), SDS 1% and 7 µL of the proteinase K (200 µg/mL). These micro tubes were maintained in water bath at 50ºC for 12 hours. The DNA was precipitated with 600 µL of NaCl (5 M) and centrifuged at 12.000 rpm for 10 minutes. The supernatant with the DNA was transferred to other micro tube (600 µL), precipitated with 700 µL of ethyl alcohol and maintained at –20ºC for 1 hour. The DNA was centrifuged and rinsed with 700 µL of 70% ethyl alcohol. The pellets under environmental temperature for about 20 minutes were dried. Thereafter, they were re-suspended in 80 mL of the buffer TE (10 mM Tris pH 8.0 and 1 mM EDTA), treated with 7 µL of RNAse (30 mg/mL) in water bath at 37ºC for 1 hour, and stored at –20ºC in the horizontal freezer.

The DNA was quantified in the Shimadzu spectrophotometer with absorbance at 260 nm. The samples were diluted to the concentration of 10 ng/µL. The DNA quality was checked using agarose gel electrophoresis buffered with TBE 1X (500 mM Tris-HC1, 60 mM boric acid and 83 mM EDTA) at 70 volts for 1 hour.

Amplification and DNA electrophoresis

The genomic DNA was amplified in the reaction volume of 15 ml using the buffer Tris-KCl 1X (Tris-HCl 20 mM pH 8.4 and KCl 50 mM), 2.5 mM $MgCl_2$, 0.46 mM primer (oligonucleotides), 0.2 mM from every dNTPs, one unit of Taq DNA Polymerase and 10 ng of DNA. The RAPD reactions were amplified in the "Eppendorf Mastercycler® Gradient" thermocycler, programmed to 40 cycles, initial step of denaturation at 92ºC for 4 minutes and a final step of extension at 72ºC for 5 minutes. Every cycle consisted of 40 seconds at 92ºC, 1.5 minutes at 40ºC and 2 minutes at 72ºC.

60 primers from the Operon Kit (Operon Technologies Inc. in Alameda, California, USA) were evaluated. However, only eight selected primers with a good standard to the amplification were used. The product from the amplification

was separated in agarose gel at 1.4%. 15 ml of the amplified product and 2 ml of the sampling buffer (40% sucrose and 0.25% bromophenol blue) were used in horizontal electrophoresis. The electrophoresis was carried out at 70 volts for 4 hours (3 V/cm) using the buffer TBE 1X (500 mM Tris-HCl, 60 mM boric acid and 83 mM EDTA). Every reaction had a negative control (N) where all the previous components but DNA were added to the solution. A bath with ethidium bromide at 0.5 mg/ml for 30 minutes was used to reveal the gel. Thereafter, the gels were photographed using the EDAS system (Kodak 1D Image Analysis 3.5).

Data analysis

Fragments of identical molecular size were used to form the similarity matrix based on the Jaccard coefficients coded as 1 for the presence or 0 for the absence of these fragments. The percentage of polymorphic fragments and the index of genetic diversity of Shannon were obtained from the POPGENE software version 1.31 (YEH; BOYLE; XIYAN, 1999). The software TFPGA 1.3 (MILLER, 1997) was used to estimate the identity and genetic distance (NEI, 1978) among the groups. The frequency of fragments was estimate by the exact test of Raymond and Rousset (1995). The software ARLEQUIN 3.0 (EXCOFFIER; LAVAL; SCHNEIDER, 2005) was used to determine the genetic differentiation using the estimates of the F_{st} (WEIR; COCKERHAM, 1984), the number of migrants per generation (Nm) and the molecular analysis of variance – AMOVA (EXCOFFIER; SMOUSE; QUATTRO, 1992). The molecular variance between the groups was evaluated after combining all of them in the following groups: CI14 x CI18, CI14 x CI25 and CI18 x CI25. The significance of these tests was determined by the random permutation method using from 1,000 to 10,000 permutations. The significance of the F_{st} was tested by the X^2 [$c^2 = 2n F_{st}$ (k-1); GL = (k-1) (s-1)] after Workman and Niswander (1970) in which ***n*** is the number of individuals in two groups, ***k*** is the number of alleles and ***s*** is the number of groups. The magnitude of the genetic differentiation between these groups was based on the Wright (1978) definition as having little differentiation when F_{st} is ranging between 0 and 0.05, moderate when F_{st} is ranging between 0.051 and 0.15, great when the F_{st} is ranging between 0.151 and 0.25 and very great when the F_{st} is above 0.25.

Results

Seventy-nine amplified fragments were polymorphic (75.2%) and 26 were monomorphic (24.8%). The number of clear and reproducible fragments generated per primer ranged from eight (primer OPX12) to 19 (primer OPX09). The biggest fragment (2600bp) was obtained from the primer OPX09 and the smaller (200bp) from the primers OPX09 and OPA02 (Table 1). Third-two in 105 fragments had significant differences in their frequencies ($P<0.05$). The total of ten fragments with low frequency (lower than 0.100) were found in the three groups (CI14 = 2; CI18 = 7; CI25 = 1). Twenty-five were excluded fragments – frequency of 0.000 (CI18 = 5; CI25 = 20), and four were fixed fragments – frequency of 1.000 (CI14 = 1; CI18 = 3) (Table 2).

Table 1. Nucleotide sequence in the primers, percentage of G+C bases, number of fragments (NF), number of polymorphic fragments (NPF), and size of the amplified fragments from the groups of *S. brasiliensis*.

Primers	Sequence (3' → 5')	% (G+C)	NF	NPF	Size (bp)
OPX01	CTG GGC ACG A	70	11	07	550-2500
OPX06	ACG CCA GAG G	70	13	05	400-2072
OPX07	GAG CGA GGC T	70	12	10	600-2072
OPX09	GGT CTG GTT G	60	19	17	200-2600
OPX12	TCG CCA GCC A	70	08	04	700-2072
OPX13	ACG GGA GCA A	60	13	10	400-2072
OPA02	TGC CGA GCT G	70	17	17	200-2072
OPA16	AGC CAG CGA A	60	12	09	300-2500
Total	-	-	105	79	200-2600

Source: Elaboration of the authors.

Table 2. Characterization, size and frequency of fragments with significant values using the exact test (P<0.05) on the groups of *S. brasiliensis*. *P<0.05.

Primer	Size (bp)	CI14	CI18	CI25	p
	2700	0,051*	0,097*	0,302	0,002
	2600	0,157	0,494	0,000*	0,000
OPX01	500	0,189	0,039*	0,000*	0,002
	400	0,173	0,013*	0,000*	0,003
	300	0,189	0,013*	0,000*	0,002
OPX06	2700	0,194	0,000*	0,000*	0,001
	2700	0,051*	0,293	0,000*	0,001
	2500	0,163	0,526	0,000*	0,001
	2072	0,452	1,000*	0,000*	0,000
OPX07	1900	0,106	0,613	0,476	0,002
	1700	0,367	0,726	0,000*	0,001
	1000	0,367	0,842	0,842	0,001
	800	0,225	0,646	0,000*	0,000
	2200	0,553	1,000*	0,000*	0,002
	2072	0,776	0,526	0,000*	0,004
	2000	0,163	0,776	0,842	0,000
OPX09	1000	0,476	0,258	0,000*	0,003
	800	0,311	0,106	0,000*	0,001
	500	0,367	0,553	0,120	0,002
	320	0,258	0,025*	0,000*	0,001
OPX12	2100	0,225	0,000*	0,552	0,002
	2072	0,225	0,000*	0,552	0,002
	1900	0,452	1,000*	0,000*	0,001
OPX13	1100	0,452	0,000*	0,842	0,002
	700	0,163	0,225	0,500	0,002
	500	0,293	0,367	0,613	0,001
OPA02	480	0,209	0,051*	0,000*	0,001
	380	0,194	0,013*	0,000*	0,001
	2700	0,430	0,414	0,000*	0,000
OPA16	2500	0,324	0,134	0,000*	0,000
	1400	1,000*	0,258	0,367	0,000
	1300	0,225	0,000*	0,014*	0,000

Source: Elaboration of the authors.

The percentage of polymorphic fragments (71.43 and 63.81%) and the index of Shannon (0.382 and 0.287) were higher in the CI14 and CI18 groups than in the CI25 (33.33% and 0.174). These results indicate a higher genetic variability in the first two groups.

The F_{st} showed low ancestry between CI14 x CI18, CI14 x CI25 and CI18 x CI25. These results suggest a very high and a high genetic differentiation among the groups. Corroborating this hypothesis, the Nm was low in all the groups suggesting a low gene flow. The higher value of Nm between CI14 and CI18 was 0.372 individuals per generation (Table 3).

According to the analysis of molecular variance (AMOVA), most of the genetic variation occurs within every group of *S. brasiliensis* (62.81, 72.73 and 80.17%) unlike between them (37.19, 27.27 and 19.83%). These results were confirmed by the estimates of the identity and the genetic distance (Table 4).

Table 3. F_{st}, X^2 test for the F_{st}, genetic differentiation according to Wright (1978) and number of migrants (Nm) in the different groups of *S. brasiliensis*.*P<0.05.

Groups	Number of fish	F_{st}	Wright	X^2	Nm
CI14 x CI18	80	0.372*	Very high	59.52	1.68
CI14 x CI25	47	0.273*	Very high	25.66	2.46
CI18 x CI25	47	0.198*	High	18.61	4.35

Source: Elaboration of the authors.

Table 4. Molecular analyses of variance (AMOVA), genetic distance (D) and genetic identity (I) for the different groups of *S. brasiliensis*. B.G = between group; W.G = within group *P<0.05.

Groups	Source of variation	Sum of squares	Variance components	% of variation	D	I
CI14 x CI18	B.G	86.515	6.3596	37.19*	0.067	0.935
	W.G	483.357	10.741	62.81		
	Total	569.872	17.100	100		
CI14 x CI25	B.G	163.725	3.8372	27.27*	0.075	0.928
	W.G	798.350	10.235	72.73		
	Total	962.075	14.072	100		
CI18 x CI25	B.G	35.506	2.2251	19.83*	0.143	0.867
	W.G	404.707	8.9935	80.17		
	Total	440.213	11.218	100		

Source: Elaboration of the authors.

Discussion

The RAPD analysis was appropriate to evaluate the genetic diversity within and between the groups of *S. brasiliensis* collected in the passage ladder of the UHE of Canoas I. The main problems towards the dominant performance of this technique (BENTER et al., 1995) were overcome through the amplification tests and standardization of samples carried out in conjunction with the negative controls in the different amplifications.

The percentage of polymorphic fragments and the Shannon index showed high genetic variability for the CI14 and CI18 groups unlike for the CI25. In fact, the results from the CI25 could have been

influenced by the low number of samples collected (seven fish). Therefore, there are high genetic variability within every group despite the low frequency of fragments and a significant number of excluded fragments (Table 2). These results for the wild populations are important since that the presence of heritable variations makes the fish skillful and able to reproduce and survive under selection pressure (LOWE; HARRIS; ASHTON, 2004; RAMOS, 2007). In contrast, Lopes et al. (2007) reported a lower percentage of polymorphic fragments (42.19%) for *S. brasiliensis* also collected in Canoas I, but they stated that this level was still appropriate for these wild populations.

The high genetic variability observed in the present study demonstrate that despite the restocking program of the Estação de Hidrologia e Aquicultura of the Duke Energy International, the genetic diversity of this wild population is still displaying high values. These results may indicate a higher adaptation of this species to the environmental conditions in this ecosystem (LOPERA-BARRERO et al., 2010a). However, was unable to determine whether these sampled groups are formed from individuals from previous releases of offspring. Thus, a broad sampling from wild populations in conjunction with the analysis of the fish stocks used in these programs throughout reproductive period, as well as investments in the synthesis of specific microsatellite markers that yet does not exist would be more appropriate to prove that fish collected in the passage ladder came from these programs. Moreover, this approach will confirm that the restocking programs have been effective in the preservation of the wild fish populations. As these current samples of fish will form a new stock for attending the requirement of these programs, the next fish stock will have a broad genetic base to produce offspring without inducing unnecessary genetic risks to the wild populations.

Conversely, the decline in the genetic variability may induce problems of adaptability and survivorship of these offspring, and negative influences on the ecosystem (LOPERA-BARRERO et al., 2010b). In a worst scenario, species can be extinct (AGOSTINHO; THOMAZ; GOMES, 2005). Thus, all the fish released during restocking programs should represent the wild populations (LOPERA-BARRERO, 2009), and both must have their genetic analysis evaluated (RODRIGUEZ-RODRIGUEZ et al., 2010).

The presence of the exclusive fragment in CI14 indicates that the groups tend to differentiate. This tendency was verified by the F_{st} values ($P<0.05$) which characterized the differentiation as very high (CI14 x CI18 and CI14 x CI25) and high (CI18 x CI25). The number of migrants (Nm) also corroborates this differentiation because of its low values that assume the absence of gene flow (Table 3).

Similarly, the AMOVA and the genetic distance showed that the highest fraction of the genetic variation is within all these three groups and not among them. The moderate distance between them are revealing genetic differentiation among the groups or different population structures (Table 4). The variation component within every group indicates that the sample size allowed us to find a large proportion of genetic variability in the wild population collected in the passage ladder of Canoas I. This approach is very important for the management and preservation programs (PAULA, 2006).

The results from the F_{st}, AMOVA and genetic distance observed in this study are different from the report of Lopes et al. (2007) who found low genetic differentiation (F_{st}: 0.014; genetic distance: 0.006) among groups collected in the Canoas I (17 fish collected in March, 2003 and 24 fish collected from November to March, 2004/2005). They concluded that those groups were sampled from a single population. Studies with other migratory fish as *Leporinus elongatus* (RAMOS, 2007), *Prochilodus lineatus* (PAULA, 2006) and *Piaractus mesopotamicus* (LOPERA-BARRERO et al., 2010a) collected in the same passage ladder also showed a single population structure.

The *Salminus brasiliensis* that reproduces during the rainy season has its reproductive migratory performance from October to March which is the hottest period of the year (MACHADO, 2003). Therefore, the sampling in three different times when the highest migratory populations may be between the middle and the end of the reproductive period could have induced this genetic structure that differs from previous reports in the same place. The Dourado is a long-distance migratory species (FEITOSA et al., 2004; FREITAS, 2010) that covers from 100 to 1440 km (BONETTO et al., 1971; SVERLIJ; ESPINACH ROS, 1986), and this performance permit this large migratory period within the reproductive stage.

Another factor that could have had influence on the genetic structure was the sampling place because Dias (2003), who evaluate the genetic diversity in eight locations in the middle Paranapanema between October 1993 and October 1995, stated that the complex of Canoas I had the highest relative frequencies of *S. brasiliensis* (0.5%). Similarly, Britto and Sirol (2006), who evaluated four consecutive periods (2000/2001; 2001/2002; 2002/2003; 2003/2004) in the passage ladder of Canoas I, reported increases in the percentage of Characiformes in all of them, and increases in other migratory populations comprised by medium- and big-size fish in 2001/2002 (58.73%) and 2002/2003 (88.96%). These responses corroborate the present study in which a large number of *S. brasiliensis* were collected in a short period (CI14: 81 fish; CI18: 54 fish). Small sample size (CI25: 7 fish) was collected a only at the end of the reproductive period.

The decreasing order of the sample size along these sampling periods (CI14 = 81, CI18 = 54 and CI25 = seven fish) was also reported for this same species by Lopes et al. (2007). Similarly, Lopera-Barrero et al. (2010a) reported from the complex Canoas I and Canoas II a similar decline in the population of *Piaractus mesopotamicus*. These results were attributed to the dam effects and the presence of depleted downstream populations. Nonetheless the quantity of 135 fish collected in the first two periods of five days, the current results permitted us to state that the ladder has been an efficient passage for fish to go up the dams in the middle Paranapanema River. Otherwise, more research is still necessary to determine the efficacy of these ladders on the natural reproduction of migratory fish species as the *S. brasiliensis* because Lopes et al. (2007) stated that the migratory species are not reaching the reproductive process after transposing the Canoas complex.

Conclusions

In summary, the current results indicated high variability within the population groups and genetic differentiation among them. The fish stocks from these samples represent a large genetic base that will allow the fish technicians to release juveniles without genetic risks to wild populations. Genetic monitoring using the molecular markers as the RAPD or microsatellites on the wild populations, brood stocks and progenies that have participation in restocking programs is fundamental to avoid declines in the genetic variability, prevent its effects on the wild populations, and allow for insights into the preservation, management and reproduction of the *S. brasiliensis*.

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