

## Genetic diversity of migratory fish populations of the Rio Grande Reservoir (São Paulo, Brazil)

### Diversidade genética de populações de peixes migratórios do reservatório do Rio Grande (São Paulo, Brasil)

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#### Abstract

In recent years, anthropogenic factors such as pollution, overfishing, and construction of hydroelectric plants have significantly impacted natural fish populations. Research focusing on genetically evaluation of these impacts is necessary to objectively target conservation programs. The aim of this study was to evaluate the genetic diversity of Curimba (*Prochilodus lineatus*), Pacu (*Piaractus mesopotamicus*), and Piracanjuba (*Brycon orbignyanus*) populations from the Água Vermelha Reservoir, Rio Grande-SP. Microsatellite loci were amplified, producing 56, 24, and 26 alleles for the populations of the three species, respectively. The number of alleles per locus ranged from three to ten for *P. lineatus*, two to five for *P. mesopotamicus*, and two to four for *B. orbignyanus*. The observed heterozygosity ( $H_o$ ) was higher in the *P. lineatus* population (0.547), relative to the *P. mesopotamicus* and *B. orbignyanus* populations (0.473 and 0.527, respectively). The mean values of  $H_o$  were lower than the average expected heterozygosity ( $H_e$ ) in the three species, being corroborated by the positive inbreeding coefficient ( $F_{is}$ ). Deviations from the Hardy-Weinberg equilibrium (HWE) were found in five, three, and two loci for *P. lineatus*, *P. mesopotamicus*, and *B. orbignyanus*, respectively. Wilcoxon tests revealed recent bottlenecks in the three species, evidenced by a significant excess of heterozygotes ( $p < 0.05$ ) detected only in the Infinite Allele Model (IAM). In conclusion, adequate genetic variability was observed in the three populations with the presence of heterozygous deficits.

**Key-words:** *Brycon orbignyanus*. Conservation. Genetic variability. Microsatellites. *Piaractus mesopotamicus*. *Prochilodus lineatus*.

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## Resumo

Nos últimos anos, fatores antrópicos como poluição, sobrepesca e construção de hidrelétricas têm impactado significativamente as populações naturais de peixes. Pesquisas que permitam avaliar geneticamente esse impacto são necessárias para orientar objetivamente programas de conservação. O objetivo do presente estudo foi avaliar a diversidade genética de populações de Curimba (*Prochilodus lineatus*), Pacu (*Piaractus mesopotamicus*) e Piracanjuba (*Brycon orbignyanus*) provenientes do reservatório de Água Vermelha, Rio Grande-SP. Foram amplificados loci microssatélites que produziram 56, 24 e 26 alelos para as populações das três espécies, respectivamente. O número de alelos por locus variou de três a dez para *P. lineatus*, dois a cinco para *P. mesopotamicus* e dois a quatro para *B. orbignyanus*. A heterozigosidade observada ( $H_o$ ) foi mais elevada na população de *P. lineatus* (0,547) em relação às populações de *P. mesopotamicus* e *B. orbignyanus* (0,473 e 0,527, respectivamente). A média dos valores de  $H_o$  foram inferiores à média de  $H_e$  nas três espécies sendo corroborado pelo Fis positivo observado. Foi encontrado desvio do equilíbrio de Hardy-Weinberg (HWE) em cinco loci para *P. lineatus*, três loci para *P. mesopotamicus* e dois em *B. orbignyanus*. A análise de *bottleneck* revelou excesso de heterozigotos ( $p < 0,05$ ) pelo teste de Wilcoxon nas três espécies apenas no modelo *Infinite Allele Model* (IAM). Foi observada adequada variabilidade genética nas três populações com a presença de déficit de heterozigotos.

**Palavras-chave:** *Brycon orbignyanus*. Conservação. Microssatélites. *Piaractus mesopotamicus*. *Prochilodus lineatus*. Variabilidade genética.

Reservoirs are constructed in most of the Brazilian hydrographic basins to meet the growing demand for energy. However, they represent a barrier to migratory species and may compromise the population structure of several aquatic species (REVALDAVES et al., 2016). Long-distance migratory fishes are especially affected since, during the reproductive period, they migrate to the spawning sites located upstream and thus require free river stretches (AGOSTINHO et al., 2008). Furthermore, due to the decrease in reproduction and population growth rate, the possibility of decreasing genetic variability by bottleneck effects, genetic drift, and inbreeding increases considerably (LAROCHE; DURAND, 2004).

Previous studies have shown that the fragmentation of rivers by the presence of dams may have negative effects on the genetic structure of Brazilian migratory fish populations (RAMOS et al., 2012; ASHIKAGA et al., 2015; RIBEIRO et al., 2016). However, notably, this is not the only factor that endangers the populations of these fishes, as they may also be affected by overfishing, pollution, river banks deforestation, and habitat destruction, among others (LOPERA-BARRERO et al., 2015,

2016).

In rivers with large numbers of reservoirs, such as the Rio Grande, in São Paulo (SP), Brazil, a decline in migratory fish populations has been detected in the past decades (AGOSTINHO et al., 2016). Among the native species of economic importance in this river, Pacu (*Piaractus mesopotamicus*), Curimba (*Prochilodus lineatus*), and Piracanjuba (*Brycon orbignyanus*) are the major long-distance migratory species (WAGNER et al., 2012; IBGE, 2015). Few studies have evaluated the genetic diversity of these species in the Rio Grande, for example, Lopera-Barrero et al. (2015) and Ribeiro et al. (2015), who evaluated *P. lineatus* and *P. mesopotamicus*, respectively. In addition, there has been no study on *B. orbignyanus* populations in this river. Thus, the objective of the present study was to evaluate the genetic diversity of *P. lineatus*, *P. mesopotamicus*, and *B. orbignyanus* populations in the Água Vermelha Reservoir, Rio Grande, SP.

Sixty-eight caudal fin samples of  $\sim 1$  cm<sup>2</sup> were collected from *P. lineatus* (36 samples), *P. mesopotamicus* (25 samples), and *B. orbignyanus* (7 samples). All samples were obtained from the Água Vermelha Hydroelectric Power Plant

Reservoir (19°52'04"S 50°20'43"W), located in the Rio Grande (São Paulo). Sampling was supervised by the company AES Brasil. The fin fragments were stored in microtubes with 70% alcohol until the analysis. The study was approved by the Committee on Ethics in Animal Experimentation (protocol n° 203/2012 of the State University of Londrina).

DNA was extracted following the NaCl extraction protocol described by Lopera-Barrero et al. (2008). Subsequently, DNA samples were quantified in PICODROP® (Picodrop Limited, Hinxton, UK) and diluted to a final concentration of 20 ng/μL. DNA integrity was evaluated on a 1% agarose gel stained with SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad CA, USA).

The DNA was amplified to a final reaction volume of 15 μL, using 1X of Tris-KCl buffer, 2.0 mM of MgCl<sub>2</sub>, 0.8 μM of each primer (Forward and Reverse), 0.2 mM of each dNTP, one unit (for *P. mesopotamicus* and *B. orbignyanus*) or half unit (for *P. lineatus*) of Platinum Taq DNA Polymerase, and 20 ng of each sample. For *P. lineatus* DNA samples, PCR was performed according to the methodology described by Souza et al. (2018), using eight microsatellite loci: Par12, Par14, Par21, Par43, Par80 (BARBOSA et al., 2006, 2008), PI01, Pli30, and Pli60 (YAZBECK; KALAPOTHAKIS, 2007). For *B. orbignyanus*, PCR was performed according to Castro et al. (2017), using eight microsatellite loci: BoM5, BoM13 (BARROSO et al., 2003), Bh5, Bh6, Bh8, Bh13, Bh16 (SANCHES; GALETTI, 2006), and Par80. Finally, the amplification conditions for *P. mesopotamicus* was according to the methodology described by Ribeiro et al. (2015), using seven loci: Pme2, Pme4, Pme5, Pme14, Pme20, Pme28, and Pme32 (CALCAGNOTTO et al., 2001). The reactions were performed on a Veriti® thermal cycler (Applied Biosystems®, Austin, TX, USA). Subsequently, the amplification products were submitted to electrophoresis on 10% polyacrylamide gel (acrylamide: bisacrylamide - 29:1), denatured in urea (6 M) and ran in 1X TBE buffer (90 mM of Tris-Borate and 2 mM of EDTA) at

180 V and 250 mA for 8 h. Allele visualization was achieved by staining with silver nitrate (BASSAM et al., 1991). The alleles size was calculated using a 100-bp DNA ladder (Invitrogen).

The observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosities, as well as the Hardy-Weinberg equilibrium (HWE) were calculated using the POPGENE 1.31 software (YEH et al., 1999). The inbreeding coefficient or fixation index ( $F_{is}$ ) was calculated by means of the FSTAT 2.9.3 software (GOUDET, 2002). The possible reduction of the effective population size was verified with the Bottleneck 1.2.02 software (CORNUET; LUIKART, 1996), considering the mutation models: IAM (Infinite Allele Model), TPM (Two-phase Mutation Model), and SMM (Stepwise Mutation Model). Statistical significance was evaluated with the Wilcoxon test.

The *P. lineatus* population produced a total of 56 alleles, ranging from 3 (Pli60) to 10 (Pli30) alleles per locus (Table 1). The fragments size ranged from 160 bp (Par12) to 305 bp (Pli30) and was in accordance with the sizes previously reported (BARBOSA et al., 2006, 2008; YAZBECK; KALAPOTHAKIS, 2007). Regarding the *P. mesopotamicus* population, 24 alleles were produced for the seven evaluated loci. The Pme32 locus produced the lowest number of alleles, with only two alleles, while the Pme28 produced the highest, with five alleles (Table 1). Fragment size ranged from 185 bp (Pme5) to 280 bp (Pme28), similar to that found in previous studies (CALCAGNOTTO et al., 2001; POVH et al., 2010). Finally, 26 alleles were obtained in the *B. orbignyanus* population, from 2 (Par80) to 4 (Bh8, Bh13 and Bh16) alleles per locus (Table 1), with fragments size ranging from 90 bp (BoM13) to 225 bp (Bh5). It is important to emphasize that, for this species, heterologous primers of related species were used. These primers were previously tested and produced good amplification and reproducibility standards, agreeing with earlier research (CASTRO et al., 2017).

Mean  $H_o$  values were lower than the mean  $H_e$  values in the three species, being corroborated by the observed positive  $F_{is}$ . Mean  $F_{is}$  was higher for *P. lineatus* (0.384), where with all loci presenting heterozygous deficits, and lower for *B. orbignyanus* (0.044), with only five loci showing heterozygous deficits. Deviations from the Hardy-Weinberg equilibrium ( $p < 0.05$ ) were observed in five (Par12, Par14, Par21, Pli30, and Pli60), three (Pme5, Pme14 and Pme20), and two (BoM13 and Bh13) loci for *P. lineatus*, *P. mesopotamicus*, and *B. orbignyanus*, respectively (Table 1). This deviation may have been caused by inbreeding, bottleneck

effects or a combination of both. The Bottleneck analysis showed heterozygotes excess ( $p < 0.05$ ) in the three populations only in the IAM, with *P. lineatus* presenting the lowest  $p$  value (0.01) in the Wilcoxon test (Table 2). According to Cornuet and Luikart (1996), populations that have experienced a recent reduction in effective population size tend to have an  $H_o$  greater than the  $H_e$  in the equilibrium of mutation and drift. In view of this, it is probable that the *P. lineatus* population was more influenced by recent population reductions and/or inbreeding, which resulted in the deviation of HWE observed in most loci.

**Table 1.** Intra-population diversity indexes in the three fish species captured in the Água Vermelha Reservoir.

<i>Prochilodus lineatus</i>					
Locus	$H_o$	$H_e$	Na	$F_{is}$	HWE
Par12	0.500	0.889	8.0	0.423	0.000*
Par14	0.500	0.693	7.0	0.553	0.009*
Par21	0.357	0.796	6.0	0.469	0.000*
Par43	0.769	0.812	7.0	0.111	0.461
Par80	0.714	0.820	7.0	0.286	0.180
Pli1	0.769	0.859	8.0	0.153	0.388
Pli30	0.615	0.868	10.0	0.380	0.001*
Pli60	0.154	0.520	3.0	0.698	0.018*
Mean	0.547	0.782	7.0	0.384	0.132
<i>Piaractus mesopotamicus</i>					
Locus	$H_o$	$H_e$	Na	$F_{is}$	HWE
Pme2	0.720	0.739	4.0	0.006	0.446
Pme4	0.600	0.659	3.0	0.071	0.186
Pme5	0.280	0.458	3.0	0.376	0.028*
Pme14	0.640	0.638	3.0	-0.024	0.002*
Pme20	0.320	0.409	4.0	0.202	0.000*
Pme28	0.333	0.566	5.0	0.398	0.172
Pme32	0.417	0.337	2.0	-0.263	0.225
Mean	0.473	0.544	3.429	0.109	0.151
<i>Brycon orbignyanus</i>					
Locus	$H_o$	$H_e$	Na	$F_{is}$	HWE
BoM5	0.429	0.670	3.0	0.312	0.128
BoM13	0.143	0.648	3.0	0.763	0.001*
Bh5	0.500	0.682	3.0	0.200	0.227

continue

continuation

Bh6	0.429	0.385	3.0	-0.200	0.955
Bh8	1.000	0.747	4.0	-0.441	0.594
Bh13	0.429	0.582	4.0	0.208	0.040*
Bh16	0.571	0.659	4.0	0.067	0.506
Par80	0.714	0.495	2.0	-0.556	0.197
Mean	0.527	0.609	3.250	0.044	0.331

Ho: observed heterozygosity; He: expected heterozygosity; Na: number of alleles; Fis: coefficient of inbreeding; HWE: Hardy-Weinberg equilibrium;

\* Significant values ( $P < 0,05$ ).

Despite the observed heterozygous deficit, the mean values of Ho were similar to those found by other authors in the Euclides da Cunha and Limoeiro reservoirs from the Rio Pardo, São Paulo (LOPERA-BARRERO et al., 2015) and slightly higher than those found by Carvalho-Costa et al. (2008) in *Prochilodus costatus* populations from the Três Marias Reservoir, Minas Gerais. Similarly, the Ho values of the *P. mesopotamicus* population was in accordance with that found in Pantanal populations of the same species (CALCAGNOTTO; DESALLE, 2009). Finally, the Ho of *B. orbignyanus* was also similar to that found by other studies for natural

stocks (ASHIKAGA et al., 2015) or captive stocks (CASTRO et al., 2017). However, *B. orbignyanus* requires special attention since it is highly sensitive to environmental changes, which makes it endangered in certain regions (ASHIKAGA et al., 2015). Overall, it can be considered that the heterozygosity indexes were satisfactory, indicating the existence of genetic variability in the three populations of this reservoir. However, the results do not rule out the need for continuous genetic evaluations at this site, considering that the observed heterozygote deficit indicates a possible genetic decline in future generations.

**Table 2.** Signal test for heterozygote excess/deficiency in the three fish species captured in the Água Vermelha Reservoir.

Populations	N	IAM <sup>a</sup>		TPM <sup>b</sup>		SMM <sup>c</sup>	
		H <sub>d</sub> /H <sub>e</sub>	P	H <sub>d</sub> /H <sub>e</sub>	P	H <sub>d</sub> /H <sub>e</sub>	P
<i>P. lineatus</i>	36	01/07	0.01*	02/06	0.38	04/04	0.74
<i>P. mesopotamicus</i>	25	02/05	0.04*	02/05	0.29	03/04	1.00
<i>B. orbignyanus</i>	7	02/06	0.04*	03/05	0.38	03/05	0.84

N: Number of individuals per population

<sup>a</sup>Infinite Allele Model <sup>b</sup>Two-phase Mutation Model <sup>c</sup>Stepwise Mutation Model

\* Significant values from the Wilcoxon tests ( $P < 0.05$ ).

The impact of anthropic factors over natural populations has been frequent in recent years (ASHIKAGA et al., 2015), with hydroelectric reservoirs being one of the causes of the reduction of natural fish stocks due to habitat fragmentation. Previous studies have shown that the presence of reservoirs may promote differences in fish genetic

diversity due to the interruption of migration (BARROCA et al., 2012) while the time of dam implantation could influence the structure of these populations (LOPERA-BARRERO et al., 2016; FERREIRA et al., 2017). In addition, overfishing, pollution, and environmental destruction must also be highlighted as important factors that negatively

impact these stocks and could lead to population declines. The Água Vermelha Hydroelectric Plant was inaugurated in 1978 and it is expected that, during the last 40 years of operation, geographic isolation has affected fish populations. However, further studies are needed to ascertain the magnitude of these impacts, as well as to make comparisons with samples collected downstream of the dam in order to ascertain the differentiation and genetic structure of these populations.

In conclusion, adequate genetic variability was observed in the three populations, and the presence of a heterozygous deficit was detected. Further studies are needed to evaluate the likelihood of future reductions in variability and changes in genetic structure.

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