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ARTICLES

Full Length Research Paper

Genetic relationships between captive and wild subpopulations of *Arapaima gigas* (Schinz, in Cuvier, 1822)

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***Arapaima gigas*, also known as pirarucu, is endemic to the Amazon basin. There is currently considerable effort being made to cultivate this species to reduce pressure on natural populations. We characterized the diversity and genetic structure of subpopulations of wild and captive *A. gigas* based on 19 microsatellite loci. Captive subpopulations of *A. gigas* exhibited less diversity than wild individuals. We also verified the existence of outlier loci under selective pressure in both subpopulations, indicating the occurrence of local adaptation in some individuals of *A. gigas*. Furthermore, we identified a high-level genetic structure among the subpopulations, with no mixing between subpopulations. The lack of mixing between populations, the local adaptation, and the genetic structure indicate that these subpopulations should be managed and isolated to prevent captive individuals from escaping into the wild, which would reduce the diversity of the wild subpopulations. We suggest a program for the genetic management of captive subpopulations to avoid the random loss of genetic variability and the selection of characteristics that are undesirable for commercial and conservation purposes.**

Key words: Allelic diversity, osteoglossidae, conservation and management, heterezygosity, osteoglossiformes.

INTRODUCTION

The future of aquaculture largely depends upon the effective management of the genetic resources of farmed fish. Maintaining an appropriate level of genetic diversity is critical to aquaculture production and value, and is necessary to ensure the availability of affordable products for human consumption in poor areas worldwide. Unfortunately, most farmed fish species in Brazil have not been fully domesticated, and their wild relatives are

preferentially used for breeding programs and related research. Furthermore, there are many species (possibly hundreds) that might have potential for aquaculture (Pullin, 2007), including *Arapaima gigas*, which is a potential candidate for Brazilian aquaculture, particularly in Northern Brazil.

A. gigas (pirarucu) is endemic to the Amazon basin and is the longest freshwater fish in the world, reaching 3 m in length and more than 200 kg in weight (Castello, 2004). Because of its high economic importance in the fisheries of Northern Brazil, *A. gigas* has recently been introduced into fish farms to facilitate its reproduction and rearing in captivity. This effort has increased in recent years due to the ability of *A. gigas* to adapt to a confined environment and improvements in facility management. Because *A. gigas* is the largest freshwater scaled fish in the world, it has high commercial value and has been overexploited for more than two decades (Viana et al., 2007). Near the principal cities of the Brazilian Amazon, *A. gigas* is considered commercially extinct, and fishermen have reported difficulties in catching *A. gigas* in certain areas (Goulding, 1980; Castello, 2008; Oliveira et al., 2005).

The International Union for the Conservation of Nature (IUCN) has included *A. gigas* on the red list of endangered species (Ortiz et al., 2007) and classified it in Appendix II of the Convention on the International Trade of Endangered Species of Wild Fauna and Flora (CITES), which lists species that are not necessarily extinct but may become extinct if their trade is not controlled (Viana et al., 2007).

To prevent overfishing and preserve the species, the Brazilian Institute of Environment and Natural Resources (IBAMA), a Brazilian government agency, established a minimum size of *A. gigas* for capture (1.5 m total length) in 1989. In 1990, the IBAMA enforced a fishing ban from December 1st to May 31st, which corresponds to the reproductive phase of the species. Since 1996, IBAMA and the Amazonas State Environmental Agency (SDS - Sustainable Development State Secretary) have prohibited the fishing and commercialization of *A. gigas*; fish from managed areas and fish farms are exempted from this prohibition (Arantes et al., 2007). These conservation actions were implemented concurrently with the creation of Sustainable Development Reserves (SDR) in Amazonas State, where fishing occurs with the direct involvement of local fishermen in the management of populations of this species. Therefore, annual portions (quotas) are permitted for the capture of *A. gigas* during a certain period of the year, according to the minimum size limitations. These actions have resulted in reduced fish extraction from the wild environment and increased the interest of fish farmers in the artificial management of breeders.

A new species of *Arapaima* was discovered in the Central Amazon of Brazil (Stewart, 2013a), and it was believed that the *Arapaima* was considered monotypic since 1868, with *A. gigas* is the only recognized species (Stewart, 2013b). This species was collected near the confluence of the Solimões and Purus Rivers, and comparisons with the pirarucus Development Mamirauá shows that there are at least two species of *Arapaima* in the Amazon State.

The cultivation of freshwater fish in Brazil is expanding rapidly due to the availability of favorable areas for cultivation, the existence of an enormous river network, and the high adaptive potential of many native species in captivity. However, aquaculture practices have reduced the genetic variability of captive animals as a result of unassisted breeding or the use of a small number of founders in fish farms (Wasko et al., 2004). Both practices alter genotype frequencies over generations and result in adaptation of the species to the confinement conditions (Santos et al., 2012).

The demand for *A. gigas* is high; therefore, large-scale data on the genetic features of wild and cultivated populations are needed. The sizes of founder populations are generally limited by farming constraints, which results in the use of a few individuals as broodstock. This practice may lead to erosion of the genetic diversity of the stocks, thereby compromising industrial performance. Due to the relatively short history of large-scale *A. gigas* commercial culture, the phenotypic effects of this erosion are not yet clear.

The present study is the first to address the genetic variability of captive *A. gigas* subpopulations. This study aimed to (i) define the genetic structure of *A. gigas* populations in a selected areas by measuring the genetic heterogeneity among wild and captive populations and (ii) estimate the degree of genetic erosion of reared stocks by comparing their genetic variability with that of geographically close wild stocks.

MATERIALS AND METHODS

Ethics committee

The group leader, Adalberto Luis Val was granted a license for capture under the umbrella project ADAPTA (SISBIO License #29837-4). Samples from wild subpopulations were collected according to the guidelines set by the IBAMA of the Ministry of Environment. All samples were collected from anesthetized animals according to the guidelines from Brazilian Council for Ethics in the use of Experimental Animals (CONCEA) and the Ministry of Science, Technology and Innovation.

Collection of samples

Wild *A. gigas* individuals were captured from the SDR during the legal period for managed fishing with the help of a harpoon, which was the only gear allowed. Local fishermen are licensed to harpoon the fish body (never the head) and, subsequently place fish in ice boxes to commercialize them. The spear catch is a traditional (cultural) way used by locals to minimize damage in fish meat. Captive individuals were captured with trawls with permission from two private farm onwers and with the permission Ceara State Aquaculture Facility. Because these fish were intended for the local

Figure 1. Geographical locations of the *Arapaima gigas* sample collection sites in the Brazilian Amazon (North) and Northeastern Brazil: (A) Departamento Nacional de Obras Contra as Secas (DNOCS) Pentecoste - Ceará; (B) Fishfarm Porto Velho (PPV), Porto Velho - Rondônia, (C) Sustainable Development Reserve Mamirauá (SDRM) Tefé - Amazonas; (D) Sustainable Development Reserve Fonte Boa (SDRFB), Fonte Boa - Amazonas and (E) Fishfarm FrigoPesca (PFP-1, -2 and -3), Manacapuru - Amazonas.

market, they were anesthetized and euthanized in ice water prior to the collection of tissue samples. A total of 10 g of white muscle was collected from each *A. gigas* sample using the tweezers process for the stilettos and then transferred to sterile eppendorf tubes and immersed in liquid nitrogen. The samples were stored at a low temperature (-80°C) until subsequent DNA analysis.

Samples of white muscle were collected from 218 wild (two subpopulations) and captive (five subpopulations) *A. gigas* individuals from Northern and Northeastern Brazil (Figure 1). The wild individuals were collected from the SDR in Mamirauá (SDRM; 25 samples) (01° 49' 00" S and 65° 42' 00" W) and Fonte Boa (SDRFB; 18 samples) (02° 30' 52" S 66° 05' 33" W) in Amazonas. All samples of captive individuals were taken from F_1 progeny and acquired from three fish farms: FrigoPesca (Amazonas), from which samples were collected three different times (every 6 months) between 2009 and 2010 (PFP-1: 47 samples, PFP-2: 18 samples, and PFP-3: 32 samples) (03° 17' 39" S and 60° 38' 04" W); Porto Velho (Rondonia State) (PPV: 48 samples) (08° 45' 43" S and 63° 54' 07" W); and Estação de Piscicultura do Departamento Nacional de Obras Contra as Secas (Ceará State) (DNOCS: 30 samples) (03° 47' 29" S and 39° 15' 58" W).

DNA extraction and PCR amplification

Total genomic DNA was extracted from the 218 tissue samples using the protocol described by Sambrook et al. (1989) with some modifications. Approximately, 100 mg of tissue was digested overnight at 37°C in 0.7 ml of lysis buffer (6 M urea, 10 mM Tris-HCl, 125 mM NaCl, 1% SDS, 10 mM EDTA, pH 7.5); 10 µl of proteinase K (Fermentas - Thermo Fisher Scientific - Suwanee, GA

USA), RNase A (Invitrogen - Life Technologies- Grand Island, NY) and 1,4-dithiothreitol (Biosynth® Chemistry and Biology Rietlistr, Switzerland) were then added. After digestion, the samples were washed with phenol-chloroform-isoamyl alcohol (Invitrogen) and isopropanol on ice. The pellet was resuspended in 50 µl DNase-free H_2O. The quality of the extracted DNA was verified by electrophoresis in a 1.5% agarose gel using GelRed® (Invitrogen) and visualization using an L-PIX Molecular Image transilluminator (Loccus Biotecnologia - São Paulo, Brazil). The DNA was quantified using a NanoDrop® 2000 spectrophotometer (Thermo Scientific - Suwanee, GA USA).

The *A. gigas* samples were genotyped using the 10 microsatellite loci identified by Farias et al. (2003) (AgCTm1, AgCTm3, AgCTm4, AgCTm5, AgCTm7, AgCTm8, AgCAm4, AgCAm16, AgCAm18, and AgCAm26) and the 9 loci identified by Molecular Ecology Resources Primer Development (Consortium et al., 2011) (AG_01, AG_02, AG_03, AG_07, AG_09, AG_11, AG_12, AG_13, and AG_15). Polymerase chain reactions (PCR) were performed using a 96-well Veriti™ Thermal Cycler (Applied Biosystems - Life Technologies - Grand Island, NY) in a final reaction volume of 10 µl. Each PCR contained 1.5 µl of genomic DNA (50 ng/µl), 1.0 µl of forward primer (0.4 mM), 1.5 µl of reverse primer (0.4 mM), 1.0 µl 6-FAM fluorescent M13 primer [14] (0.4 mM), and 5.0 µl PCR Master Mix 2X (Fermentas) containing Taq DNA polymerase (0.05 U), $MgCl_2$ (1.5 mM), and dNTPs (0.4 mM). The PCR cycling conditions were as follows: 94°C for 3 min; 20 cycles at 94°C for 20 s, 56 to 58°C for 20 s, and 68°C for 30 s; 25 cycles at 94°C for 20 s, 53°C for 20 s, and 68°C for 30 s, and a final extension at 72°C for 10 min. The amplified PCR products were confirmed by electrophoresis in a 1.5% agarose gel using GelRed® (Invitrogen) and visualization using an L-PIX Molecular Image transilluminator (Loccus

Biotecnologia). The amplified products were genotyped on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems) using the GeneScan Liz-500 (-250) size standard (Applied Biosystems) to determine fragment length. The alleles were scored based on the consistent pattern of their stutter peaks and the peak intensity in each individual at each locus using GeneMapper v4.0 (Applied Biosystems).

Data analysis

Genetic diversity

The genetic diversity of the *A. gigas* subpopulations was estimated based on the following parameters: the observed (H_O) and expected (H_E) heterozygosities (Fstat v2.9.3.2) (Goudet, 2001); the number of alleles per locus (Genetix v4. 5.2) (Belkhir et al., 2004); the number of private alleles or the fixation index (F_{IS}) (GDA v1.1) (Lewis and Zaykin, 2000); the polymorphism information content (PIC) (Cervus v3.0.3) (Kalinowski et al., 2007); and the effective number of alleles per locus (A_E). To calculate the effective number of alleles, we used the following mathematical formula: $A_E = 1/(1-H_E)$. Micro-Checker v2.3 software (van Oosterhout et al., 2004) was used to test for the presence of null alleles, and their frequencies were calculated using Cervus v3.0.3 software (Kalinowski et al., 2007). The linkage disequilibrium (*LD*) and Hardy-Weinberg equilibrium (*HWE*) were calculated using the Fstat software, with sequential Bonferroni corrections to minimize the errors of type I microsatellite markers (Rice, 1989). The significance level was α = 0.05.

Genetic differentiation

The levels of genetic differentiation among sampling sites were analyzed using the *F* statistics of Wright (F_{IT}, F_{IS} and F_{ST}) (Weir and Cockerham, 1984) using the software Fstat and Arlequin v3.5 (Excoffier and Lischer, 2010). The indices of genetic differentiation, F_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995), were applied to better address the mutational models for the microsatellite data (e.g., IAM for F_{ST} (Kimura and Crow, 1964) and SMM for R_{ST} (Kimura and Otha, 1978). We also applied 3 estimators of genetic differentiation, G_{STest} (Nei and Chesser, 1983), G'_{STest} (Hedrick, 2005), and D_{est} (Jost, 2008). These estimators were calculated using Smogd v1.2.4 software (Crawford, 2010). Analysis of molecular variance (AMOVA) (Excoffier and Slatkin, 1995) was performed for the 7 *A. gigas* subpopulations using Arlequin software.

Analysis of population structure

Structure v2.3.1 software (Pritchard et al., 2000; Falush et al., 2003) was used to verify the level of genetic structure among the *A. gigas* subpopulations under the ancestral admixture model, which correlates gene frequencies between subpopulations. Subsequently, the data generated a burn-in of 50,000 steps per 200,000 using the Markov chain Monte Carlo (MCMC) test for inference. We used the Structure Harvest v0.6.8 software (Earl and Holdt, 2012) to check the value of ΔK (according to Evanno et al., 2005), which represents the highest level of hierarchy of the genetic divisions. Furthermore, we conducted a run for each test with K = 1 and K = the number of sampled populations +2, with 3 replicates for each K. To complement the analysis of genetic structure, we performed a factorial correspondence analysis (FCA) (Benzecri, 1973) of the genetic distances between subpopulations using Genetix software, allowing the program to identify potential outlier individuals. The values of F_{ST} (Weir and Cockerham, 1984) and the

genetic distance (D_A) (Nei, 1978; Cavalli-Sforza and Edwards, 1967) in pairs were constructed after comparing the subpopulations of *A. gigas*. A neighbor-joining tree (NJ) was constructed using GDA software based on genetic distance according to Nei (1978).

Bottlenecks and detection of outlier loci (F_{ST}-outlier method)

We analyzed the occurrence of bottleneck populations (with an excess of heterozygotes) in the wild and captive *A. gigas* subpopulations using Bottleneck software (Cornuet and Luikart, 1996; Piry et al., 1999). This analysis was used to detect potential bottleneck populations of no more than $2N_E$-$4N_E$ for past generations, depending on the severity of the bottleneck and the rate of change of the loci analyzed. In this study, we applied 2 mutational models [Stepwise mutation model (SMM) and Two phase model (TPM)]. These models are the most appropriate for evaluating microsatellite data (di Rienzo et al., 1994) and were implemented at 95% (SMM) and 5% (TPM) for the various stages of mutation, ranging 12% among several steps, as recommended by Piry et al. (1999).

Outlier loci were detected using 2 different approaches. First, a coalescent-based simulation approach was used to identify outlier loci with unusually high and low F_{ST} values by comparing the observed F_{ST} values with the values expected under neutrality (Beaumont and Nichols, 1996), as implemented in the Lositan selection workbench (Antao et al., 2008). We performed an initial run with 100,000 simulations on all loci using the mean neutral F_{ST} as the preliminary value. A more accurate estimate of the mean neutral F_{ST} was obtained after the first run by excluding all loci outside the 95% confidence interval because their distribution could result from selection rather than neutral evolution. This refined estimate was used for a final set of 100,000 simulations of all loci. In addition, we used the method described by Vitalis et al. (2001), which investigates outliers in a pairwise fashion based on subpopulation-specific *F*-statistics. The coalescent simulations were performed with Detsel v1.0 (Vitalis et al., 2003). The null distributions were generated using the following parameters: population size before the split $N_0 = 500$; mutation rate $\mu = 0.0001$ and 0.00001; ancestral population size $N_e = 500$, 1000 and 10000; time since the bottleneck $T_0 = 50$, 100 and 1000; and time since the population split $t = 100$. The outliers were determined based on an empirical p value for each locus array of 50 × 50 square cells (Vitalis et al., 2001).

RESULTS

Genetic diversity of wild and captive subpopulations

Summaries of the genetic and allelic diversities of the *A. gigas* subpopulations are presented in Tables 1 and S1 (Supporting information). We observed the presence of null alleles for loci AG_03 (PPV) and AG_15 (PPV, RDSFB and RDSM). A total of 146 alleles were observed for the 19 microsatellites markers, with an average of 7.68 alleles per locus. The total number of alleles ranged from 49 (2.58 alleles/loci; DNOCS) to 88 (4.63 alleles/loci; SDRM). We also detected the occurrence of 49 private alleles (Table 2), 23 of which had a frequency of greater than 5%. The SDRM (wild) subpopulation showed higher values for the mean number of alleles (MNA = 4.632) and allelic richness (AR = 4.258), whereas the group from DNOCS (captive) showed the

Table 1. Genetic diversity indices for wild and captive subpopulations of *Arapaima gigas* based on 19 microsatellite loci.

Origin	Subpopulations	Allelic diversity					Genetic diversity			
		N	TNA	MNA	NEA	NPA	AR	H_E	H_O	F_{IS}
Captive	PFP-1	47	51	2.684	1.621	5	2.587	0.383	0.473	-0.225**
	PFP-2	18	59	3.105	2.066	9	3.060	0.516	0.695	-0.317**
	PFP-3	32	52	2.737	1.852	4	2.680	0.460	0.551	-0.179**
	DNOCS	30	49	2.632	1.730	3	2.562	0.422	0.542	-0.266**
	PPV	48	50	2.684	1.684	5	2.567	0.406	0.482	-0.177**
Wild	SDRFB	18	75	3.947	2.004	7	3.815	0.501	0.559	-0.085[(1)]**
	SDRM	28	88	4.632	2.268	16	4.258	0.559	0.591	-0.035[(1)]**

N, sample size; *TNA*, total number of alleles; *MNA*, mean number of alleles; *NEA*, number of effective alleles; *NPA*, number of private alleles; *AR*, allelic richness; H_E, genic diversity (expected heterozygosity in Hardy-Weinberg equilibrium); H_O, observed heterozygosity; F_{IS}, inbreeding coefficient of population; (n), number of loci that deviated from Hardy-Weinberg equilibrium (HWE); level of significance, **$p < 0.05$.

Table 2. Values of the genetic differentiation indices for the 7 subpopulations of *Arapaima gigas* based on 19 microsatellite loci.

Locos	PIC	F_{IT}	F_{IS}	F_{ST}	R_{ST}	G_{Stest}	G'_{Stest}	D_{est}
AgCTm1	0.454	0.381*	-0.106	0.441*	0.544*	0.358*	0.534*	0.275*
AgCTm3	0.657	0.157*	-0.155	0.270*	0.430*	0.204*	0.526*	0.405*
AgCTm4	0.707	-0.214	-0.425	0.148*	0.855*	0.143*	0.495*	0.410*
AgCTm5	0.823	0.347*	-0.064	0.387*	0.440*	0.306*	0.828*	0.752*
AgCTm7	0.235	0.214*	0.097	0.130*	0.160*	0.113*	0.148*	0.039*
AgCTm8	0.557	0.225*	-0.430	0.458*	0.411*	0.397*	0.645*	0.411*
AgCAm4	0.299	-0.210	-0.572	0.230*	0.230*	0.192*	0.285*	0.115*
AgCAm16	0.848	0.420*	0.091	0.362*	0.347*	0.305*	0.860*	0.798*
AgCAm18	0.198	-0.008	-0.080	0.067*	0.083*	0.052*	0.068*	0.017*
AgCAm26	0.623	-0.080	-0.174	0.216*	0.152*	0.162*	0.426*	0.315*
AG_01	0.756	0.277*	-0.110	0.349*	0.335*	0.306*	0.754*	0.646*
AG_02	0.447	0.179*	0.012	0.169*	0.156*	0.174*	0.343*	0.205*
AG_03	0.628	0.036	-0.298	0.257*	0.106*	0.190*	0.463*	0.337*
AG_07	0.653	0.128*	-0.192	0.268*	0.192*	0.219*	0.545*	0.418*
AG_09	0.600	0.297	-0.034	0.320*	0.433*	0.265*	0.577*	0.424*
AG_11	0.462	0,040	-0.238	0.225*	0.326*	0.203*	0.370*	0.210*
AG_12	0.733	0.243*	-0.224	0.382*	0.366*	0.330*	0.745*	0.620*
AG_13	0.726	-0.213	-0.501	0.192*	0.135*	0.162*	0.486*	0.387*
AG_15	0.452	0.337*	0.110*	0.255*	0.408*	0.257*	0.470*	0.286*
All	0.571	0.156	-0.185	0.288	0.327	0.228	0.504	0.277

PIC, Polymorphism information content; F_{IT}, fixation index of the total population; F_{IS}, fixation index of the subpopulations; F_{ST}, fixation index result for the comparison of the subpopulations to the total populations; R_{ST}, analogous to F_{ST} of microsatellite markers; G_{Sest}, estimator of relative differentiation; G'_{Sest}, standardized measure of genetic differentiation; D_{est}, current estimate of differentiation; level of significance, *$p < 0.05$.

lowest values (MNA = 2.632 and AR = 2.562).

The indices of genetic diversity in this study ranged from 0.473 to 0.695 (H_O), 0.383 to 0.559 (H_E), 0.198 to 0.848 *PIC*, and -0.317 to -0.035 (F_{IS}). Of the 19 studied loci, 13 were highly informative (Table 2). However, 6 loci were monomorphic in some subpopulations of *A. gigas* (Table S1). Only the wild subpopulations had F_{IS} values

close to 0 (Table 1), indicating that these subpopulations tended to approach Hardy-Weinberg equilibrium ($F_{IS} \sim 0$; $H_O \sim H_E$). The loci AG_09 (RDSFB) and AG_15 (RDSM) significantly deviated from Hardy-Weinberg equilibrium (HWE) ($P < 0.05$, Bonferroni correction 0.0004). However, we did not detect linkage disequilibrium (LD) for the analyzed pairs of loci ($p > 0.05$).

Table 3. F_{ST} (above) and genetic distance values (below) for *A. gigas* subpopulations based on 19 microsatellite loci.

Variables	PFP-1	PFP2	PFP3	DNOCS	PPV	SDRFB	SDRM
PFP1	-	0.278	0.255	0.338	0.364	0.318	0.299
PFP2	0.341(0.341)	-	0.270	0.265	0.284	0.223	0.230
PFP3	0.281(0.000)	0.439(0.281)	-	0.280	0.341	0.242	0.241
DNOCS	0.422(0.000)	0.377(0.433)	0.374(0.396)	-	0.312	0.298	0.266
PPV	0.477(0.422)	0.390(0.000)	0.502(0.000)	0.397(0.000)	-	0.261	0.299
SDRFB	0.433(0.000)	0.371(0.440)	0.360(0.000)	0.458(0.377)	0.332(0.000)	-	0.135
SDRM	0.440(0.477)	0.458(0.000)	0.408(0.439)	0.429(0.000)	0.481(0.371)	0.207(0.458)	-

The genetic distances calculated according to Nei (1978) are shown; the distances calculated according to Cavalli-Sforza and Edwards (1967) are shown within parentheses.

Table 4. Analysis of molecular variance (AMOVA) for the 7 subpopulations of *Arapaima gigas* based on 19 microsatellite loci.

Source of variation	% var	F-stat	F-value	Standard error	p-value
Within individuals	0.866	F_{IT}	0.134	0.044	-
Between individuals	-0.081	F_{IS}	-0.103	0.043	1.000
Between populations	0.215	F_{ST}	0.215	0.016	0.001*

*Indicates a significant difference at $p < 0.05$.

Genetic differentiation and population structure

The indices of genetic differentiation (F_{ST}, R_{ST}, G_{Stest}, G'_{Stest}, and D_{est}) were significant for all examined loci ($p < 0.05$). The locus AgCAm18 was the only marker that showed low (D_{est} = 0.017) and moderate (R_{ST} = 0.083) genetic differentiation. The average value of F_{ST} (0.288) was very close to the G_{Stest} (0.228) and D_{est} (0.277) indices (Table 2). The F_{ST} values between pairs of *A. gigas* subpopulations ranged from 0.135 to 0.364, revealing that the level of genetic differentiation between the analyzed pairs varied from high to extremely high (Table 3). The AMOVA revealed that the distribution of genetic variability was 21.5% among the subpopulations and 86.6% among individuals of the subpopulations (Table 4).

We verified the occurrence of genetic structure among the *A. gigas* subpopulations with distinct groups (Figure 2); the clusters were based on the data inferred from differences in allelic frequencies. The average association coefficient (*q*) for each subpopulation (K = 7) showed that the individuals of *A. gigas* are distributed (Table S2) in specific agglomerations (q > 0.9), resulting in a high-resolution *q* value and indicating that no subpopulation was mixed. The FCA yielded values of 6.61 and 6.32% for Axes 1 and 2, respectively; the dispersion graph of those parts is shown in Figure 3. Notably, there was a clear separation of subpopulations in the wild, with Axis 1 > 0 and Axis 2 < 0, and the formation of two *A. gigas* populations: one group consisting of the subpopulations PFP-1, PFP-2, PFP-3, and DNOCS (group 1; Axis 1 < 0), and a second group consisting of the PPV, SDRM, and SDRFB subpopulations (group 2, Axis 1 > 0) (Figure 3). The separation of the subpopulations into these two distinct groups by FCA was consistent with the data obtained for the NJ tree based on the genetic distances between the subpopulations (Figure 4).

Bottlenecks and detection of outlier loci (F_{ST}-outlier method)

To detect bottlenecks, we eliminated the loci that drifted from Hardy-Weinberg equilibrium because the presence of these loci could strongly influence the final results and because of the effect of selection on the over-dominant loci with an excess of heterozygotes (Luikart and Cornuet, 1998). Furthermore, we verified the minimum number of microsatellite markers needed for an analysis of recent genetic bottlenecks, as the model for mutational markers could influence the final results if an inappropriate number of SSR loci were used.

We detected a recent population bottleneck in all analyzed subpopulations of *A. gigas*. Both the Sign and Wilcoxon tests showed a significant excess of heterozygotes for the TPM mutational model. However, only the captive subpopulations showed an excess of heterozygotes using the Sign test. The mutational model SMM was significant only for the subpopulations PFP-2 and PFP-3 using the Wilcoxon test.

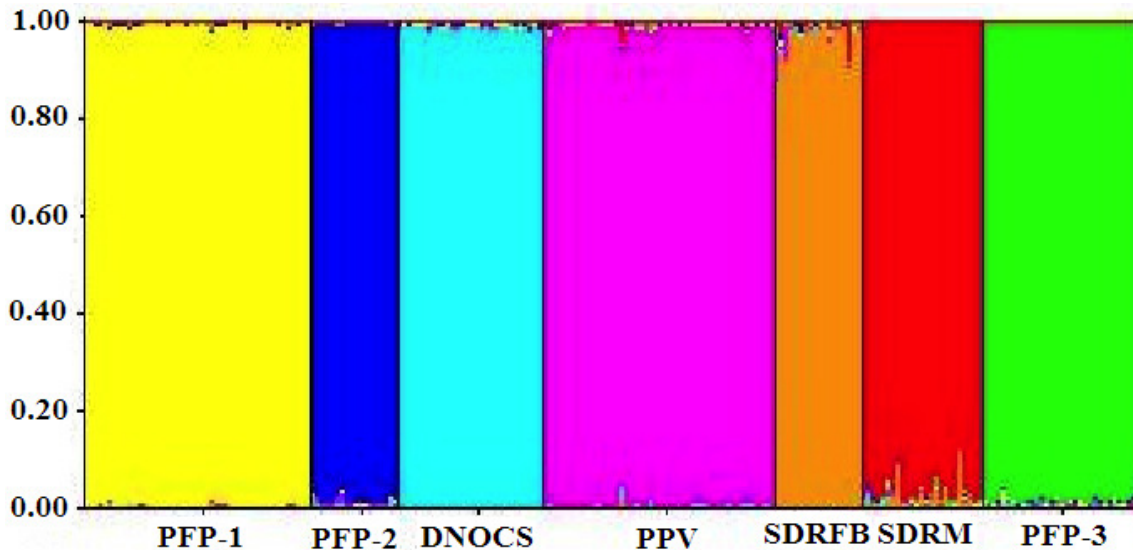We observed 10 outlier loci in the *A. gigas* subpopulations;

Figure 2. Structural analysis results for the 7 subpopulations based on 19 microsatellite loci. K = 7.
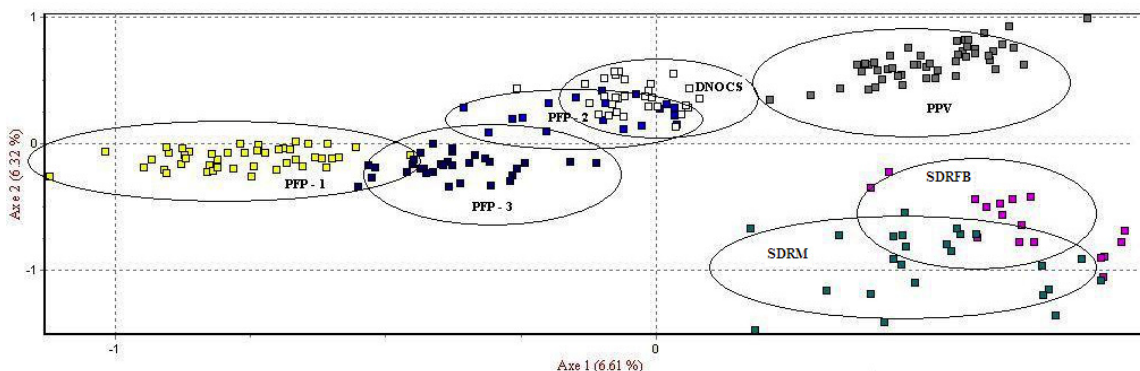

Figure 3. Factorial correspondence analysis (FCA) of the 7 subpopulations based on 19 microsatellite loci.
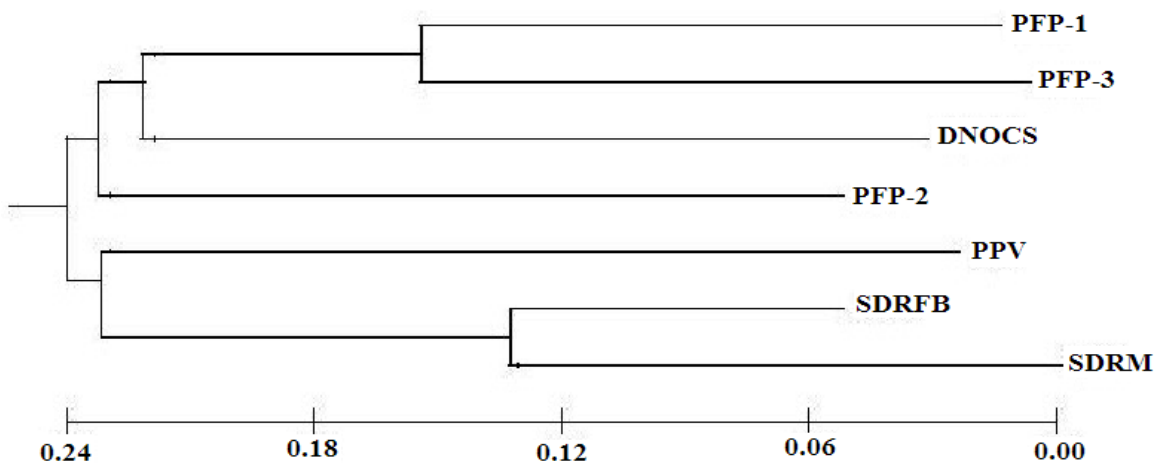

Figure 4. Neighbor-joining dendrogram for wild and captive subpopulations of *Arapaima gigas* based on 19 microsatellite loci.
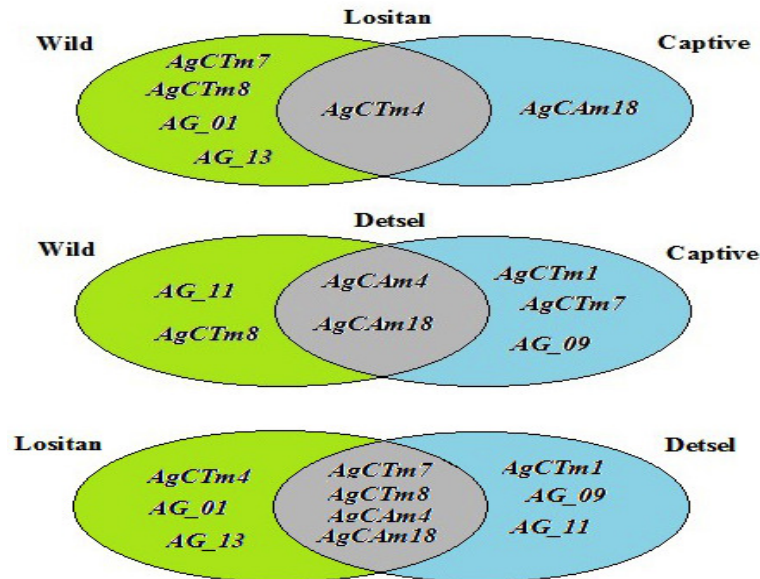
Figure 5. Outlier loci detected among the 7 studied subpopulations according to Beaumont and Nichols (1996) and Vitalis et al. (2001) using Lositan (Antao et al. 2008) and Detsel v1.0 (Vitalis et al. 2003), respectively.

four of these loci were identified as common to all subpopulations by Lositan and Detsel software (Figure 5), which suggests that these four loci may be related to the local adaptation of *A. gigas*. The Lositan program identified four outlier loci with balanced selective pressure for the 2 subpopulations of wild and captive specimens. Furthermore, we detected a locus with positive selective pressure for wild individuals, while the remaining loci were considered neutral markers (Figure S1).

DISCUSSION

Genetic differences between wild and captive subpopulations

According to Ellegren and Sheldon (2008), populations are usually in evolutionary equilibrium; however, evolutionary responses may be hidden due to deterioration or loss of the environment. In addition, the connectivity between populations plays a crucial role in the maintenance of genetic variation, particularly in small populations strongly affected by genetic drift (Broquet et al., 2010). Genetic analyses of 7 subpopulations of *A. gigas* using 19 microsatellite markers revealed that most of the markers were highly polymorphic, with 13 loci showing values of PIC > 0.5 (Table 2). The results also revealed the occurrence of moderate genetic diversity in *A. gigas* subpopulations, indicating that the evolutionary potential of the wild individuals from the SDR is greater than that of the captive individuals, as expected (Table

1). Hedrick (2001) reported that the pattern of variation within a given variable population generally consists of neutral alleles, that is, the equilibrium is predicted by a reduction of variation through genetic drift and an increase in variation by random mutation. Therefore, even when a population is small, selection may act on the variation in a particular gene, but genetic drift acts more strongly than selection on the allele frequencies.

An excess of heterozygotes was observed in all *A. gigas* populations (Table 1). We also observed decreased heterozygosity at the AgCAm16 locus in all the domesticated and wild populations, which suggests a recent evolutionary history because this locus is predicted to be under intensive selective pressure. According to Barroso et al. (2005), deficiency in heterozygous loci may occur due to positive selection, improvement of the relatedness between individuals, and population structure. We observed higher allelic richness in wild subpopulations (SDRFB and SDRM) compared to the captive subpopulations; the observed values are consistent with those reported by Silva (2008) for individuals of *A. gigas* in the Jarauá and Maraã lakes (SDRM). Araripe et al. (2013) investigated the wild subpopulations of *A. gigas* in a large geographical area; based on analyses of 7 informative microsatellite regions, they proposed that the genetic differentiation between subpopulations should increase with increasing geographical distance between them and that the differences among populations separated by geographical distances greater than 1300 km could be caused by historical bottlenecks in population size, in

light of the sedentary behavior of the species.

Here, we observed that the average genetic differentiation among all subpopulations (F_{ST}) was 21.5% ($p < 0.05$), demonstrating the high discrimination of the seven studied subpopulations (Figure 2) and the absence of mixtures (Table S2). This genetic differentiation suggests that the captive subpopulations are increasingly isolated due to lack of gene flow between fish farms, which merits further consideration. However, gene flow is also limited between individuals of the wild subpopulations of the SDR. According to Teixeira (2008), *A. gigas* individuals caught in the wild may not represent the total population because a group may have descended from one pair of individuals. According to Glover et al. (2011), genetic differences have been observed between Atlantic salmon fry in fish sites populated for commercial reproduction from farms of the same company, indicating that the captive environment accelerates genetic differentiation. However, Hedrick (1999) reported that the number of highly polymorphic microsatellite F_{ST} might be underestimated and that such an underestimate could influence the observed proportion of heterozygotes in subpopulations compared to the total population. In this context, Hartl and Clark (2010) reported that the estimated F statistic must correct the effects of sampling and the limited number of subpopulations, and suggested that new indices would provide a correction of the values of F_{ST}.

The recent bottleneck in captive subpopulations is certainly due to the founder effect, which results from the use of a small number of individuals to establish reproductive stocks in fish farms. Furthermore, the lack of allelic richness in captive subpopulations may have contributed to the genetic signature of a new bottleneck (Broquet et al., 2010). These results reinforce the idea that the small number of breeding stations in fish farms may contribute to the genetic deterioration of captive populations.

The bottleneck detected in wild subpopulations is likely due to intense fishing activity in recent years. Furthermore, the practice of selective fishing in the SDR has likely decreased breeding inventories and, subsequently, the effective population size. A decrease in wild fish stocks was reported in the mid-1990s, when the *A. gigas* production reached 207.5 tons, compared with the previous decade, when production was 1,751 tons (Venturiere and Bernardino, 1999). Luikart and Cornut (1998) reported that natural populations show changes in the frequency of heterozygotes when a stock experiences a recent bottleneck.

We observed that the *A. gigas* subpopulations are undergoing selection, leading to local adaptation to the new environmental conditions that the species has been recently exposed to (Figure 5). We observed four loci with balanced and positive selection in the wild groups. In the captive groups, we identified two loci with balanced selection. However, when all groups were considered, we

observed six loci with positive selection, indicating the existence of local adaptive processes in the wild and captive subpopulations. According to Orr (1998), genotyping many loci is likely important to identify complex adaptive differences between populations. The adaptive complexity provided by genotypic differentiation is directly related to balanced selection, which is common in confined populations but rare in wild populations. Furthermore, balanced selection, which favors heterozygotes, is more frequent than selection favoring homozygotes, thereby maintaining this variation (Amos and Balmford, 2001). The balanced selection of wild *A. gigas* populations observed in this study is likely due to a recent bottleneck experienced by these subpopulations due to over-fishing.

Local adaptations might be lost through intraspecific hybridization; therefore, actions that increase the amount of genetic exchange among locally adapted populations could be harmful. According to Utter (2004) and Einum and Fleming (1997), the release of individuals from hatcheries or their escape from aquaculture ponds could harm wild populations through intraspecific hybridization and the loss of local adaptations. Nunes et al. (2011) suggested that the identification of loci with adaptive importance is a key step toward understanding the process of speciation in natural populations because these loci are responsible for phenotypic variations that affect the fitness of the species in different environments. According to Sønstebø et al. (2007) and Vasemägi et al. (2005), analysis of the genetic differences between the captive broodstocks and wild populations is also important because the specific genes of each population reflect local adaptability; consequently, the survival of juveniles will be affected if the populations are mixed. Our results indicate that this species requires management and conservation.

Implications for management and conservation

There is a global need to preserve genetic resources by maintaining species and their genetic diversities. The identification of actions that will minimize the effects of genetic changes (or losses) within biological populations is one of the primary mechanisms for maintaining variable and genetically healthy populations (Boettcher et al., 2010). According to He et al. (2012), microsatellite markers, in combination with recent statistical methodologies, represent a useful tool for the conservation and management of endangered species. For *A. gigas* subpopulations, allelic richness should be maintained and conserved, with a particular focus on preserving the existing rare alleles within populations, as suggested by Pérez-Ruzafa et al. (2006). Genetic information is also needed to aid the understanding of the current level of conservation of endangered species (Laikre et al., 2009).

Conservation projects focusing on the management and conservation of the genetic diversity of endangered species must consider the likely environmental changes (Boettcher et al., 2010) caused by stochastic events or human activity. Our results suggest that subpopulations of *A. gigas* have had independent evolutionary trajectories, and thus effective conservation measures should focus on preserving the genetic integrity of each subpopulation separately. Therefore, we suggest a more detailed management of breeding groups and periodic monitoring of wild populations.

Hrbek et al. (2005) proposed the use of a large group of matrices of *A. gigas* (founders) in fish farms, regardless of their geographical origin, thereby minimizing the potential loss of genetic diversity due to founder effects. Furthermore, it has been suggested that confined populations be supplemented with wild individuals from selected areas to maintain high genetic diversity in confinement systems. However, before we consider maintaining or restoring the genetic diversity, we should be alert to individuals collected from nature that served as new breeding on fish farms, as these new breeding, can they bring in parasites from fish farms that might jeopardize the survival of older breeding and consequently reduce the effective size and negatively affect the genetic diversity of these populations.

In addition, we suggest the rotation of *A. gigas* groups within and between fish farms to maintain the evolutionary potential of the species and avoid greater adaptation to captive systems. *A. gigas* fingerlings from several wild and captive breeders could also be distributed to fish farmers. However, the formation of closely related couples should be avoided to reduce consanguinity, which could compromise the genetic health of *A. gigas* lots for future distribution.

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Conflict of Interest

The author(s) have not declared any conflict of interest.

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SUPPLEMENTARY

Table S1. Basic parameters for genetic diversity associated with subpopulations of *Arapaima gigas* based on 19 microsatellite loci.

Loci	Subpopulations						
	Captive					Wild	
	PFP-1	PFP-2	PFP-3	DNOCS	PPV	SDRFB	SDRM
AgCTm1							
H_O	0.540	0.944	0.040	0.000	0.381	0.133	0.300
H_E	0.470	0.577	0.039	0.000	0.363	0.124	0.460
A	2.000	5.000	2.000	1.000	2.000	2.000	3.000
A_E	1.887	2.364	1.040	1.000	1.570	1.142	1.852
F_{IS}	-0.136	-0.619	0.000	-	-0.038	-0.037	0.370
AgCTm3							
H_O	0.667	0.714	0.875	0.654	0.512	0.786	0.348
H_E	0.552	0.766	0.650	0.587	0.414	0.566	0.401
A	3.000	5.000	4.000	3.000	3.000	4.000	4.000
A_E	2.232	4.274	2.857	2.421	1.706	2.304	1.669
F_{IS}	-0.195	0.103	-0.327	-0.095	-0.226	0.355	0.154
AgCTm4							
H_O	1.000	1.000	0.926	1.000	0.775	0.875	0.947
H_E	0.636	0.611	0.593	0.680	0.537	0.793	0.813
A	4.000	4.000	3.000	4.000	3.000	7.000	7.000
A_E	2.747	2.571	2.457	3.125	2.160	4.831	5.348
F_{IS}	-0.563	-0.615	-0.625	-0.454	-0.434	-0.071	-0.139
AgCTm5							
H_O	0.667	0.722	0.478	0.654	0.244	0.611	0.920
H_E	0.554	0.619	0.593	0.499	0.219	0.775	0.818
A	3.000	3.000	4.000	3.000	3.000	6.000	10.000
A_E	2.242	2.625	2.457	1.996	1.280	4.444	5.494
F_{IS}	-0.193	-0.139	0.214	-0.242	-0.102	0.238	-0.104
AgCTm7							
H_O	0.000	0.000	0.000	0.083	0.364	0.556	0.417
H_E	0.000	0.000	0.000	0.079	0.469	0.438	0.448
A	1.000	1.000	1.000	2.000	3.000	3.000	3.000
A_E	1.000	1.000	1.000	1.086	1.883	1.779	1.812
F_{IS}	-	-	-	-0.022	0.236	-0.241	0.091
AgCTm8							
H_O	0.526	0.875	0.577	0.040	0.650	1.000	0.000
H_E	0.410	0.492	0.482	0.039	0.466	0.500	0.000
A	3.000	2.000	2.000	2.000	2.000	2.000	1.000
A_E	1.695	1.968	1.930	1.040	1.873	2.000	1.000
F_{IS}	-0.271	-0.765	-0.179	0.000	-0.383	-1.000	-
AgCAm4							
H_O	0.000	0.500	0.222	0.923	0.674	0.000	0.840
H_E	0.000	0.375	0.198	0.497	0.447	0.000	0.487
A	1.000	2.000	2.000	2.000	2.000	1.000	2.000
A_E	1.000	1.600	1.247	1.988	1.808	1.000	1.949

Table S1. Contd.

F_{IS}	-	-0.300	-0.106	-0.852	-0.500	-	-0.714
AgCAm16							
H_O	0.425	0.533	0.556	0.458	0.585	0.706	0.550
H_E	0.429	0.560	0.578	0.478	0.608	0.808	0.714
A	3.000	3.000	3.000	3.000	3.000	8.000	5.000
A_E	1.751	2.273	2.370	1.916	2.551	5.208	3.496
F_{IS}	0.021	0.082	0.058	0.063	0.050	0.156	0.254
AgCAm18							
H_O	0.000	0.357	0.259	0.423	0.275	0.000	0.250
H_E	0.000	0.304	0.226	0.429	0.237	0.000	0.224
A	1.000	3.000	2.000	3.000	2.000	1.000	3.000
A_E	1.000	1.437	1.292	1.751	1.311	1.000	1.289
F_{IS}	-	-0.140	-0.130	0.033	-0.147	-	-0.092
AgCAm26							
H_O	0.610	0.938	0.731	0.625	0.462	0.824	0.636
H_E	0.495	0.623	0.659	0.640	0.380	0.644	0.548
A	3.000	3.000	3.000	3.000	3.000	6.000	4.000
A_E	1.980	2.652	2.932	2.778	1.613	2.809	2.212
F_{IS}	-0.220	-0.480	-0.089	0.044	-0.201	-0.251	-0.140
AG_01							
H_O	0.456	0.929	0.581	0.440	0.727	0.733	0.591
H_E	0.416	0.600	0.564	0.343	0.664	0.624	0.606
A	3.000	3.000	3.000	2.000	3.000	3.000	3.000
A_E	1.712	2.500	2.293	1.522	2.976	2.660	2.538
F_{IS}	-0.087	-0.523	-0.013	-0.263	-0.083	-0.141	0.049
AG_02							
H_O	0.372	0.533	0.370	0.440	0.349	0.438	0.524
H_E	0.313	0.580	0.417	0.385	0.318	0.482	0.622
A	3.000	3.000	2.000	2.000	2.000	2.000	3.000
A_E	1.456	2.381	1.715	1.626	1.466	1.930	2.646
F_{IS}	-0.176	0.115	-0.123	-0.088	0.125	0.182	0.130
AG_03							
H_O	0.575	1.000	0.967	0.720	0.405	0.938	0.579
H_E	0.434	0.635	0.499	0.518	0.544	0.691	0.460
A	3.000	4.000	2.000	3.000	3.000	6.000	4.000
A_E	1.767	2.740	1.996	2.075	2.193	3.236	1.852
F_{IS}	-0.314	-0.553	-0.933	0.371	0.267	-0.327	-0.234
AG_07							
H_O	0.537	1.000	0.500	0.800	0.540	0.706	0.550
H_E	0.429	0.653	0.530	0.540	0.422	0.600	0.671
A	3.000	4.000	3.000	2.000	3.000	4.000	5.000
A_E	1.751	2.882	2.128	2.174	1.730	2.500	3.040
F_{IS}	-0.240	-0.511	0.077	-0.466	-0.269	-0.146	0.205
AG_09							
H_O	0.619	0.750	0.692	0.375	0.024	0.125	0.909
H_E	0.520	0.553	0.644	0.520	0.047	0.602	0.549

Table S1. Contd.

A	3.000	3.000	3.000	3.000	3.000	4.000	3.000
A_E	2.083	2.237	2.809	2.083	1.049	2.512	2.217
F_{IS}	-0.178	-0.328	-0.055	0.298	0.500	0.804*	-0.644
AG_11							
H_O	0.286	0.600	0.875	0.733	0.200	0.000	0.920
H_E	0.272	0.460	0.655	0.518	0.180	0.000	0.755
A	3.000	3.000	3.000	3.000	2.000	1.000	6.000
A_E	1.374	1.852	2.898	2.075	1.220	1.000	4.082
F_{IS}	-0.039	-0.273	-0.316	-0.402	-0.099	-	-0.199
AG_12							
H_O	0.415	0.429	0.577	0.593	0.780	0.750	0.783
H_E	0.362	0.400	0.467	0.450	0.535	0.589	0.751
A	3.000	3.000	3.000	3.000	3.000	6.000	11.000
A_E	1.567	1.667	1.876	1.818	2.150	2.433	4.016
F_{IS}	-0.133	-0.033	-0.218	-0.300	-0.449	-0.241	-0.019
AG_13							
H_O	1.000	0.882	1.000	0.962	0.909	1.000	0.920
H_E	0.725	0.493	0.627	0.499	0.598	0.650	0.748
A	4.000	2.000	4.000	2.000	3.000	4.000	6.000
A_E	3.636	1.972	2.681	1.996	2.488	2.857	3.968
F_{IS}	-0.370	-0.778	-0.583	-0.923	-0.511	-0.519	-0.211
AG_15							
H_O	0.300	0.500	0.240	0.370	0.310	0.444	0.250
H_E	0.255	0.506	0.339	0.308	0.262	0.633	0.539
A	2.000	3.000	3.000	3.000	2.000	5.000	5.000
A_E	1.342	2.024	1.513	1.445	1.355	2.725	2.169
F_{IS}	-0.164	0.044	0.311	-0.185	-0.171	0.323	0.554*
Total							
H_O	0.473	0.695	0.551	0.542	0.482	0.559	0.591
H_E	0.383	0.516	0.460	0.422	0.406	0.501	0.559
A	2.684	3.105	2.737	2.632	2.684	3.947	4.632
A_E	1.621	2.066	1.852	1.730	1.684	2.004	2.268
F_{IS}	-0.225	-0.317	-0.179	-0.266	-0.177	-0.085	-0.035

*Locos with deviation for Hardy-Weinberg equilibrium (HWE), $p < 0.05$ (Bonferroni correction; 0.0004).

Table S2. Member proportions of each population according to the seven clusters determined with Structure Software.

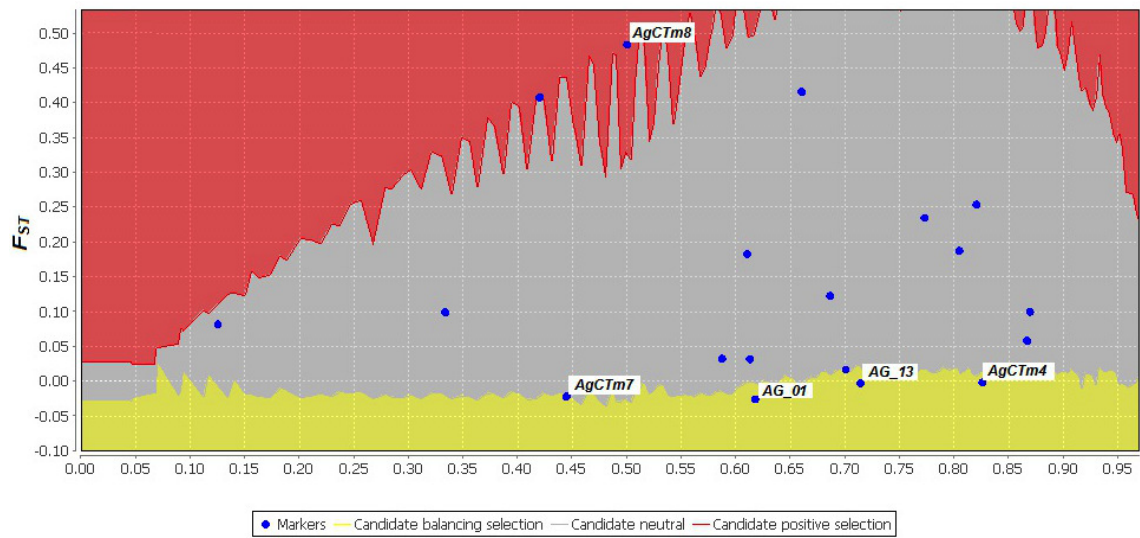| Populations/cluster	1	2	3	4	5	6	7
PFP-1	0.002	0.003	0.003	**0.984**	0.002	0.003	0.002
PFP-2	0.003	0.003	**0.978**	0.004	0.005	0.006	0.003
PFP-3	0.002	**0.980**	0.003	0.004	0.003	0.004	0.003
DNOCS	0.002	0.003	0.003	0.003	0.003	**0.983**	0.002
PPV	0.003	0.003	0.003	0.003	**0.981**	0.004	0.003
SDRFB	0.012	0.005	0.004	0.003	0.011	0.004	**0.961**
SDRM	**0.962**	0.004	0.004	0.004	0.004	0.004	0.019

(Contribution > 0.9 in bold).

Figure S1. Outlier loci with positive selective pressure for wild individuals.

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