Genetic variability of *Prochilodus lineatus* in artificial and semi-natural reproduction

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**ABSTRACT**

*Prochilodus lineatus* is a South American fish of great economic and social importance that is impacted by high fishing pressure. Restocking programmes have tried to be effective in recovering natural populations; however, genetic and reproductive methodologies are necessary to make feasible the survival of released fingerlings. The genetic variability of *P. lineatus* subjected to semi-natural and artificial reproduction was evaluated. Caudal fin samples from 25 breeders subjected to an artificial reproduction were collected at different sex ratios (1\#:1\$ and 2\#:1\$), and 138 fingerlings originated from these spawning. Similarly, samples were collected from 10 breeders (1\#:1\$) subjected to a semi-natural reproduction, and 70 fingerlings were generated from this spawning. Ten microsatellite loci were evaluated, producing 142 alleles. High interpopulation variability for broodstock and fingerlings was observed in both reproductive systems. A deficit of heterozygotes through the inbreeding coefficient ($F_{is}$) was obtained. Moreover, a deviation in the Hardy–Weinberg equilibrium of the fingerlings in both systems was detected. $F_{st}$ values indicated a moderate genetic differentiation in 1:1 artificial reproduction system and low differentiation in 2:1 seminatural reproduction system. Despite the high genetic variability, genetics analyses in the next generations of fingerlings will be necessary considering the likelihood of a decrease in genetic variability.

**ARTICLE HISTORY**

Received 7 May 2017 Revised 19 July 2017 Accepted 31 July 2017

**KEYWORDS**

Broodstock; conservation; Curimba; microsatellite; restocking programs

**Introduction**

The Curimba (*Prochilodus lineatus*) (Valenciennes, 1837), a migratory fish species of great economic and ecological importance in South America, is present in the Paraná and Paraguay River basins, which include Argentina, Bolivia, Uruguay, Paraguay and several Brazilian states (Castro and Vari 2003). Moreover, Curimba was also recently introduced to aquaculture out of the native range (Kalous et al. 2012).

According to data obtained from the Food and Agriculture Organization (FAO 2016), species of the *Prochilodus* genus lead the list of the main target species of extractive fishing in Brazil. As *Prochilodus* species are easily captured, the fishing of *P. lineatus* in the basins is a common practice of riparian populations as an important commercial activity (Machado and Foresti 2012). Furthermore, anthropogenic factors, such as the construction of hydropower plants and the dumping of pollutants have exerted pressure on the natural populations of this species (Agostinho et al. 2007; Reynalte-Tataje et al. 2013).

Previous studies have reported genetic similarities between geographically distant populations of *P. lineatus* (Sivasundar et al. 2001), possibly influenced by great swimming capacity and its ability to migrate upstream. However, other studies report genetic differentiation could be possibly influenced by hydropower plants (Lopera-Barrero et al. 2016). As an alternative to mitigate these effects, restocking programmes are used in Brazilian rivers as a strategy for the conservation of several endangered species (Agostinho et al. 2007), which include *P. lineatus*. Within this context, genetic monitoring in these programmes is of fundamental importance for the targeting of reproductive activities (Ribeiro et al. 2016).
Different reproductive systems can be used to obtain fingerlings in restocking programmes and can infer differences in the genetic variability of progeny, stress and mortality of broodstocks (Lopera-Barrero et al. 2014). Among the main reproductive models used, the most remarkable are artificial and semi-natural systems. The artificial reproduction system does not need of spawning tanks and increased facility in the management of gametes, however, a higher mortality is generally observed when this technique is used (Reynalte-Tataje et al. 2013) and it can cause a decrease of genetic variability (Lopera-Barrero et al. 2014). In the semi-natural reproduction, better fertilisation and hatching rates, conservation of genetic variability and lower mortality rates of broodstocks are expected compared to the artificial reproduction (Reynalte-Tataje et al. 2013).

Another important strategy adopted in these programmes is the ratio of males and females used for spawning. In Rhomdia quelen, studies have indicated that reproduction with different sex ratios may influence the genetic diversity of the progeny (Goes et al. 2017). However, the effect of different sex ratios and reproductive systems on P. lineatus progeny is not known.

The objective of the present study was to use microsatellite markers to evaluate the genetic variability of different sex ratios of P. lineatus subjected to semi-natural and artificial reproductive systems.

Materials and methods

Reproductive systems

The reproduction of the fish in the artificial and semi-natural reproductive systems was performed at the Hydrobiology Station (21°19’01.2”S 49°47’22.8”W) located in the city of Promissão, State of São Paulo, Brazil. The molecular analyses were performed in the laboratories of the universities located in Londrina and Maringá cities, State of Paraná, Brazil. The methodologies were approved by the Ethics Committee on the use of Animals of the State University of Londrina (CEUA_UEL n. 17156.2012.50).

A total of 35 Curimba breeders were selected from the Hydrobiology Station, and these fish were subsequently divided according to the reproduction system and sex ratio: five males and five females (1:1 ratio) and 10 males and five females (2:1) in the artificial reproduction system; and five males and five females (1:1) in the semi-natural reproduction system. The selection criterion was based on the physical condition of the animals, such as abdominal bulging and hyperaemia of urogenital papilla. A biopsy of each breeder was performed to calculate the hormonal dosage required to induce reproduction. For the induction of reproduction (by hypophysation), the procedure was followed according to the methodology adapted from Pereira et al. (2009).

Broodstocks from the artificial reproductive system were placed separately (males and females) in concrete tanks containing 2 m³ of water. A semen pool (semen from all males) was used for fertilisation of the immediate collected oocytes; the spawning procedure was performed according to the methodology of Lopera-Barrero et al. (2014). The spawning in the semi-natural reproductive system were performed using a procedure identical to the 1:1 artificial system; however, immediately following hypophysation, the broodstocks were placed in circular tanks (4 m diameter and 1.5 m depth) with a forced water circulation system to simulate natural environmental conditions (i.e. river current). Thus, semi-natural spawning occurred between several couples, enabling a random fertilisation process. After spawning and fertilisation, the eggs were orientated towards the collector station through a tube located in a central area of the tank, thereby facilitating egg collection and increasing the suitability of the eggs to cylindrical-conical incubators (captivation of 200L with continuous water flow), where the formation and hatching of the larvae occurred.

DNA extraction and amplification

For DNA extraction, a total of 35 caudal fin samples were collected from broodstocks (for both systems), 69 fin samples were obtained from fingerlings in the artificial system (1:1), 69 fin samples were obtained from fingerlings in the artificial system (2:1) and 70 fin samples were obtained from fingerlings in the semi-natural system. Caudal fin samples were collected from the fingerlings at 90 days after the eggs hatched. DNA was extracted using an extraction protocol containing NaCl (Lopera-Barrero et al. 2008). Subsequently, the DNA quality was determined using agarose gel electrophoresis (0.01). DNA quantification was performed standardising the samples through dilution to a final concentration of 20 ng μL⁻¹.

The amplification was performed in a final reaction volume of 15 μL containing 1× Tris–KCl buffer, 2.0 mM of MgCl₂, 0.8 μM of each primer (Forward and Reverse), 0.2 mM of each dNTP, a half unit of Platinum Taq DNA Polymerase and 20 ng of DNA. Initially, the
DNA was denatured at 95 °C for five minutes, followed by 35 cycles of denaturation at 94 °C for 60 seconds, annealing for 60 seconds and extension at 72 °C for 60 seconds, with a final extension at 72 °C for 20 minutes. A total of 10 microsatellite loci was evaluated: Par12, Par14, Par15, Par21, Par43, Par80 (Barbosa et al. 2006, 2008), PI01, PIi30, PIi43 and PIi60 (Yazbeck and Kalapothakis 2007). The reactions were performed on a Veriti® thermal cycler (Applied Biosystems®, Austin, TX). The amplified samples were subjected to 10% polyacrylamide gel electrophoresis at 180 V and 250 mA for eight hours. The image was submitted to Adobe Photoshop CC (64 Bit) software; the gel electrophoresis was aligned and the allele sizes were calculated using a 100 bp DNA ladder (Invitrogen, Carlsbad, CA).

Statistical analyses
The observed heterozygosity (Ho), expected heterozygosity (He) and the Hardy–Weinberg equilibrium (Hw) were calculated for each locus using POPGENE 1.32 software (Yeh et al. 1999). The AMOVA (analysis of molecular variance) and genetic differentiation (Fst) between parental and progeny were obtained using Arlequin 3.0 software (Excoffier et al. 2005). The classification by Wright (1978) was used, where values between 0.00 and 0.05; 0.051 and 0.15; 0.151 and 0.25 and >0.25 indicate low, moderate, high and elevated genetic differentiation, respectively. The inbreeding coefficient (Fis) was calculated using FSTAT 2.9.3 software (Goudet 2005).

Results and discussion
A total of 142 alleles were found in the 10 amplified loci. The total number of alleles per locus ranged from seven (PIi60) to 20 (PIi30), and the sizes (bp) ranged from 115 bp (Par15) to 390 bp (Par30). The mean number of alleles/locus was 14.2. The mean values for the observed (Ho) and expected heterozygosity (He) were higher in the progeny compared to the parents (broodstocks) in both reproductive systems and characterised by high genetic variability (Hatanaka et al. 2006; Lopera-Barrero et al. 2016). The highest and lowest average Ho values were obtained in the fingerlings from the 1:1 semi-natural system (0.720) and in the broodstocks from the 1:1 artificial system (0.575). The Shannon index values (I) also showed high genetic variability for all groups (Table 1).

The size of and number of alleles per locus was similar to that reported in other studies of the Prochilodus genus (Barbosa et al. 2006; Yazbeck and Kalapothakis 2007; Barbosa et al. 2008). The variability indicated that the broodstocks were formed with individuals that had elevated genetic diversity, enabling the formation of progenies with high genetic variation through spawning and in all reproductive systems.

Within this context, the semi-natural reproductive system produced progeny with the highest genetic variability. Other studies comparing these systems on other species reported similar results. Lopera-Barrero et al. (2014) compared the artificial and semi-natural reproductive systems in spawning of 20 Brycon orbignyanus broodstocks (10♂ and 10♀), and observed greater genetic variability in the progeny from the semi-natural system than from the artificial system (Ho = 0.945 and the Shannon index, I = 0.924; Ho = 0.823 and I = 0.886, respectively). The broodstock ratios in the spawning (1:1 and 1:2) had low influence on the genetic variability of the progeny, considering that a small increase of this variability was observed in all groups. This result suggests that the variability was more related to the initial genetic composition of the parents than to the number of breeders.

A deviation from the Hardy-Weinberg equilibrium (p < .05) was observed for the fingerlings from all systems and sex ratios (Table 1). In restocking.

| Locus | 1:1 artificial reproduction | 1:1 semi-natural reproduction |
|-------|-----------------------------|-----------------------------|
| Mean  | Brood. | Fing. | Brood. | Fing. |
| Ho    | 0.575 | 0.609 | 0.600 | 0.614 |
| He    | 0.716 | 0.775 | 0.824 | 0.859 |
| I     | 1.071 | 1.559 | 1.809 | 1.770 |
| Fst   | 0.250* | 0.310* | 0.280* | 0.281* |
| Hw    | ns     | *     | ns     | *     |

Table 1. Observed Heterozygosity (Ho), expected heterozygosity (He), the Shannon index (I), coefficient inbreeding (Fis), probability test of the Hardy–Weinberg equilibrium (Hw) and genetic differentiation (Fst) with classification by Wright (1978), for breeders and fingerlings of P. lineatus.
programmes only a few couples can produce thousands of descendants that make up a progeny, therefore, the likelihood of the emergence of genetic bottlenecks is evident (Machado-Schiaffino et al. 2007), even more when only part of the broodstock is replaced by animals from other rivers or basins, resulting in a potential genetic bottleneck provoked over generations. In this context, it is important to emphasize that the intrapopulation genetic variability was high in all groups, indicating that despite the heterozygote deficit observed, high variability was maintained in the fingerlings. However, the inbreeding coefficient (Fis) \( p < .05 \) indicated a heterozygotes deficit, particularly in the fingerlings from artificial systems 1:1 and 2:1. These results indicate the likelihood of a decline in genetic variability for the next generation of fingerlings. Before a decline in genetic variability occurs, the replacement of the broodstocks (always based on genetic analysis) is suggested, thus adding a new genetic pool to the composition of the next progenies.

The AMOVA results showed that this genetic variation was higher within the groups than between the groups. The genetic differentiation was moderate for the individuals from the artificial 1:1 group and low for the individuals from the artificial 2:1 and semi-natural 1:1 groups (Table 1). This fact can be justified by a greater reproductive dominance in 1:1 artificial reproduction, causing an unequal contribution of the broodstocks in the genetic composition of the fingerlings. However, the low genetic differentiation in the 2:1 artificial and 1:1 semi-natural groups, may presume a more homogenous contribution of the parents.

According to Frankham et al. (2008), actions that inform the genetic diversity of the released fingerlings in the natural environment are fundamental to direct the reproductive activities in restocking programmes, since the adaptability provided by the genetic variability of the animals that will be released into the river basins must be adequate to enable the reproduction and perpetuation of the species at this site. Our results show that the genetic variability of offspring of *P. lineatus* was high in both reproductive systems and spawning ratios. However, genetic monitoring of the fingerlings that will be obtained in the next generations is evidently required, considering the likelihood of a decrease in genetic variability resulting from elevated inbreeding coefficient values.

**Acknowledgements**

The authors thank AES Tiete for providing the physical structure and the animals involved in the present work.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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