GENETIC IMPLICATIONS OF RESTOCKING PROGRAMS ON WILD POPULATIONS OF STREAKED PROCHILOD

Prochilodus lineatus

ABSTRACT

Genetic diversity of wild and farmed populations is crucial, both for conservation of fish resources and fish culture development. To infer the genetic diversity and population structure of Streaked prochilod Prochilodus lineatus, individuals were sampled between 2007-2009 from four fish farms and from the Upper Uruguay River Basin, both in southern Brazil. Population structure was identified in both farmed and wild individuals through seven microsatellite loci. Bayesian analysis indicated three main groups, including two from fish farms. Pairwise genetic differentiation showed spatial structure between and within wild and farmed populations; however, the sampling design did not allow testing temporal structure according to isolation-by-time (IBT), which means that populations can breed within the same geographic distribution, but reproduce at different times. Cultivated individuals presented lower diversity, allelic richness and effective population size, but higher inbreeding rates, compared to wild populations. These characteristics constitute warning signs against indiscriminate restocking of natural Prochilodus lineatus populations, a species sensitive to fragmented habitats, with farmed fish.

Key words: fishing resources; curimbatá; freshwater fish; population genetics; rebuilding.

INTRODUCTION

Genetic diversity levels and genetic composition of wild populations and farmed fish are fundamental to conservation strategies designed to improve the genetic composition of fish used in restocking projects (Bondioli et al., 2017; Duong and Scribner, 2018). However, reproduction within the confines of a commercial fish farm stands in stark contrast to the complexity of natural reproduction events that occur in large Neotropical rivers, especially in long-migration fish (Ribolli et al., 2016). More specifically, the low number of parents and non-genetic characterization of broodstock in fish farm situations can lead to genetic problems, such as inbreeding and fixation of harmful alleles. These
problems can be aggravated by the use of improper methods during the stages of reproduction like the use of imbalanced broodstock sex ratios and pooling of gametes (Tave, 1999; Ribolli and Zaniboni-Filho, 2009). In addition, domestication can involve selection of only some important alleles for aquaculture, thus losing adaptive characteristics that would be essential to the adaptation of restocked wild fish (Prado et al., 2018).

*Prochilodus lineatus* (Valenciennes, 1836) is a large migratory Characiformes with distribution in the Paraná-Paraguay and Paraíba do Sul River Basins (Castro and Vari, 2004) and the Uruguay River Basin (Zaniboni-Filho and Schulz, 2003). Referred to as streaked prochilod, sábalo, curimba, curimbatá and grumatão, *P. lineatus* is considered a main resource for fisheries in the Paraná River (Sverlij et al., 1993; Baigún et al., 2013) and Uruguay River (Espinach Ros et al., 1998; Schork et al., 2013). Together with other freshwater species, *P. lineatus* is an important economic resource for riverine families, mainly in tropical countries (Allan et al., 2005; Hoeinghaus et al., 2009).

Similar to other migratory species, degradation of natural river waters and damming can impose limits on its otherwise long migration of up to 1,100 km (Espinach Ros et al., 1998), thus posing the most serious threats to *P. lineatus* populations of the Upper Uruguay River Basin. In order to compensate for the decline of natural stocks, restocking practices have been urged by compensation for damming of rivers, imposing fines for environmental damage, or even voluntary restocking, to increase fishing. Notwithstanding such attempts, Brazilian legislation, as regulated by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA; Normative Instruction No. 146), does not concern itself with the genetic characteristics of the broodstock used for repopulation or the genetic composition of individuals used in restocking. *P. lineatus* is known to be a docile, easily handled species that displays dominant reproductive characteristics, high fecundity; therefore, reproduction in hatcheries can be easily achieved in small fish farms through artificial means. With only a couple of broodstock, it is possible to produce thousands of larvae of *P. lineatus* (Viveiros et al. 2010).

However, such key genetic parameters as effective population size (*Ne*) and genetic diversity are fundamental determinants of adaptive potential in a wild population (Hedrick, 2005).

---

**Figure 1.** Map of sampling sites of wild and farmed *Prochilodus lineatus*. Wild individuals from the Upper Uruguay River: DBG (downstream from Barra Grande HPP), DMA (downstream from Machadinho HPP), and DIT (downstream from Itá HPP); fish farm stations (FF): FF1 = São Leopoldo/RS, FF2 = Ijuí/RS, FF3 = Ajuricaba/RS, and FF4 = Chapecó/SC.
Thus, limited genetic diversity and effective population size of broodstock may pose a risk to wild populations restocked under these conditions (Small et al., 2009; Fonseca et al., 2017; Prado et al., 2018). In addition, restocking programs that avoid introduction of genotypes unrepresentative of the augmented population, low genetic diversity and inbreeding can have negative effects (Ward, 2006; Roques et al., 2018).

Therefore, this study aimed to evaluate the genetic consequences of restocking wild populations with farmed fish by comparing of genetic diversity and spatial structure between farmed and wild genetic populations of *P. lineatus* from the Uruguay River Basin, southern Brazil.

### MATERIAL AND METHODS

#### Sampling

Adult individuals of *P. lineatus* were sampled from wild and fish farms of Santa Catarina and Rio Grande do Sul States (Figure 1). Wild individuals were sampled downstream from the Barra Grande Dam (DBG, N = 13), downstream from the Machadinho Dam (DMA, N = 44), and downstream from the Itá Dam (DIT = 49), all situated in the Upper Uruguay River Basin. Farming samples were collected from four fish farms denoted as FF1 (Fish Farm from Ajuricaba/RS, N = 10), FF2 (Fish Farm from Júri/RS, N = 17), FF3 (Fish Farm from São Leopoldo/RS, N = 11), and FF4 (Fish Farm from Chapecó/SC, N = 13).

Non-lethal sampling was performed between 2007 and 2009 in collaboration with the Laboratório de Biologia e Cultivo de Peixes de Água Doce (LAPAD) of the Universidade Federal de Santa Catarina (UFSC) and local fishermen. Fish samples were morphologically identified according to Castro and Vari (2003). A fragment of fin clips was removed from each individual, identified and then preserved in 96% ethanol. Voucher number MZUEL 11729 (deposited in the Ichthyological Collection of Universidade Estadual de Londrina-Parana, Brazil).

#### DNA extraction and amplification

Total DNA purification was performed using a CTAB protocol (2% CTAB, 20 mM EDTA, 0.1 M Tris, 1.4 mM NaCl), followed by a sodium acetate and isopropanol-induced precipitation step (Sambrook and Russell, 2001).

A portion of the mitochondrial large ribosomal subunit (16S) was amplified using primers 16SAR (CCGGCTGTATATCAAAAACAT) and 16SBR (CCGGTCTGAACCTCAGATCCGT) (Kessing et al., 1989). Polymerase chain reactions (PCR) used approximately 10 ng of template DNA, 1 unit of Taq polymerase (GE Life Sciences), 1.5 mM MgCl2 in 20 µL of 1x PCR buffer (GE Life Sciences). Polymerase chain reactions were performed using seven polymorphic microsatellite loci (SSRs) developed for *Prochilodus argenteus* (Carvalho-Costa et al., 2008) and used in *P. lineatus* (Par12 (AAAC)7, Par14 (TGTC)5, Par21 (ATGA)6, Par43 (GA)6(CA)2(CAGA)4(GA)21, Par80 (CT)37, Par82 (CT)27; Barbosa et al., 2006; Barbosa et al., 2008), and one locus developed for *Prochilodus lineatus* (Pi60; Yazbeck and Kalapothakis, 2007). PCR reactions included 15 ng template DNA, 3.6 mM of each starter primer, 200 µM dNTPs, 1.5 µL 10X buffer, 1.5 mM MgCl2, and 1 U Taq DNA polymerase (Invitrogen) in a 15 µL total volume. The amplification conditions were as follows: initial denaturation at 95 °C for 5 min, 35 amplification cycles of 94 °C for 1 min, specific annealing temperature for 1 min (Par12, 54°C; Par14 and Par21, 48°C; Par43, 50°C Par80 and Par82, 52°C; Pli60, 67°C) and 72 °C for 1 min, followed by final elongation at 72 °C for 20 min. Microsatellite amplification products were submitted to 1% polyacrylamide gel electrophoresis and visualized by silver nitrate staining.

#### Data analyses

The amplified 16S sequences were edited with the SEQMAN 7.0 program (DNASTAR Inc.) and aligned with the CLUSTALW algorithm (Thompson et al., 1994) provided with the MEGA 5.1 program (Tamura et al., 2011). Standard nucleotide (π) and haplotype (h) diversity indices were estimated using the DNASp 5.1 program (Librado and Rozas, 2009). Pairwise Kimura 2-parameter distances (K2P; Kimura, 1980) were used to build neighbor-joining (NJ) and maximum likelihood (ML) trees using the MEGA program.

Microsatellite genotyping was performed using the TL 100 program (TotalLab Ltd.). The presence of null alleles was investigated with the MICRO-CHECKER program (Van Oosterhout et al., 2004). GENEPOP, v.1.2 (Raymond and Rousson, 1995) was used to test for departure from Hardy-Weinberg equilibrium (HWE), as well as linkage disequilibrium. Mean number of alleles per locus (A), number of private alleles, mean observed (Ho), and expected (He) heterozygosity values were calculated in GENEALEX 6.5. The allelic polymorphism information content (PIC) was calculated using CERVUS 3.0 (Kalinsky et al., 2007). Allelic richness, inbreeding reactions, were included in all PCR amplifications. Both strands of PCR products were purified with a GFX™ PCR DNA and Gel Band Purification Kit (GE Life Sciences), according to the manufacturer’s instructions, and sequenced in an ABI 3500 automatic sequencer with the same sets of primers as those used for the PCR reaction. All haplotype sequences obtained were deposited in GenBank (Accession Numbers MK312666 and MK312666). Additionally, sequences from GenBank were included in our phylogenetic analyses (*P. lineatus*: Z22696, Meyer et al., 1993; U34024, Ortí and Meyer, 1997; *P. nigricans*: AY788075, Calcagnotto et al., 2005; *P. reticulatus*: HQ171358, Oliveira et al., 2011; *Psectrogaster rhomboides*: FJ944746, and *Semaprochilodus insignis*: FJ944756).
coefficients (FIS; Weir and Cockerham, 1984) and the p-values for heterozygote excess (PL) and deficit (PS) were calculated using FSTAT 2.9.3.2 (Goudet, 2001). The percentage of population assignment into each sampled population was estimated using GENEALEX 6.5, and the effective population size (Ne) for each sampled population was calculated based on the linkage disequilibrium (LD) method (Waples and Do, 2008) using NEESTIMATOR 2.0 (Do et al., 2014).

Overall genetic structure was calculated using the analysis of molecular variance (AMOVA) within and among all populations sampled through permutation tests with 1000 replicates, using ARLEQUIN 3.5 (Excoffier and Lischer, 2010). Pairwise FST values (Weir and Cockerham, 1984) were calculated between wild and farmed sampling populations using FSTAT 2.9.3.2 (Goudet, 2001). To evaluate the population structure between wild and cultivated individuals sampled, we used a Bayesian cluster analysis implemented in STRUCTURE 2.3.3 software (Pritchard et al., 2000; Falush et al., 2003) whereby individuals were assigned to clusters without a priori information, while assuming an admixed model of population structure and correlated allele frequencies. The most likely number of genetic clusters (K) was estimated by six independent runs each for K = 1 to 9 with 600,000 Markov Chain Monte Carlo (MCMC) repetitions and using 300,000 initial interactions as the burn-in period. The optimal value of K was estimated by the Evanno method (AK, Evanno et al., 2005), using STRUCTURE HARVESTER (Earl, 2012), which can be found at “http://taylor0.biology.ucla.edu/structureHarvester/”. The spatial population structure was also investigated to identify clusters of genetically related individuals using Discriminant Analysis of Principal Components (DAPC) available in the adegenet package (Jombart et al., 2010), implemented in R software (R Development Core Team, 2017). Evidence of genetic clusters (K) was examined in DAPC using find.clusters function and Bayesian Information Criterion (BIC). We tested values of K= 1-14, with ten runs at each value of K. The BIC values were visually examined to identify values of K (Jombart et al., 2010). The DAPC function was then executed using this grouping, retaining axes of Principal Components Analysis sufficient to explain >80% of total variance of data.

To guide the best crosses and crosses that should be avoided in order to maintain the genetic variability of progenies, we performed relationship assessment of farmed broodstock using COANCESTRY v.1.0.1.5 software (Wang, 2011).

RESULTS

16S sequencing

After aligning and editing the sequences, the 16S gene was much conserved and presented only one polymorphic site among P. lineatus individuals. The haplotype and nucleotide diversities were 0.325 and 0.0006, respectively. The average nucleotide proportions were 31:22:24:23 (A:T:C:G). Sequences from two individuals retrieved from GenBank were used to confirm the identity of P. lineatus specimens with sequences from two other Prochilodus species. Two species of the Curimatinae sub-family were used as outgroups. Both phylogenetic approaches retrieved trees with similar topologies; hence, only the ML tree is shown. Wild individuals from the Uruguay River formed a monophyletic group that diverged from 0 to 0.002 (K2P; Figure 2).
Microsatellite analysis

The microsatellites used in this study showed no presence of large allele dropouts or other deviations when analyzed by MICRO-CHECKER. Genetic diversity estimates, number of alleles, allelic richness, effective population size, observed and expected heterozygosity and inbreeding coefficient are all shown in Table 1.

**Table 1.** Genetic diversity estimates for wild and farmed *Prochilodus lineatus* populations.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Par12</th>
<th>Par14</th>
<th>Par21</th>
<th>Par43</th>
<th>Par80</th>
<th>Par82</th>
<th>Pli60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FF1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>A</td>
<td>6.0</td>
<td>3.9</td>
<td>4.9</td>
<td>6.5</td>
<td>7.4</td>
<td>7.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Ae</td>
<td>4.9</td>
<td>3.1</td>
<td>4.9</td>
<td>6.3</td>
<td>7.3</td>
<td>5.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Ho</td>
<td>0.300</td>
<td>0.889</td>
<td>0.600</td>
<td>0.900</td>
<td>0.818</td>
<td>0.625</td>
<td>0.909</td>
</tr>
<tr>
<td>He</td>
<td>0.795</td>
<td>0.673</td>
<td>0.795</td>
<td>0.840</td>
<td>0.864</td>
<td>0.820</td>
<td>0.744</td>
</tr>
<tr>
<td>HWE</td>
<td>0.000*</td>
<td>0.020</td>
<td>0.259</td>
<td>0.414</td>
<td>0.102</td>
<td>0.163</td>
<td>0.224</td>
</tr>
<tr>
<td>Fis</td>
<td>0.654*†</td>
<td>-0.267</td>
<td>0.294</td>
<td>0.019</td>
<td>0.100</td>
<td>0.300</td>
<td>0.176</td>
</tr>
<tr>
<td>Pl</td>
<td>0.001</td>
<td>0.955</td>
<td>0.070</td>
<td>0.681</td>
<td>0.290</td>
<td>0.039</td>
<td>0.963</td>
</tr>
<tr>
<td>Ps</td>
<td>1.000</td>
<td>0.179</td>
<td>0.984</td>
<td>0.687</td>
<td>0.922</td>
<td>0.993</td>
<td>0.229</td>
</tr>
<tr>
<td><strong>FF2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>A</td>
<td>5.8</td>
<td>2.0</td>
<td>4.9</td>
<td>6.6</td>
<td>6.2</td>
<td>6.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Ae</td>
<td>5.1</td>
<td>1.9</td>
<td>4.3</td>
<td>6.0</td>
<td>5.8</td>
<td>6.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Ho</td>
<td>0.938</td>
<td>0.765</td>
<td>0.353</td>
<td>0.706</td>
<td>0.706</td>
<td>0.882</td>
<td>0.467</td>
</tr>
<tr>
<td>Ar</td>
<td>5.838</td>
<td>2.000</td>
<td>4.950</td>
<td>6.660</td>
<td>6.297</td>
<td>6.396</td>
<td>5.428</td>
</tr>
<tr>
<td>He</td>
<td>0.803</td>
<td>0.472</td>
<td>0.765</td>
<td>0.834</td>
<td>0.827</td>
<td>0.836</td>
<td>0.791</td>
</tr>
<tr>
<td>HWE</td>
<td>0.863</td>
<td>0.032</td>
<td>0.000*</td>
<td>0.042</td>
<td>0.083</td>
<td>0.723</td>
<td>0.016</td>
</tr>
<tr>
<td>Fis</td>
<td>-0.136</td>
<td>-0.600</td>
<td>0.560*†</td>
<td>0.183</td>
<td>0.176</td>
<td>-0.026</td>
<td>0.438*†</td>
</tr>
<tr>
<td>Pl</td>
<td>0.970</td>
<td>1.000</td>
<td>0.001</td>
<td>0.082</td>
<td>0.082</td>
<td>0.713</td>
<td>0.002</td>
</tr>
<tr>
<td>Ps</td>
<td>0.184</td>
<td>0.020</td>
<td>1.000</td>
<td>0.975</td>
<td>0.979</td>
<td>0.560</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>FF3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>4.9</td>
<td>1.0</td>
<td>4.5</td>
<td>4.6</td>
<td>4.6</td>
<td>5.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Ae</td>
<td>4.4</td>
<td>1.0</td>
<td>2.8</td>
<td>3.8</td>
<td>3.8</td>
<td>3.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Ho</td>
<td>0.900</td>
<td>0.000</td>
<td>0.600</td>
<td>0.900</td>
<td>0.900</td>
<td>1.000</td>
<td>0.143</td>
</tr>
<tr>
<td>Ar</td>
<td>4.900</td>
<td>1.000</td>
<td>4.586</td>
<td>4.620</td>
<td>4.620</td>
<td>5.939</td>
<td>3.000</td>
</tr>
<tr>
<td>He</td>
<td>0.775</td>
<td>0.000</td>
<td>0.640</td>
<td>0.740</td>
<td>0.740</td>
<td>0.730</td>
<td>0.520</td>
</tr>
<tr>
<td>HWE</td>
<td>0.498</td>
<td>NA</td>
<td>0.378</td>
<td>0.051</td>
<td>0.053</td>
<td>0.414</td>
<td>0.021</td>
</tr>
<tr>
<td>Fis</td>
<td>-0.110</td>
<td>NA</td>
<td>0.115</td>
<td>-0.165</td>
<td>-0.165</td>
<td>-0.324</td>
<td>0.760†</td>
</tr>
<tr>
<td>Pl</td>
<td>0.866</td>
<td>NA</td>
<td>0.409</td>
<td>0.924</td>
<td>0.906</td>
<td>1.000</td>
<td>0.023</td>
</tr>
<tr>
<td>Ps</td>
<td>0.426</td>
<td>NA</td>
<td>0.864</td>
<td>0.318</td>
<td>0.330</td>
<td>0.023</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>FF4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>2.9</td>
<td>2.7</td>
<td>2.9</td>
<td>5.2</td>
<td>5.2</td>
<td>5.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Ae</td>
<td>2.9</td>
<td>2.3</td>
<td>2.9</td>
<td>4.7</td>
<td>4.7</td>
<td>4.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Ho</td>
<td>0.167</td>
<td>0.923</td>
<td>0.769</td>
<td>0.923</td>
<td>0.923</td>
<td>0.769</td>
<td>0.462</td>
</tr>
<tr>
<td>Ar</td>
<td>2.998</td>
<td>2.797</td>
<td>2.999</td>
<td>5.288</td>
<td>5.288</td>
<td>5.539</td>
<td>2.000</td>
</tr>
<tr>
<td>N</td>
<td>13</td>
<td>5</td>
<td>10</td>
<td>14</td>
<td>20</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>A</td>
<td>6.9</td>
<td>4.3</td>
<td>6.6</td>
<td>8.4</td>
<td>8.9</td>
<td>10.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Ae</td>
<td>6.8</td>
<td>3.8</td>
<td>6.6</td>
<td>9.9</td>
<td>10.6</td>
<td>16.6</td>
<td>5.9</td>
</tr>
<tr>
<td>Ho</td>
<td>0.535</td>
<td>1.000</td>
<td>0.895</td>
<td>0.775</td>
<td>0.750</td>
<td>0.795</td>
<td>0.591</td>
</tr>
<tr>
<td>He</td>
<td>0.853</td>
<td>0.735</td>
<td>0.849</td>
<td>0.899</td>
<td>0.905</td>
<td>0.940</td>
<td>0.831</td>
</tr>
<tr>
<td>HWE</td>
<td>0.000</td>
<td>0.000</td>
<td>0.535</td>
<td>0.000</td>
<td>0.006</td>
<td>0.006</td>
<td>0.000</td>
</tr>
<tr>
<td>Fis</td>
<td>0.383</td>
<td>-0.352</td>
<td>-0.041</td>
<td>0.150</td>
<td>0.184</td>
<td>0.165</td>
<td>0.299</td>
</tr>
<tr>
<td>Pl</td>
<td>0.001</td>
<td>1.000</td>
<td>0.792</td>
<td>0.008</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Ps</td>
<td>1.000</td>
<td>0.001</td>
<td>0.3612</td>
<td>0.999</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>8</th>
<th>6</th>
<th>10</th>
<th>13</th>
<th>12</th>
<th>13</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.178</td>
<td>4.888</td>
<td>5.434</td>
<td>8.175</td>
<td>7.513</td>
<td>7.088</td>
<td>6.121</td>
</tr>
<tr>
<td>Ae</td>
<td>5.9</td>
<td>3.7</td>
<td>4.7</td>
<td>9.1</td>
<td>7.8</td>
<td>7.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Ho</td>
<td>0.659</td>
<td>1.000</td>
<td>0.714</td>
<td>0.594</td>
<td>0.649</td>
<td>0.762</td>
<td>0.659</td>
</tr>
<tr>
<td>He</td>
<td>0.831</td>
<td>0.726</td>
<td>0.788</td>
<td>0.890</td>
<td>0.872</td>
<td>0.857</td>
<td>0.775</td>
</tr>
<tr>
<td>HWE</td>
<td>0.004*</td>
<td>0.000*</td>
<td>0.262</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.014</td>
</tr>
<tr>
<td>Fis</td>
<td>0.219†</td>
<td>-0.367*</td>
<td>0.108</td>
<td>0.347*†</td>
<td>0.269*†</td>
<td>0.122</td>
<td>0.162</td>
</tr>
<tr>
<td>Pl</td>
<td>0.004</td>
<td>1.000</td>
<td>0.132</td>
<td>0.001</td>
<td>0.001</td>
<td>0.032</td>
<td>0.022</td>
</tr>
<tr>
<td>Ps</td>
<td>0.999</td>
<td>0.001</td>
<td>0.928</td>
<td>1.000</td>
<td>1.000</td>
<td>0.987</td>
<td>0.994</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>5</th>
<th>5</th>
<th>6</th>
<th>8</th>
<th>5</th>
<th>8</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.021</td>
<td>4.065</td>
<td>5.503</td>
<td>7.093</td>
<td>5.000</td>
<td>6.462</td>
<td>5.413</td>
</tr>
<tr>
<td>Ae</td>
<td>1.7</td>
<td>3.3</td>
<td>4.7</td>
<td>6.9</td>
<td>3.9</td>
<td>5.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Ar</td>
<td>4.021</td>
<td>4.065</td>
<td>5.503</td>
<td>7.093</td>
<td>5.000</td>
<td>6.462</td>
<td>5.413</td>
</tr>
<tr>
<td>Ho</td>
<td>0.400</td>
<td>1.000</td>
<td>0.750</td>
<td>0.750</td>
<td>0.714</td>
<td>0.583</td>
<td>0.538</td>
</tr>
<tr>
<td>He</td>
<td>0.420</td>
<td>0.692</td>
<td>0.788</td>
<td>0.854</td>
<td>0.745</td>
<td>0.802</td>
<td>0.695</td>
</tr>
<tr>
<td>HWE</td>
<td>0.478</td>
<td>0.067</td>
<td>0.050</td>
<td>0.087</td>
<td>0.208</td>
<td>0.067</td>
<td>0.005*</td>
</tr>
<tr>
<td>Fis</td>
<td>0.100</td>
<td>-0.412</td>
<td>0.092</td>
<td>0.165</td>
<td>0.118</td>
<td>0.313</td>
<td>0.263†</td>
</tr>
<tr>
<td>Pl</td>
<td>0.477</td>
<td>1.000</td>
<td>0.358</td>
<td>0.130</td>
<td>0.443</td>
<td>0.021</td>
<td>0.065</td>
</tr>
<tr>
<td>Ps</td>
<td>0.911</td>
<td>0.009</td>
<td>0.869</td>
<td>0.972</td>
<td>0.863</td>
<td>0.993</td>
<td>0.986</td>
</tr>
</tbody>
</table>

Samples size, N; mean number of alleles, A; allelic richness, Ar; observed heterozygosity, Ho; expected heterozygosity, Ae; effective number of alleles; He; Hardy–Weinberg equilibrium, HWE; population inbreeding coefficient Fis; P values for the deficit of heterozygotes, Pl; P values for the excess of heterozygotes, Ps. *Significant values after Bonferroni correction (α = 0.007). † Null alleles. NA = Not analyzed.
The number of alleles per locus ranged from one (Par14; FF3) to 25 (Par82; DIT). The mean values of Ho and He ranged from 0.000 to 1.000. Significant deviations from the HWE is related to the presence of null alleles, resulting in a deficit of heterozygotes in some loci (Table 1). Private alleles, i.e., those found in a single population, were present in all wild populations (DIT = 25; DMA = 20 and DBG = 6) and only one farm population (FF1 = 5). Mean PIC values ranged from 0.800 (Par14) to 0.933 (Par82). Effective population size (Ne) ranged from 2.1 to 20.5 in farmed populations and from 13.3 to 191.4 in wild populations (Table 2). The population assignment test indicates that 83% of individuals were assigned to self-populations (Table 2).

**Table 2.** Effective population size (Ne) and population assignment for wild and aquaculture sampled populations of *Prochilodus lineatus*. Confidence interval CI = 95%.

<table>
<thead>
<tr>
<th>Population</th>
<th>Sample Size</th>
<th>Ne (CI 95%)</th>
<th>Self Population</th>
<th>Other Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF1</td>
<td>11</td>
<td>20.5 (6.3 - inf.)</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>FF2</td>
<td>17</td>
<td>7.4 (3.3 - 13.1)</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>FF3</td>
<td>10</td>
<td>3.2 (1.7-16.1)</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>FF4</td>
<td>13</td>
<td>2.1 (1.4 - 4.4)</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>DIT</td>
<td>49</td>
<td>191.4 (70.1 - inf.)</td>
<td>35</td>
<td>14</td>
</tr>
<tr>
<td>DMA</td>
<td>44</td>
<td>31.1 (21.5 - 51.5)</td>
<td>42</td>
<td>2</td>
</tr>
<tr>
<td>DBG</td>
<td>13</td>
<td>13.3 (4.2 - 293)</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Percentage</td>
<td>-</td>
<td>83%</td>
<td>17%</td>
<td></td>
</tr>
</tbody>
</table>

Global differentiation, including all populations sampled in the present study, by AMOVA showed low significant genetic structure in all scenarios analyzed (Table 3). Divergence was detected among wild populations (FST = 0.056, p = 0.000), among farming stations (FST = 0.051; p = 0.000), and between wild and farm *P. lineatus* (FST = 0.094; p = 0.000) (Table 4).

**Table 3.** Pairwise FST estimates (below of diagonal) and corresponding significance levels (above of diagonal) between sampled wild and farmed populations of *Prochilodus lineatus*.

<table>
<thead>
<tr>
<th></th>
<th>Farmed</th>
<th>Wild</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FF1</td>
<td>FF2</td>
<td>FF3</td>
<td>FF4</td>
<td>DIT</td>
<td>DMA</td>
<td>DBG</td>
<td></td>
</tr>
<tr>
<td>FF1</td>
<td>0.00238</td>
<td>0.00476</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td></td>
</tr>
<tr>
<td>FF2</td>
<td>0.0302</td>
<td>0.01190</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td></td>
</tr>
<tr>
<td>FF3</td>
<td>0.1326</td>
<td>0.0674</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td></td>
</tr>
<tr>
<td>FF4</td>
<td>0.1319</td>
<td>0.1475</td>
<td>0.2270</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td></td>
</tr>
<tr>
<td>DIT</td>
<td>0.0464</td>
<td>0.0531</td>
<td>0.1112</td>
<td>0.1190</td>
<td>0.00238</td>
<td>0.00238</td>
<td>0.00238</td>
<td></td>
</tr>
<tr>
<td>DMA</td>
<td>0.0862</td>
<td>0.0978</td>
<td>0.1762</td>
<td>0.1573</td>
<td>0.0461</td>
<td>0.00238</td>
<td>0.00238</td>
<td></td>
</tr>
<tr>
<td>DBG</td>
<td>0.1277</td>
<td>0.1381</td>
<td>0.2381</td>
<td>0.2418</td>
<td>0.0701</td>
<td>0.0566</td>
<td>0.0566</td>
<td></td>
</tr>
</tbody>
</table>

Indicative adjusted nominal level (5%) for multiple comparisons is $\alpha = 0.002$. Significance is in bold.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Among localities</th>
<th>Between individuals/within localities</th>
<th>Within individuals</th>
<th>Fixation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm</td>
<td>5.657</td>
<td>25.053</td>
<td>69.289</td>
<td>FST = 0.056*</td>
</tr>
<tr>
<td>Wild</td>
<td>5.273</td>
<td>17.010</td>
<td>77.716</td>
<td>FST = 0.051*</td>
</tr>
<tr>
<td>All</td>
<td>8.165</td>
<td>22.594</td>
<td>69.239</td>
<td>FST = 0.094*</td>
</tr>
</tbody>
</table>

* $p = 0.000$

Bayesian analyses for all samples without a priori information suggest three main clusters (Figure 3). It is possible to observe a sharp structure between cultivated and wild individuals, as well as genetic structure among wild fish (Figure 4). DAPC similarly identified a genetic structure in wild and farmed *P. lineatus*. The initial sharp decline in Bayesian Information Criterion (BIC) values was between K = 3 – 5 (Figure 5). When using the lowest K value (K = 5), it is also possible to
see the separation between individuals from FF4 (Cluster 1 in blue) and FF2 (Cluster 5 in red). For wild individuals, DAPC showed a distribution of the microsatellite genotypes into three main clusters (Clusters 2, 3 and 4; Figure 5), corroborating the genetic differentiation among wild sampled populations of *P. lineatus* from the Upper Uruguay River (Table 2). Discriminant functions based on DAPC analyses assigned most individuals to the genetic cluster where they were assigned a priori by K-means analyses used to infer the best-supported clustering (Figure 6). The low overlapping of the genetic clusters on the ordination plot indicated high degree of differentiation between FF4, and high overlapping of the genetic clusters of wild individuals (Figure 4b). Differentiation index FST similarly corroborated the results of DAPC, showing higher genetic differentiation between FF4 in relation to other farm populations (Table 4).

**Figure 3.** Plot of mean log-likelihood values (LnP (D)) (A) and Evanno’s DeltaK (B) generated in STRUCTURE HARVESTER based on wild and farmed *Prochilodus lineatus*.

**Figure 4.** *Prochilodus lineatus* population structure from the Bayesian cluster analysis for K = 3. Black lines separate the seven different sampled populations based on location.
**Figure 5.** Discriminant Analysis of Principal Components (DAPC) for 30 retained PC axes and two discriminate functions. Five clusters were recovered with this model (BIC scores indicating \( K = 5 \)). The bottom right graphic shows eigenvalues of the two principal components. Cluster 1 (blue) = *Prochilodus lineatus* from FF4; Cluster 2 (lavender) = individuals from DMA and DBG; Cluster 3 (yellow) = FF1, DIT and DMA; Cluster 4 (orange) = DBG, DMA and DIT; and Cluster 5 (red) = FF2 and FF3.

**Figure 6.** Panel represents whether the individuals (rows) were correctly assigned (based DAPC; Figure 5) to the genetic cluster where they were included a priori (columns). Colors represent membership probabilities to each genetic cluster (red = 1, orange = 0.75, yellow = 0.25, white = 0) and blue crosses indicate the cluster where the individuals were originally assigned.

The lowest variance for the kinship estimators was found in the triadic likelihood estimator (TrioML). Sampling variances for the kinship estimators (KE) ranged from 0.0000 to 0.5709 in FF1, from 0.000 to 0.1890 in FF2, from 0.0000 to 0.8095 in FF3, and from 0.0000 to 0.8231 in FF4. The relatedness values for simulated pairs were split into three categories, according Wang (2011): high (>0.5; full-sib and parent-offspring), intermediate (>0.25 and <0.5; half-sib or other kinship), and low (<0.25; unrelated). Considering all possible crosses, advisable matings were 90.4% (FF1), 84% (FF2), 56.9% (FF3) and 62% (FF4). Results for indicated mating crossings are showed in green, crosses to be avoided are showed in yellow, and prohibitive mating are showed in red (Figure 7).
GENETIC IMPLICATIONS OF RESTOCKING PROGRAMS...

DISCUSSION

In this study, we identified genetic divergence between and within wild and farmed populations of *P. lineatus* in southern Brazil. Wild populations showed moderate to high genetic diversity, a characteristic that seems to be shared among most Prochilodus species (Sivasundar et al., 2001; Rueda et al., 2013; Braga-Silva and Galleti Jr., 2016; Ferreira et al., 2017). On the other hand, the cultivated populations showed smaller values of genetic diversity, especially when compared to wild populations of *P. lineatus*. Farmed populations similarly displayed lower diversity and allelic richness, but higher inbreeding rates, when compared to wild individuals. These confined populations are more likely to present a reduction in genetic variability as a result of genetic drift (de Oliveira et al., 2018). Among other problems, the low values of genetic diversity increase susceptibility to disease in aquaculture programs (Doyle, 2016) and would, therefore, compromise the viability of natural populations in any restocking initiative (Allendorf and Luikart, 2009).

Farmed fish presented genetic structure distinct from that of wild populations in addition to genetic differentiation among the farmed groups studied. In particular, individuals from FF4, which is located in southeastern Rio Grande do Sul State, exhibited substantially distinct genetic composition in comparison to individuals from other fish farms studied. In general, all fish farms presented some crosses not indicated, due to the high degree of kinship among some broodstock. According to Fonseca et al. (2017), the successful restocking programs depends upon keeping levels of inbreeding low and optimizing genetic variability, resultant of genetic information of broodstock and breeding designs.

The genetic composition of the freshwater broodstock is generally formed by exchange of matrices between fish farmers, regardless of the river basin where they are found, and guidelines are available for this commercialization. In addition to compromising the genetic composition of natural populations (Vaini et al., 2016), crosses among fish from different hydrographic basins may result in non-intentional, as well as intentional, interspecific crosses, as detected in several species of fish in previous studies (Hashimoto et al., 2014a,b; Scaranto et al., 2018).

Different from recent studies that found genetic structure associated with temporal experimental design (e.g., Braga-Silva and Galleti Jr., 2016; Ribolli et al., 2017; Ribolli et al., 2018), our sample arrangement did not allow for testing IBT (isolation-by-time) in the wild populations. Nevertheless, as reported in many studies of population genetics, population structure in Neotropical migratory fish species is not rare (Pereira et al., 2009; Garcez et al., 2011; Ashikaga et al., 2015). The genetic structure of *P. lineatus* identified in the present study may be related to the geographical features of the Upper Uruguay River, which is characterized by stretches of rapids and canyons (e.g., Canyon Augusto César), forming a semipermeable barrier in periods of drought that may have contributed to genetic differentiation of populations downstream from the Machadinho Dam. The installation of Itá Dam probably preserves the genetic isolation of the natural barrier flooded by the Itá Reservoir. Additionally, since wild fish samples were collected in different years, the possible relationship between population structure and reproductive organization, as already reported in potamodromous migratory fish, cannot be ruled out (Braga-Silva and Galetti Jr., 2016, Ribolli et al., 2017).

Genetic differentiation between and within wild and farmed fish is a clear signal that restocking using farmed fish may, indeed, compromise the genetic diversity of wild stocks, resulting in genetic introgression, which occurs when exogenous genetic material is different from that of the wild population (Ryman et al., 1995; Prado et al., 2018). Furthermore, the genetic constitution of the wild population can be permanently altered by the loss of important genetic material. This happens when genes or gene complexes favored by artificial selection are not adaptable to the natural environment, thus causing erosion...
of the wild gene pool, decreasing the reproductive capability of wild fish and introducing diseases in the wild populations, or even leading to loss of the natural population (Ryman et al., 1995). Therefore, the identification of population structure is an essential precondition for developing recommendations for genetic management because the populations present in different fragments can be completely, or partially, isolated, or even be a single population (Frankham, 2008).

*P. lineatus* from fish farms showed lower genetic variability and effective population size compared to their counterparts in natural populations. Moreover, farmed individuals presented a genetic composition completely different from that of wild individuals. Thus, if these farmed fish were used for restocking purposes, the genetic variability of recipient populations could be compromised since wild populations undergo continual natural selection, selecting specific genotypes that maximize the fitness of individuals to survive in particular natural environments (Ward, 2006), in this case, *P. lineatus*.

**CONCLUSION**

The collective results of the present study should serve as a warning against restocking of natural fish populations that occur in just about all Neotropical river basins using fish, either larvae or juveniles, from commercial fish farms. Our results also show that the stock of fish farms, FF1-FF4, as analyzed in the present study, should not be used for restocking as an alternative for the recovery and maintenance of wild *P. lineatus* stocks of the Upper Uruguay River Basin. It is clear from our results that any programs that propose to set up breeding stock for restocking purposes should be cognizant of the genetic diversity of the stock to be managed, both wild and cultivated. It is also recommended that policymakers consider adding measures to legislation that include genetic analyses to any current or proposed restocking programs in order to properly preserve and/or recover wild stocks of freshwater fish, including *P. lineatus*.

**ACKNOWLEDGMENTS**

We appreciate the cooperation of fish farmers in the region of Ajuricaba, Ijuí, São Leopoldo - RS, Chapecó-SC, as well as artisanal fishermen for the fish samples. We thank the Laboratory of Developmental Physiology and Plant Genetics (LFDGV-UFS). JR thanks PNPD/CAES; EZF thanks CNPq (Grant 304949/2017-5). “This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) -Finance Code 001”. The manuscript was enriched by the comments to two peer reviewers.

**REFERENCES**


