

# Genetic diversity and aquaculture conservation for a threatened Neotropical catfish



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Due to the ecological importance of *Lophosilurus alexandri*, the present work evaluated its genetic representativeness by comparing wild stocks to broodstocks that were kept at three restocking hatcheries along the São Francisco River. A total of 97 samples were genotyped for newly developed microsatellite markers. Low levels of genetic diversity (average alleles number of 4.2 alleles) were detected in all cases, being more severe in captive groups. Significant pairwise  $F_{ST}$  and  $D_{EST}$  values, Structure, and DAPC analyses showed that wild animals were structured in two groups, and a third group was formed by captive animals, evidencing the need to adopt genetic criteria to retain genetic diversity in the hatcheries. For this reason, three full-sib families were constructed to select the best relatedness estimator for *L. alexandri* and establish a cut-off value aimed to avoid full-sibling matings in the hatcheries. Two estimators, Wang ( $R_W$ ) and Lynch & Li ( $R_{LL}$ ), were accurate in reflecting the relatedness level for full-sibs in this species. According to them, less than 50% of the potential breeding matings in the three hatcheries are advisable. The innate low diversity of *L. alexandri* highlights the importance of minimizing inbreeding and retaining genetic diversity towards the species recovery.

**Keywords:** *Lophosilurus alexandri*, Low genetic diversity, Relatedness estimator, Restocking, Sedentary behavior.

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Devido à importância ecológica de *Lophiosilurus alexandri*, o presente trabalho avaliou sua representatividade genética, comparando estoques selvagens com plantéis de reprodutores de três larviculturas ao longo do Rio São Francisco. Noventa e sete amostras foram genotipadas com marcadores microssatélites recém-desenvolvidos. Baixos níveis de diversidade genética (número médio de alelos de 4,2) foram detectados em todos os casos, sendo mais severo no cativeiro. Os valores de  $F_{ST}$  e  $D_{EST}$  par a par, as análises do Structure e DAPC mostraram a estruturação dos animais selvagens em dois grupos, e um terceiro formado pelas larviculturas, evidenciando a necessidade de adoção de critérios genéticos para retenção da diversidade genética no cativeiro. Por essa razão, três famílias de irmãos completos foram construídas para selecionar o melhor estimador de parentesco para a espécie e estabelecer os valores mínimos de corte para evitar cruzamentos indesejados. Dois estimadores, Wang ( $R_w$ ) e Lynch & Li ( $R_{LL}$ ), foram eficientes em refletir as relações de parentesco para irmãos completos nessa espécie. Segundo eles, menos de 50% dos potenciais cruzamentos são recomendáveis nas três larviculturas. A baixa diversidade genética inerente ao *L. alexandri* destaca a importância de minimizar a consanguinidade e evitar perda de diversidade genética, visando a recuperação da espécie.

**Palavras-chave:** Baixa diversidade genética, Comportamento sedentário, Estimador de parentesco, *Lophiosilurus alexandri*, Repovoamento.

## INTRODUCTION

Brazil is one of most biodiverse countries for freshwater fish species (Collen *et al.*, 2014), but its ichthyofauna has been impacted due to overfishing, riparian forest degradation, siltation, water transposition, and especially, hydroelectric plants. The São Francisco River (SF) is one of the most impacted basins with declines in fish populations, and at least 10% of the river's more than 200 species are considered threatened (Maneta *et al.*, 2009; Reis *et al.*, 2016; ICMBio, 2018), which has resulted in implementing restocking actions for these species.

One of this species is the *Lophiosilurus alexandri* Steindachner, 1876, an endemic catfish to the SF, which belongs to the order Siluriformes and family Pseudopimelodidae (Carvalho *et al.*, 2016). It is a sedentary, carnivorous, active nocturnally, and exhibits male parental care for the progenies (Sato *et al.*, 2003; Costa *et al.*, 2015).

This species has been subjected to a drastic population reduction in the submiddle and lower stretches of São Francisco River and it has been included into restocking actions for more than two decades (Lopes *et al.*, 2013). Despite the efforts, *L. alexandri* was included in the Brazilian Red List as Vulnerable, designation for species at high risk of extinction (ICMBio, 2014). Recently, *L. alexandri* was also included in the Ministry of Environment's National Action Plan (PAN São Francisco — Ordinance n.34 of May 27<sup>th</sup>, 2015), which aims to conserve threatened species of this river basin.

One neglected aspect by these restocking actions is the genetic representativeness of the broodstocks compared to the wild conspecifics. Keeping sufficient breeders and

incubators are usually prohibitive and most of the hatcheries use a limited number of them. Breeders are mostly not renewed and are replaced by their consecutive progeny generations ( $F_1$ ,  $F_2$ ,  $F_3$  etc.), therefore using a culture-based restocking model. This kind of strategy favors matings between related individuals and can lead to inbreeding depression, which affects reproductive success, survival, growth, disease resistance, etc and can reduce genetic diversity, which limits the evolutionary potential of the population (Frankham *et al.*, 2010; Keller *et al.*, 2012).

For effective conservation, genetic monitoring of wild populations and captive broodstocks is important to prevent adverse effects on the ichthyofauna (Povh *et al.*, 2008; Schreier *et al.*, 2015). This is particularly important when dealing with species with low genetic diversity, which are more prone to inbreeding and genetic drift (Coleman *et al.*, 2018). Breeding strategies must be adopted to avoid loss of genetic diversity in captive populations of threatened species, such as genetically characterizing the broodstocks (Carvalho *et al.*, 2012), introducing new wild breeders (Whiteley *et al.*, 2015), and slowing down the process of inbreeding by selecting matings based on the degree of relatedness (Wang, 2014).

Microsatellite markers are widely used in population genetic monitoring of species subjected to restocking actions in Brazil, such as for the *Pseudoplatystoma corruscans* (Spix & Agassiz, 1829) (Dantas *et al.*, 2013), *Prochilodus argenteus* Spix & Agassiz, 1829 (Coimbra *et al.*, 2017), *Steindachneridion parahybae* (Steindachner, 1877) (Fonseca *et al.*, 2017), *Conorhynchus conirostris* (Cuvier, 1829) (Carvalho, Beheregaray, 2018), *Salminus franciscanus* Lima & Britski, 2007 and *Brycon orthotaenia* Günther, 1864 (Coimbra *et al.*, 2020). The markers are also used to infer the genetic relatedness owed to recent common ancestry (Wang, 2017). Selection of mating pairs based on molecular relatedness estimates have been used worldwide by hatcheries running conservation programs, such as for the *Paralichthys olivaceus* (Temminck & Schlegel, 1846), *Pagrus major* (Temminck & Schlegel, 1843), *Verasper moseri* Jordan & Gilbert, 1898, *Onchorhynchus keta* (Walbaum, 1792), among others (Fisch *et al.*, 2015).

The use of estimators to determine genetic relatedness between captive-grown breeders could allow selection of those with the lowest degree of genetic similarity, resulting in progenies with greater genetic variability. Genetic relatedness estimators, such as Queller, Goodnight (1989), Ritland (1996), and Lynch, Ritland (1999), based on co-dominant markers, can be used to identify animal relationships. Despite the variety of existing estimators, the best estimator for a certain species can only be found after tests are conducted with the different estimators in groups whose relationships, full-sib or half-sib, are known (Ortega-Villaizan *et al.*, 2011).

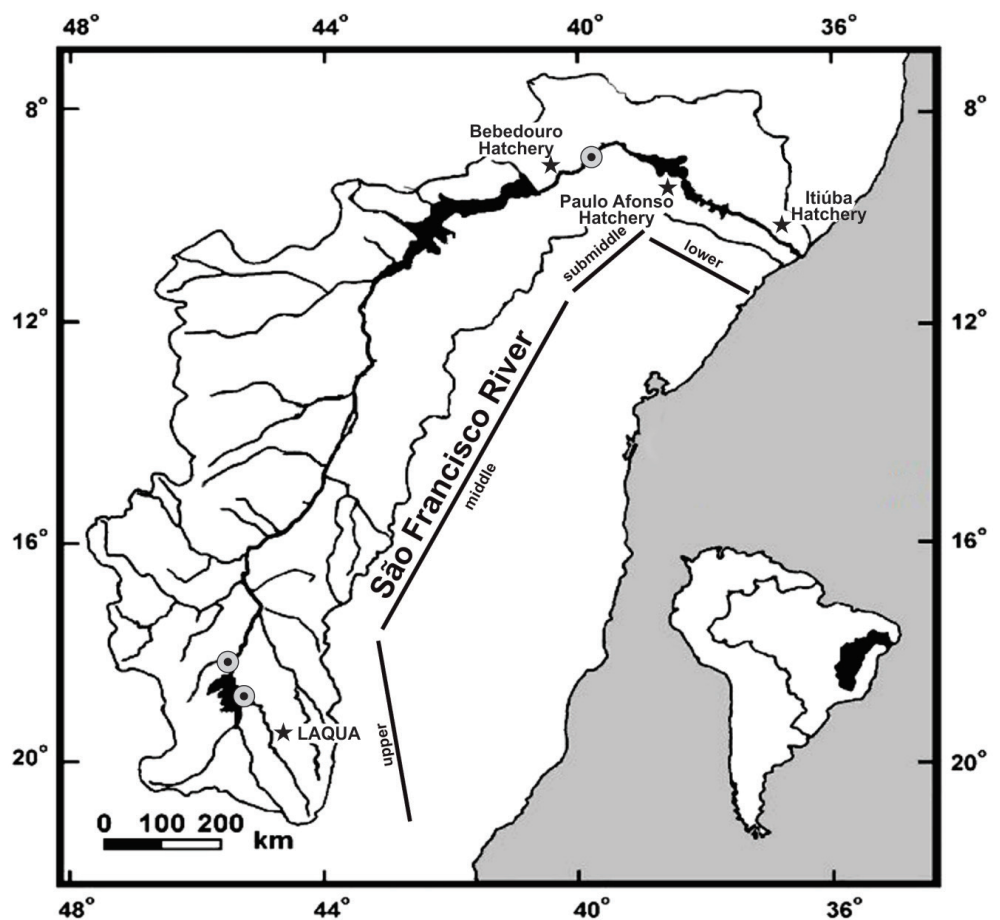
For the conservation of *L. alexandri*, analyses of the genetic diversity remains to be investigated and three hypotheses must be tested: (1) a panmictic population exists along the São Francisco River basin, (2) captive broodstocks of *L. alexandri* have similar genetic diversity to the wild conspecifics; (3) a relatedness estimator can be used to select the best matings.

In this study, we developed and validated a set of microsatellite markers to evaluate the genetic variability and structure of wild animals and captive broodstocks of *L. alexandri*. In addition, these markers were also used to identify the best genetic relatedness estimator among *L. alexandri* full-sib families, towards a conservation aquaculture program.

## MATERIAL AND METHODS

**Sampling.** For the population genetics approach, fin clips from 55 individuals of *L. alexandri* from three hatcheries along the São Francisco River were collected. Twenty of these were from Paulo Afonso Hatchery of Chesf (São Francisco Hydroelectric Company) in the submiddle stretch of the river, 23 were from the Bebedouro Hatchery of Codevasf (Development Company of São Francisco and Parnaíba Valleys) also in the submiddle stretch of the São Francisco River, and the remaining 12 were from the Itiúba Hatchery of Codevasf in the lower stretch of the river. Forty-two wild animals were sampled, 21 from the submiddle stretch, in the vicinity of Santa Maria da Boa Vista-PE, and 21 from the upper São Francisco River, in the reservoir of Três Marias and downward the reservoir (Fig. 1).

In order to select the best genetic relatedness estimator, three full-sibling families were constructed: FS1 from Bebedouro hatchery, FS2 from Itiúba hatchery, and FS3 from LAQUA (Laboratório de Aquacultura at the Universidade Federal de Minas Gerais). Each mating pair was tagged with PIT tags and was kept in an individual tank with



**FIGURE 1** | Map showing the São Francisco River Basin with locations of the three restocking hatcheries (★) of *Lophiosilurus alexandri* and an experimental laboratory (LAQUA). The wild samples taken in the upper and submiddle stretches are denoted by ⊙.

sand bottom and flowing water. The eggs were collected and transferred to a trough incubator with flowing water, where the fingerlings were maintained until reaching a size of 1 cm. Fifty-three fin clips from the three progenies were collected: 20 samples from FS1, 20 from FS2, and 13 from FS3.

In the genetic diversity analysis, none of the full-sibs was included, only the breeders were used.

One voucher specimen used in this study was deposited in the collection of the Coleção Ictiológica at the Museu de Ciências Naturais da PUC Minas (Voucher number: MCNIP 3217).

**DNA extraction.** DNA extraction was conducted based on the phenol, chloroform, isoamyl alcohol protocol described by Sambrook *et al.* (1989). The DNA samples were quantified by fluorimeter (Quantus - Promega), and their integrity and quality were evaluated using 0.8% agarose gel electrophoresis and a NanoVue™ Plus Spectrophotometer (GE Healthcare), respectively.

**Microsatellites.** Muscle fragments were obtained from a freshly captured *L. alexandri*. The voucher specimen was fixed in 10 % formalin and later preserved in 70 % ethanol (voucher: LGC6088 at Museu de Ciências Naturais da PUC Minas). Genomic DNA was extracted using a modified salting-out method (Sunnucks, Hales, 1996) and nebulized for 6 min to obtain 200–600bp fragments. Fragments were sequenced using a Nextera kit in a sixth of an Illumina HiSeq 2000 lane.

A set of 178 potentially amplifiable microsatellite loci (PAL) were developed for *L. alexandri* using the Misa algorithm (Beier *et al.*, 2017) from the scaffold data assembled by SOAP *de novo* (Li *et al.*, 2008). Primer pairs were designed for each locus using Primer3 (Rozen, Skaletsky, 2000) and tested against the scaffolds using *in silico* PCR software to verify whether a single product was found. Selected PALs consisted of primers, which produced *in silico* amplicons with no more than 10 non-ACTG bases. The markers were named with the abbreviation of the species name “Lalex” followed by a number.

From these PALs, 55 loci were initially tested with samples from wild *L. alexandri* and only those loci that showed polymorphism were subsequently analyzed in captive animals. Four different repeat motifs were tested: 42 dinucleotide motif with eight repeats, one dinucleotide motif locus with nine repeats, ten loci with trinucleotide motifs and six repeats, and two tetranucleotide motif loci with five repeats.

Forward primers received the M13 tail (5'-TGTAACGACGGCCAGT-3') at the 5' end. PCR amplification followed a modified Schuelke (2000) protocol, with a final volume of 10µl containing 20ng DNA, 10mM Tris-HCl pH 8.4, 50mM KCl, 2.5mM MgCl<sub>2</sub>, 2µM forward primer, 8µM reverse primer, 8µM fluorochrome (FAM, NED, PET or VIC), 200µM of each dNTP and 1U *Taq* DNA polymerase. The PCR amplification conditions were: 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 45 seconds, followed by eight cycles of 94°C for 30 seconds, 53°C for 45 seconds and 72°C for 45 seconds, plus final extension at 72°C for 10 minutes. Capillary electrophoresis separated PCR products on the ABI 3500 Genetic Analyzer (Applied Biosystems) using GeneScan 600 LIZ v2.0 (Applied Biosystems) as a molecular weight marker.



**Genetic data analysis.** Polymorphism information content (PIC) was calculated, only for the 42 wild samples, using CERVUS v 3.0.7 software (Kalinowski *et al.*, 2007), and the markers were classified as highly informative for PIC values greater than 0.5, as reasonably informative for values between 0.25 and 0.5, and as slightly informative markers for values less than 0.25 (Botstein *et al.*, 1980). In order to predict the probability of two randomly selected individuals from a population holding the same genotype (Waits *et al.*, 2001), the multi-loci probability of identity (PID) was calculated for all 10 loci and the 42 wild samples, using the GIMLET software (Valière, 2002) and under two relatedness scenarios: “unrelated”, which corrects for sampling a small number of individuals, and “sibs”, which assumes that the population is composed of siblings.

GENEPOP v 4.0 software (Raymond, Rousset, 1995) was used to obtain the number of alleles ( $N_a$ ), observed and expected heterozygosity ( $H_o$  and  $H_e$ , respectively), linkage disequilibrium, allele frequency, and inbreeding coefficient ( $F_{IS}$ ). Potential genotyping errors caused by stuttering, alleles dropouts, and null alleles ( $A_n$ ) occurrence were evaluated by Micro-Checker v. 2.2.3 (Oosterhout *et al.*, 2006). The genetic differentiation was pairwise evaluated between populations by  $F_{ST}$  and the Jost’s estimator ( $D_{EST}$ ) (Jost, 2008), implemented in Arlequin v. 3:01 (Excoffier *et al.*, 2005) and GenAlEx 6.51 (Peakall and Smouse, 2012), respectively. The analysis of molecular variance (AMOVA) was calculated in two scenarios: (1) only for the captive groups, and (2) for the wild groups, using Arlequin v. 3:01 (Excoffier *et al.*, 2005). The deviations of the Hardy-Weinberg Equilibrium (HWE) were estimated for all loci and groups using Arlequin v. 3:01 (Excoffier *et al.*, 2005). P-values of multiple tests were corrected by Bonferroni (Rice, 1989).

A Bayesian approach with STRUCTURE v. 2.3.4 was used to investigate the genetic structure of the population using its under admixture model, which assumes that individuals may have different ancestor genotypes with contributions from different populations (Pritchard *et al.*, 2000). The analysis in STRUCTURE v. 2.3.4 was set for a burn-in period of 10,000 interactions, followed by Markov Chain Monte Carlo (MCMC) with 100,000 replicates and four replicates for each hypothetical value of k ( $k = 1-10$ ).

Structure Harvester v. 0.6.7 software (Earl, vonHoldt, 2012) was used to coordinate the results and assume the most likely number of K (number of clusters - genetic groups), which was estimated through the log-likelihood data ( $\ln(P(X/K))$ ) (Falush *et al.*, 2003) and the best  $\Delta K$  was based on the rate of change in the log probability of the data (Evanno *et al.*, 2005).

Discriminant Analysis of Principal Components (DAPC) (Jombart *et al.*, 2010) was also used to investigate the population structure and was run in the Adegenet package (Jombart, 2008) in R platform, v. 4.0.2 (R Core Team, 2020). This multivariate analysis is used to identify and describe genetic clusters, while optimizing the variation among them. DAPC does not require populations to be in Hardy-Weinberg Equilibrium or linkage disequilibrium loci (Jombart *et al.*, 2010).

For genetic relatedness analysis, seven microsatellite markers, those that were polymorphic among parental breeders, were used to analyze the parents and progenies from three full-sib families (FS1, FS2 and FS3). The COANCESTRY software (Wang, 2011) was used to generate pairwise relatedness values (dyads) in each full-sib family, according to the five following relatedness estimators: Wang  $R_{wv}$  (Wang, 2002), Lynch

& Li  $R_{LL}$  (Lynch, 1988; Li *et al.*, 1993), Lynch, Ritland  $R_{LR}$  (1999), Ritland  $R_{xy}$  (1996), and Queller, Goodnight  $R_{QG}$  (1989). In order to choose the best estimator, a mean pairwise relatedness value was calculated for the three full-sib families and compared to the expected reference value for full-siblings (0.5).

The same estimators were applied to the wild samples from the submiddle and upper São Francisco stretches to ensure that estimations were accurate, since an absence of relatedness is expected among wild animals. Further, a minimum value of genetic relatedness of full-sib will be taken as a cut-off value for parentage.

In addition, a simulation of potential breeding pairs within the three hatcheries (Bebedouro, Paulo Afonso and Itiúba) was conducted using the best estimators and their respective cut-off values.

## RESULTS

A total of 16,819 tandem repetitive sequences were recovered using the Misa software into contigs assembled by SOAP *de novo*. After filtering potential amplifiable microsatellite loci, we produced 178 primer pairs for these loci for this catfish (shown in Tab. S1).

Of the 55 microsatellite loci analyzed, 10 showed polymorphisms, 41 were monomorphic, and four did not amplify. The ten polymorphic loci were in HWE, showed PCR amplification consistency, and there was an absence of linkage disequilibrium when genotyped for the wild samples. Polymorphism information content (PIC) values ranged from 0.202 to 0.821 and the number of alleles ranged from two (Lalex5 and Lalex19) to nine (Lalex38) (Tab. 1). The resulting values of multi-loci probability of identity (PID) were  $3.4 \times 10^{-7}$  and  $2.4 \times 10^{-3}$  for unrelated and sibs scenarios, respectively.

Genetic variability of the 10 microsatellite loci for each of the five sampling sites is described in Tab. 2. A total of 49 alleles were found, 37 of which were present in the upper SF samples, 32 in the submiddle SF, 24 in Bebedouro, 27 in Paulo Afonso, and 23 in the Itiúba samples. The average number of alleles varied between 2.30 and 3.70 per sampling group. Thirteen alleles were private, seven of which belonged to the wild samples from the upper SF, two from wild samples from the submiddle SF, two from Bebedouro, and one each from Paulo Afonso and Itiúba hatcheries. Null alleles were found in Lalex25, Lalex30, and Lalex38 but not systematically in all samples. No evidence was found of either the misidentification of stutters as alleles or dropouts.

The average observed and expected heterozygosities ( $H_o$  and  $H_e$ ), respectively ranged from 0.350 to 0.525 and from 0.393 to 0.481, respectively. Hardy-Weinberg (HWE) equilibrium deviations were significant after Bonferroni correction for Lalex1 (Bebedouro and upper SF), Lalex9 (upper SF), Lalex25 (upper SF), Lalex30 (Bebedouro), Lalex38 (submiddle SF), but none of the loci deviated from HWE in the wild samples. Among the 50 estimates (10 loci x 5 sampling sites) of HWE deviation, only six were significant.

Regarding the inbreeding coefficient ( $F_{IS}$ ), mean values were all positive, ranging between 0.044 and 0.198, except for the upper SF that was -0.092. Overall  $F_{ST}$  among the hatcheries was around 0.10 and the pairwise estimates between them ranged from 0.03 to 0.16 ( $p < 0.05$ ). The  $F_{ST}$  between wild groups (submiddle and upper SF) was

**TABLE 1** | Characteristics of the ten polymorphic microsatellite markers of *Lophiosilurus alexandri*.  $N_a$  number of alleles; PIC - polymorphism information content;  $T_m$  °C melting temperature.

Locus	Primer sequences (5' - 3')	Motif	Allele size (bp)	$N_a$	PIC	$T_m$ °C
Lalex1	F: GGACGTCATACCTTCTGTCC	(AT)8	119 - 125	3	0.469	60
	R: CTGAACACACTCAGAGGAAAGC					
Lalex5	F: CCGTAACAGGCTCGGTTTT	(CT)8	135 - 139	2	0.202	60
	R: AGAGACAGGCTCGGGAACA					
Lalex9	F: CTATTACGGCTTTTCTTGG	(CT)8	132 - 146	5	0.675	60
	R: CTCATCTTGAGGAAACGTTGG					
Lalex15	F: CACTTCATGGTCTGGCTTTG	(AC)8	146 - 164	5	0.604	60
	R: CACTGAAAGCACTATTGACAGCA					
Lalex19	F: GAAATGAAACAGGCCAGGAA	(TC)8	194 - 200	2	0.293	60
	R: TGTAGAAGAGTAGACCACGAACTGA					
Lalex25	F: AGACATGTTGCTGCCCTCCA	(TG)8	184 - 286	4	0.279	60
	R: CTATAGAAACAGAAGCGCTAA					
Lalex30	F: CACCGATTTCAGAACTGAAGG	(CG)8	177 - 203	6	0.668	60
	R: ATGCAGAAAAATCGCAAACA					
Lalex37	F: TGGGTAAACGCAAAACAATC	(CAG)6	259 - 265	3	0.368	62
	R: GAGACAGACACTGGACTTTGA					
Lalex38	F: GCGAAAATCGCAGGTTTCT	(AT)9	277 - 295	9	0.821	62
	R: ACGCATATTCACGCACAAGA					
Lalex58	F: CAGAACCGTCTGTCAACC	(TTC)6	282 - 288	3	0.444	60
	R: CCAACCCGTCCTCCTAACT					

0.25. The pairwise  $F_{ST}$  and  $D_{EST}$  values between the two hatcheries that restock the submiddle (Paulo Afonso and Bebedouro) and their wild conspecifics (submiddle SF) varied between 0.30 and 0.34 and 0.32 and 0.33, respectively. Overall,  $D_{EST}$  among the hatcheries was around 0.07, and the pairwise between them ranged from 0.016 to 0.10 ( $p < 0.05$ ) (Tab. 3). The AMOVA results for captive samples showed that most of the variance (86.6%) found could be explained by differences within the hatcheries and not among them (10.5%) ( $p < 0.05$ ). For the wild samples, 78.7% of the variance could be explained by differences within groups and 27.4% for differences between them.

The structure analysis was done in two scenarios, firstly for all groups (captive and wild) and secondly only for the wild samples, in both cases the highest likelihood of  $K$  values suggested two genetic clusters ( $K = 2$ ). All suggested clusters were present in the five sampling sites, however, at different proportions. For the first scenario, cluster 1 (red) was predominant among wild animals and was present in more than 96% of the individuals from the submiddle and upper SF. For captive samples, cluster 2 (green) was more abundant and was present in more than 98% of the samples (Fig. 2A). For the second scenario, there was a distinction between samples from the submiddle and upper SF. Cluster 2 (green) was predominant in the submiddle samples (98.6%) and cluster 1 (red) was primary presented in the upper SF samples (Fig. 2B).

Results of DAPC identified the presence of five clusters ( $k = 5$ ), with an overlap of cluster clusters 1, 3, and 5 by captive animals. DAPC showed a clear separation of the three groups: upper SF, submiddle SF, and captive samples (Fig. 3).



**TABLE 2 |** Genetic diversity for wild (upper and submiddle stretches of the São Francisco River) and captive (Bebedouro, Paulo Afonso, and Itiúba) *Lophosilurus alexandri* samples. N = number of individuals,  $N_a$  = alleles number,  $A_n$  = null alleles,  $H_e$  = expected heterozygosity,  $H_o$  = observed heterozygosity,  $F_{IS}$  = inbreeding coefficient, HWE = Hardy-Weinberg equilibrium deviation, ns = not significant, \* p < 0.05, adjusted by Bonferroni correction,  $K = 50$ .

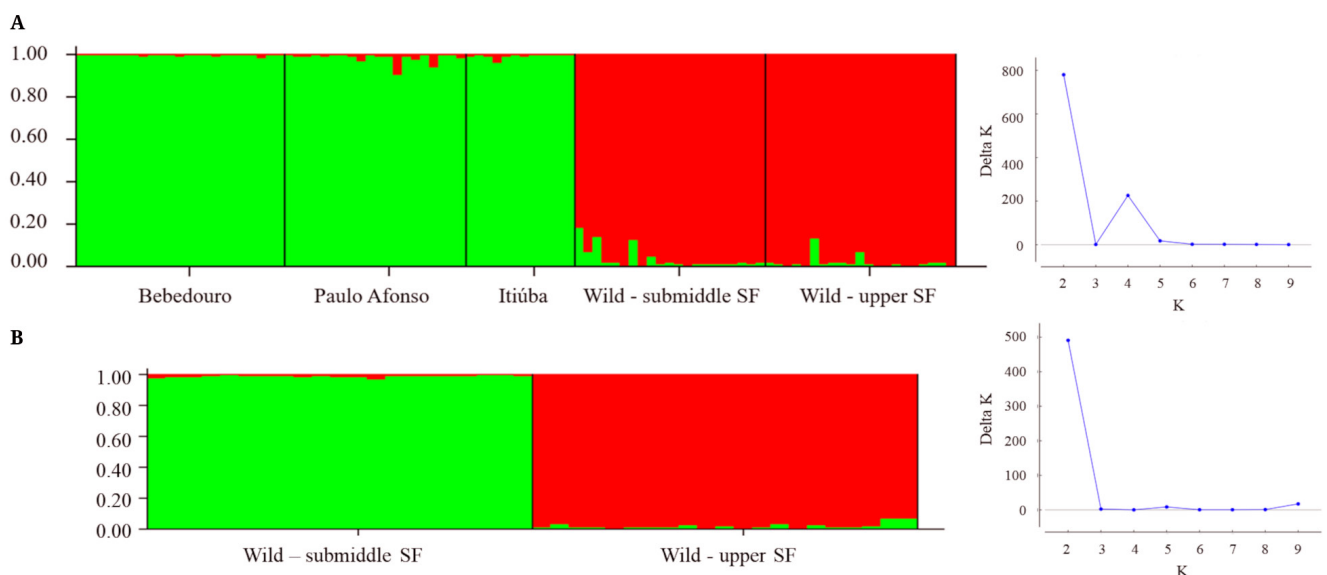
Samples	Lalex1	Lalex5	Lalex9	Lalex15	Lalex19	Lalex25	Lalex30	Lalex37	Lalex38	Lalex58	Average
<b>Bebedouro</b>											
N	23	23	23	23	23	23	23	23	23	23	23
$N_a$	3	1	2	1	2	3	5	2	3	2	2.40
$A_n$	No	No	No	No	No	No	No	No	No	No	-
$H_e$	0.598	0	0.322	0	0.476	0.544	0.653	0.414	0.603	0.322	0.393
$H_o$	0.913	0	0.391	0	0.391	0.565	0.609	0.565	0.522	0.391	0.435
$F_{IS}$	-0.545	1	-0.222	1	0.182	-0.040	0.070	-0.375	0.137	-0.222	0.098
HWE	*	ns	ns	ns	ns	ns	*	ns	ns	ns	-
<b>Paulo Afonso</b>											
N	19	20	20	20	20	20	20	20	18	18	19.5
$N_a$	3	2	5	1	2	4	3	2	3	2	2.70
$A_n$	No	No	No	No	No	No	Yes	No	Yes	No	-
$H_e$	0.679	0.050	0.660	0	0.262	0.664	0.549	0.512	0.541	0.413	0.433
$H_o$	0.684	0.050	0.550	0	0.300	0.550	0.300	0.550	0.278	0.444	0.371
$F_{IS}$	-0.009	0	0.171	1	-0.152	0.176	0.460	-0.077	0.494	-0.079	0.198
HWE	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-
<b>Itiúba</b>											
N	12	12	12	12	12	12	12	12	9	11	12
$N_a$	3	2	2	2	2	3	2	2	3	2	2.30
$A_n$	No	No	No	No	No	No	No	No	No	No	-
$H_e$	0.627	0.083	0.344	0.228	0.228	0.685	0.228	0.522	0.569	0.416	0.393
$H_o$	0.917	0.083	0.250	0.250	0.250	0.417	0.083	0.667	0.222	0.364	0.350
$F_{IS}$	-0.494	0	0.283	-0.100	-0.100	0.402	0.645	-0.294	0.624	0.130	0.110
HWE	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-
<b>Wild - submiddle SF</b>											
N	19	21	21	21	21	21	21	21	19	21	20.6
$N_a$	3	2	3	2	2	2	4	2	9	3	3.20
$A_n$	No	No	No	No	No	No	No	No	Yes	No	-
$H_e$	0.605	0.251	0.361	0.136	0.438	0.251	0.696	0.438	0.886	0.473	0.453
$H_o$	0.947	0.286	0.286	0.143	0.238	0.286	0.619	0.524	0.368	0.381	0.408
$F_{IS}$	-0.592	-0.143	0.213	-0.053	0.462	-0.143	0.113	-0.202	0.591	0.198	0.044
HWE	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	-
<b>Wild - upper SF</b>											
N	20	21	21	21	20	21	20	20	21	21	20.6
$N_a$	3	2	3	3	2	4	5	3	9	3	3.70
$A_n$	No	No	No	No	No	Yes	Yes	No	No	No	-
$H_e$	0.537	0.215	0.553	0.535	0.262	0.336	0.747	0.415	0.784	0.424	0.481
$H_o$	1.000	0.238	0.952	0.810	0.300	0.095	0.500	0.400	0.619	0.333	0.525
$F_{IS}$	-0.905	-0.111	-0.754	-0.532	-0.152	0.721	0.337	0.038	0.215	0.218	-0.092
HWE	*	ns	*	ns	ns	*	ns	ns	ns	ns	-

**TABLE 3** | Estimated pairwise  $F_{ST}$  (below diagonal) and pairwise  $D_{EST}$  (above diagonal) values for captive and wild samples of *Lophiosilurus alexandri*.

	Bebedouro	Paulo Afonso	Itiúba	Wild – submiddle SF	Wild – upper SF
Bebedouro	-	<b>0.102</b>	<b>0.100</b>	<b>0.335</b>	<b>0.352</b>
Paulo Afonso	0.150*	-	0.016	<b>0.321</b>	<b>0.263</b>
Itiúba	0.159*	0.036	-	<b>0.313</b>	<b>0.302</b>
Wild - submiddle SF	0.343*	0.305*	0.319*	-	<b>0.238</b>
Wild - upper SF	0.340*	0.252*	0.297*	0.250*	-

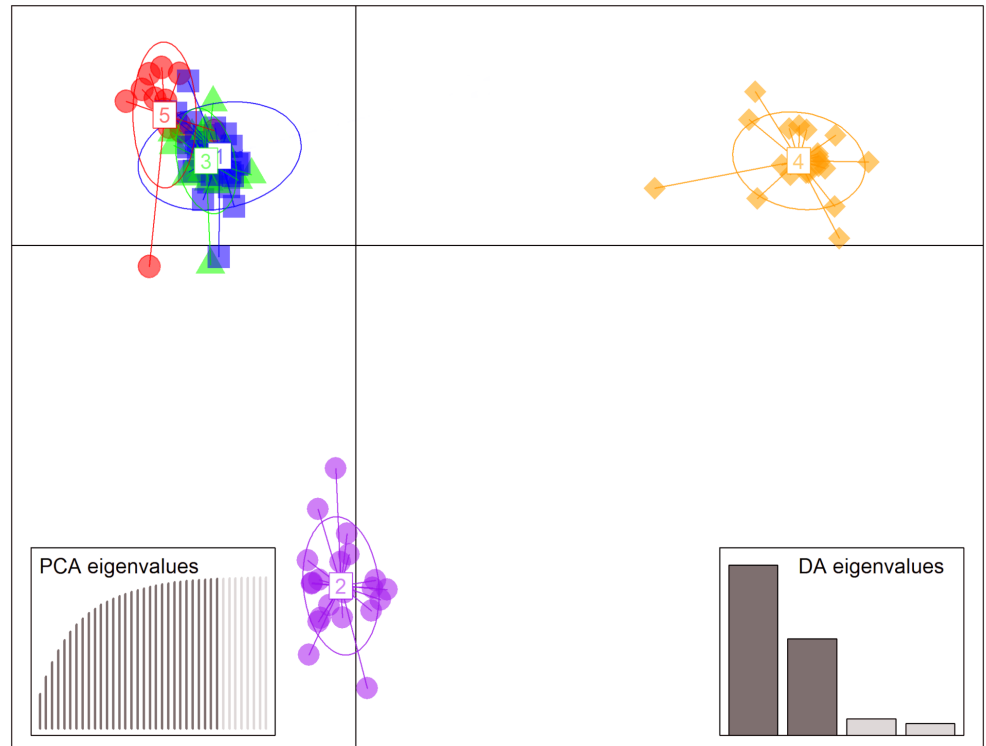
$F_{ST}$ : \* Significance level  $p < 0.05$ , adjusted by Bonferroni correction.

$D_{EST}$ : Significant values at  $p < 0.05$ , based on 999 permutations using GenALEX version 6.501 software, are highlighted in bold.

**FIGURE 2** | Attributions of *Lophiosilurus alexandri* genotypes from **A.** captive broodstocks (Bebedouro, Paulo Afonso, and Itiúba) and wild samples taken from two stretches of the São Francisco River (upper and submiddle) and **B.** only for the wild samples. Each vertical bar represents a different individual and the length is proportional to the inferred group, cluster 1 (red) and cluster 2 (green). Respective estimated K value through log-likelihood. Y axis for delta (K) values and X axis for different K values tested.

Regarding the genetic relatedness, the  $R_{LR}$ ,  $R_{xy}$  and  $R_{QG}$  estimators showed negative mean pairwise relatedness values for all three full-sib families and were discarded. However,  $R_w$  and  $R_{LL}$  gave positive average values between 0.51 and 0.64 for the three full-sib families and negative average values for the wild samples (Tab. 4).

The distribution of pairwise relatedness in intervals for the three full-sib families and wild samples under the  $R_w$  and  $R_{LL}$  estimators is represented in Fig. 4. The extremes of distribution pairwise values into full-sibs were 0.12 and 1 for the  $R_w$  estimator and 0.03 and 1 for  $R_{LL}$ , which the minimum extremes were used as cut-off values for full-sib relatedness. The distribution of pairwise relatedness values for the wild samples showed



**FIGURE 3** | Scatterplot of the Discriminant Analysis of Principal Components (DAPC) of *Lophiosilurus alexandri* genotypes from captive broodstocks (Bebedouro, Paulo Afonso, and Itiúba (clusters 1, 3, and 5) and wild samples taken from two stretches of the São Francisco River (upper (cluster 2) and submiddle (cluster 4)). Clusters are shown by different colors and inertia ellipses, while dots represent individuals. Eigenvalues of the analysis are displayed in inset.

that there were an average of 46.4% positive pairwise values for the upper SF, while for the submiddle SF there were 45.2%.

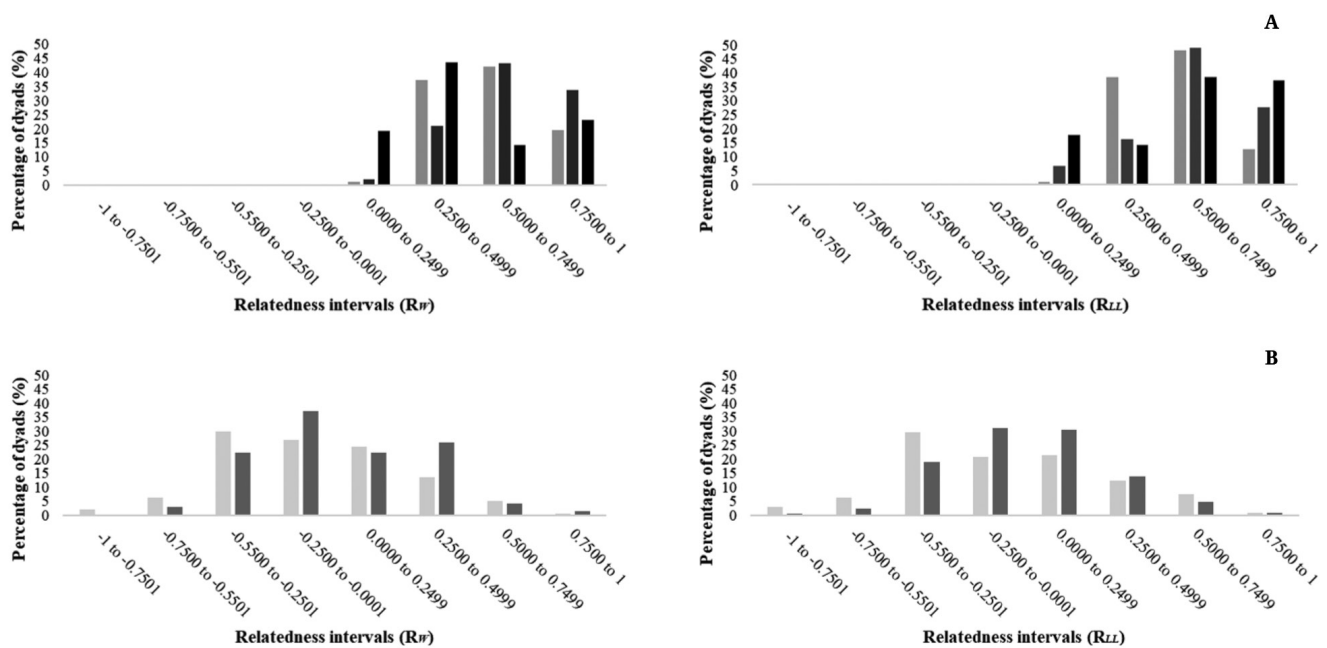
$R_w$  and  $R_{LL}$  were chosen to conduct a simulation of breeding pairs in each hatchery (Bebedouro, Paulo Afonso and Itiúba), using 0.12 and 0.03 as cut-off values, respectively. For  $R_w$ , the Bebedouro hatchery (BEB) indicated 58.46% (76/130) advisable matings, while in the Paulo Afonso (PA) hatchery there were 64% (64/100) advisable matings, and in Itiúba (IT), there were 69.45% (25/36) advisable ones. Yet for  $R_{LL}$ , Bebedouro showed 37.7% (49/130), Paulo Afonso 53% (53/100) and Itiúba 58.34% (21/36) advisable matings (Fig. 5).

## DISCUSSION

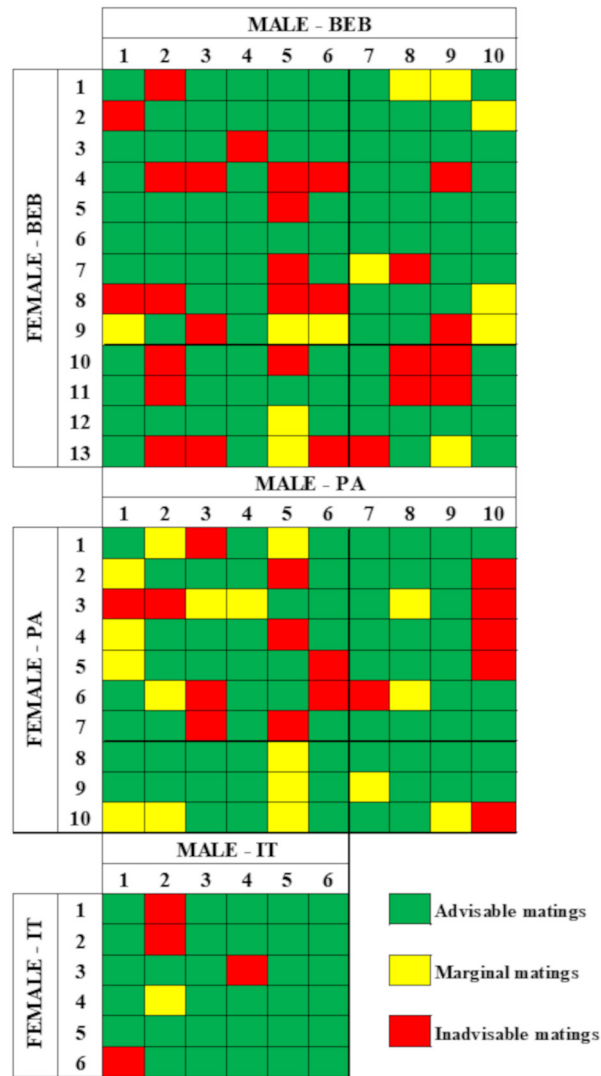
**Microsatellites.** This is the first study that developed and used microsatellite markers to examine the genetic diversity of the threatened Neotropical catfish *L. alexandri*. According to Botstein *et al.* (1980) classification of PIC values, of the ten loci genotyped of the wild samples from the submiddle and upper São Francisco (mean PIC: 0.482), four were highly informative (Lalex1, Lalex9, Lalex15, and Lalex30), five were reasonably informative (Lalex1, Lalex19, Lalex25, Lalex37, and Lalex58), and one was

**TABLE 4** | Genetic relatedness estimators for three full-sibling families (Bebedouro (FS1), Itiúba (FS2), and LAQUA (FS3) and for wild animals of *Lophosilurus alexandri* from the upper and submiddle São Francisco River.

Sampling sites	Indexes (mean values $\pm$ standard deviation)				
	Wang	Lynch & Li	Lync & Rd	Ritland	Queller & Gt
FS1	0.58 $\pm$ 0.03	0.54 $\pm$ 0.04	-0.05 $\pm$ 0.17	-0.02 $\pm$ 0.04	-0.15 $\pm$ 0.34
FS2	0.64 $\pm$ 0.03	0.60 $\pm$ 0.04	-0.05 $\pm$ 0.26	-0.02 $\pm$ 0.03	-0.09 $\pm$ 0.29
FS3	0.54 $\pm$ 0.07	0.51 $\pm$ 0.09	-0.08 $\pm$ 0.35	-0.05 $\pm$ 0.07	-0.07 $\pm$ 0.41
Wild – submiddle SF	-0.001 $\pm$ 0.10	-0.04 $\pm$ 0.11	-0.05 $\pm$ 0.08	-0.04 $\pm$ 0.08	-0.04 $\pm$ 0.10
Wild – upper SF	-0.06 $\pm$ 0.14	-0.03 $\pm$ 0.16	-0.05 $\pm$ 0.07	-0.05 $\pm$ 0.06	-0.05 $\pm$ 0.13

**FIGURE 4** | Relatedness estimators Wang ( $R_W$ ) and Lynch & Li ( $R_{LL}$ ) calculated for **A.** the three full-sib families (■ FS1, ■ FS2 and ■ FS3) and **B.** for the wild samples (■ upper and ■ submiddle São Francisco River stretches) of *Lophosilurus alexandri*.

slightly informative (Lalex5). Similarly, Hughes *et al.* (2015) were able to determine the structuring and a low genetic diversity in the Australian lungfish *Neoceratodus forsteri* (Kreffft, 1870) after the authors used a set of 11 microsatellite markers in which two were highly informative, six were reasonably informative, and three were slightly informative (mean PIC: 0.345). In the present study, although not all loci were highly informative under PIC classification, the multi-loci probability of identity (PID) values were below the threshold value for codominant markers 0.01 (Waits *et al.*, 2001), which is necessary to accurately distinguish individual genotypes, even in the sibs scenario. This indicates that the novel microsatellite set described herein is effective in assessing genetic diversity, population structure, and relatedness in the *L. alexandri*.



**FIGURE 5 |** Candidates breeding pairs in the three hatcheries, Bebedouro (BEB), Paulo Afonso (PA) and Itiúba (IT), assessed by Coancestry (Lynch & Li estimator).

**Genetic diversity.** A low number of alleles (two to nine), with an average of 4.2 alleles, and expected heterozygosity ( $H_e$ ) (0.393 to 0.481) were found. These values were well below those reported by Martinez *et al.* (2018), which found an average number of alleles per locus of 14.81 for freshwater fish species.

The low diversity found in *L. alexandri* is not common in other Siluriformes. Shibatta *et al.* (2013) described 11 microsatellite loci for *Microglanis cottoides* (Boulenger, 1891) (Siluriformes, Pseudopimelodidae) with high levels of variability (number of alleles ranging from 2 to 20, mean of 8.3 alleles per locus, and  $H_e$  from 0.119 to 0.931). For *Zungaro jahu* (Ihering, 1898) (Siluriformes, Pimelodidae), another migratory catfish, the number of alleles ranged from three to 10, with an average number of 6.7 alleles per locus, and  $H_e$  between 0.3298 and 0.8670 (Carrillo-Avila *et al.*, 2009). Similarly, in a study with *Pseudoplatystoma corruscans* (Siluriformes, Pimelodidae) six microsatellites

showed an average of 7.8 alleles,  $H_o$  between 0.6082 and 0.7171 and a  $H_e$  between 0.7143 and 0.7810 (Dantas *et al.*, 2013). Saulo-Machado *et al.* (2011) found polymorphism in all 15 analyzed loci for *Pseudoplatystoma punctifer* (Castelnaud, 1855) (Siluriformes, Pimelodidae), a migratory Amazon catfish (Buitrago-Suárez, Burr, 2007), with the number of alleles ranging from 2 to 18 and  $H_e$  from 0.025 to 0.931.

It is possible that the low number of polymorphic loci found in *L. alexandri* might be related to its sedentary behavior. The Australian lungfish *Neoceratodus forsteri* (Sarcopterygii, Neoceratodontidae), which also has sedentary habits and parental care (Pusey *et al.*, 2004; James *et al.*, 2010), show similar values of genetic diversity in terms of average number of alleles and heterozygosities (Hughes *et al.*, 2015). Other Brazilian freshwater fish species with limited displacement and parental care to whom low genetic diversity has been reported include the *Arapaima gigas*, with an average of 3.57 alleles per locus, ranging from 1 to 5 and  $H_e$  from 0.026 to 0.504 (Vitorino *et al.*, 2017), and the *Geophagus brasiliensis* (Quoy & Gaimard, 1824) which showed an average of 5.33 alleles per locus and  $H_e$  from 0.534 to 0.706 (Ferreira *et al.*, 2015). Also, studies with the Neotropical catfish species *Neoplecostomus yapo* Zawadzki, Pavanelli & Langeani, 2008 (Philippson *et al.*, 2009) and *Hypostomus ancistroides* (Ihering, 1911) (Sofia *et al.*, 2008) using other markers related the low genetic diversity to the sedentary habits of these species.

Despite the low number of alleles detected in the present study, a decrease (20.3%) in the average number of alleles present in the captive animals, when compared to the wild conspecifics from the submiddle, could be observed. Coimbra *et al.* (2017) found a significant loss (from 30 to 50%) in the average number of allelic richness between wild and hatchery samples of *Prochilodus argenteus*. Similarly, Iwersen *et al.* (2019) and Matsumoto, Hilsdorf (2009) analyzed the genetic diversity in *Prochilodus lineatus* (Valenciennes, 1837) and *Brycon insignis* Steindachner, 1877, respectively, and observed a reduction of almost 30% in the number of alleles between wild and hatchery broodstocks. A review conducted by Araki, Schmid (2010) in nearly 50 restocking actions worldwide reported that the decreased number of alleles found in captive breeders compared to those wild conspecifics was the most common feature.

Genetic diversity is used as an essential criterion for evolutionary potential. As long as the population retains genetic diversity, there will be evolutionary adaptation to deal with a variable environment (Frankham *et al.*, 2010; Allendorf *et al.*, 2012). Restocking processes based on culture-based methods, lead to mating closely related individuals and to inbreeding (Keller *et al.*, 2012). The three hatcheries of *L. alexandri* showed high mean inbreeding ( $F_{IS}$ ) values between 0.098–0.198, while the wild upper sampling site presented a negative number (–0.092). The submiddle sampling site, in turn, presented a positive value (0.044). Also, private alleles were more abundant in the upper than in the submiddle stretch. The submiddle stretch of the São Francisco River has in one extreme the reservoir of Sobradinho, and at the other end the reservoirs of Itaparica, Moxotó and Paulo Afonso Complex (I, II, III and IV), with no perennial rivers (ANA, 2003), which makes it the most fragmented stretch of the river. In this cascade of reservoirs, Sobradinho (the second largest lake in South America) is the first one in this region to retain the highest rate of nutrients and sediments, thus creating important modifications on primary productivity downstream, with severe implications for fish communities (Santos *et al.*, 2020). Furthermore, the submiddle stretch is affected by long periods of



droughts (Stolf *et al.*, 2012), contrasted with the upper region, which has 48 perennial tributaries (ANA, 2003). The wild samples from the upper stretch were mostly collected in the dendritic reservoir of Tres Marias, which despite being a reservoir, is rich in nutrients due to marginal pond grasses that become submerged during the rainy season and decompose, providing food for the fish community (Sato, Sampaio, 2005). Therefore, the two wild sampling sites (upper and submiddle São Francisco) constitute contrasting areas in terms of nutrient and sediment input that might explain differences in genetic diversity level. Hence, it is possible to consider that there are at least two units of conservation in this river, one in the upper and another in the submiddle stretch. Additional samples taken in the middle and lower stretches may reveal other units along a gradient system.

The continuous restocking of *L. alexandri* in the submiddle stretch with high levels of inbreeding may reduce population persistence and make the species even more vulnerable. The innate low genetic diversity detected in the *L. alexandri* makes the challenge of restocking actions even more difficult.

In order to avoid the loss of alleles and maximize genetic diversity, it is of utmost importance that restocking actions use an abundant number of breeders (fully genotyped) and that matings be selected under a relatedness cut-off value.

**Genetic structure.** The genetic difference found between wild animals from the upper and the submiddle SF might be related to the sedentary behavior and parental care of this species. Sedentary behavior and short displacements have been pointed to as limiting factors for gene flow, resulting in population structure in many freshwater species, such as *Arapaima gigas* (Schinz, 1822) (Torati *et al.*, 2019), *Geophagus brasiliensis* (Ferreira *et al.*, 2015), *Ichthyoelephas longirostris* (Steindachner, 1879) (Landínez-García, Márquez, 2016), and *Mogurnda adspersa* (Castelnau, 1878) (Hughes *et al.*, 2012). *Lophiosilurus alexandri* inhabits sandy bottoms where it buries itself waiting for its prey (Costa *et al.*, 2015), rarely showing displacement. The sedentary behavior and the distance of nearly 1,500 km between the upper and submiddle sampling sites in the São Francisco River might also have acted to favor a genetic structuring, limiting gene flow.

The genetic structure emphasizes the necessity of having distinct broodstocks for each stretch of the river in conservation aquaculture programs and that the exchange of breeders between hatcheries from different stretches should be avoided.

Considering the levels of genetic differentiation (Tab. 3, Fig. 3), in terms of inbreeding and number of alleles found between hatcheries (Paulo Afonso and Bebedouro) that restock the submiddle SF and the corresponding wild sample, the use of these captive breeders should not be recommended for conservation purposes. The continuing use of these broodstocks for restocking can cause genetic erosion of wild stocks and decrease the adaptive genetic variation and individual fitness, thus limiting the evolutionary potential of the population (Bijlsma, Loeschcke, 2012; Carvalho *et al.*, 2012). Therefore, we suggest replacing the existing captive broodstock with wild animals (fully genotyped), as well as adopting a capture-based restocking method to guarantee the maintenance of genetic diversity and support long-term conservation of the species, or alternatively selecting breeding pairs under a relatedness cut-off value.

**Relatedness.** The developed microsatellites were also useful in identifying the best

genetic relatedness estimators ( $R_W$  and  $R_{LL}$ ) in three full-sib families. Considering the distribution of pairwise relatedness of the three full-sib families, it was possible to determine that 0.12 and 0.03 are the cut-off values for full-sib relatedness, according to  $R_W$  and  $R_{LL}$ , respectively. Considering the innate low diversity of the species,  $R_{LL}$  should be used for selecting matings in captive systems. The practice of using relatedness estimators has been successfully applied in other culture-based restocking programs, such as for the coho salmon (Conrad *et al.*, 2013) and sockeye salmon (Kozfkay *et al.*, 2008).

The distribution of pairwise relatedness in wild samples indicated that there is a considerable number of pairwise combinations with some degree of relatedness even in the natural environment. It is possible to speculate that the life strategy (sedentary habit with parental care) of *L. alexandri* might interfere in the genetic relatedness and matings between related individuals that would happen more frequently than expected by chance.

The availability of *L. alexandri* in the wild is still incipient even after two decades of restocking actions for the species. Reports related to the monitoring of artisanal fishing in the submiddle and lower São Francisco River from June 2017 to April 2018 revealed an average monthly yield of 52.69 kg of *L. alexandri*, compared to 783.5 kg for *Prochilodus argenteus* and 2,861.3 kg for *Myleus micans* (Lütken, 1875), other species that are equally subjected to restocking actions (Chesf, 2017, 2018).

Based on the low genetic diversity recovered from wild and hatchery broodstocks, we recommend that greater efforts should be taken to minimize the genetic loss in captivity and further genetic erosion of wild populations in restocking actions. Towards a conservation aquaculture program for *L. alexandri*, the implementation of the “culture-based restocking” method requires a strategy based on the use of a minimum number of animals to form the founding broodstock, the physical tagging of breeders, the genetic monitoring of breeders and progenies (prior to being released) and the adoption of a genetic relatedness estimator.

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## Neotropical Ichthyology



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### ETHICAL STATEMENT

The research was carried out under the license number, CEUA-UFRPE- 24/2017, from the Animal Ethics Committee of the Universidade Federal Rural de Pernambuco (UFRPE).

### COMPETING INTERESTS

The authors declare no competing interests.

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