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Communication

[*Comunicação*]

Utilization of microsatellite markers to form families of "pejerrey" *Odontesthes bonariensis* in a genetic breeding program

[*Utilização de marcadores microssatélites para formação de famílias de "pejerrey"* Odontesthes bonariensis *em um programa de melhoramento genético*]

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The freshwater "pejerrey" (*Odontesthes bonariensis* Valenciennes, 1835) is a South American *atherinid* species with economic importance due to its high economic and nutritional value (Tsuzuki *et al*., 2007). This species originally colonized lakes and lagoons of Argentina and Rio Grande do Sul state (Brazil). Also, it has been successfully stocked in basins and dams in other areas of Brazil, Argentina an other South American countries, in view of its fishing values (Piedras *et al*., 2009). The high quality of this fish has created an incentive for its cultivation in regions distant from its native occurrence, like Japan and Italy (Baigún *et al*., 2009).

The starting point for the success of *O. bonariensis* is the consolidation of a genetic breeding program. Therefore, it is necessary to acquire breeders for the foundation of families; and the analysis of the founding genetic population is necessary to avoid or minimize effects of endogamy and loss of genetic variability (Tave, 1999). The maintenance of genetic variability in management practices in aquaculture is another factor to be considered, since this parameter confers adaptability and resistance, affecting growth and reproduction (Povh *et al.*, 2009).

Molecular markers of the microsatellite types are being used to analysis of endogamy coefficients, population differentiation indexes and stock variability (Yue *et al*., 2009; Patel Das *et al*., 2010), showing efficacy and sensibility, giving support to acquaculture management programs. Genetic variability and divergence were identified, through polymorphism of six microsatellite markers, in two populations of *O. bonariensis* raised under cultivation practices and with a potential source of reproducers for genetic improvement.

Six clones of microsatellites of *O. bonariensis* were selected out of 23 clones maintained in the GenBank, and used for genetic analysis (Koshimizu *et al*, 2009). The primers were designed with the aid of PerlPrimer (Ferreira *et al*., 2009), in which the size of distinct fragments, of each *locus*, allowed the optimization of genotyping through the pairs combination of *loci* (Table 1).

The polymorphism of the microsatellite *loci* was evaluated from 80 samples of two populations of *O. bonariensi*: 40 samples were collected at the Chascomús lake (Section of Buenos Aires, Argentina – 35° 35'S, 58° 02'W) and 40 samples at the Chasqueiro dam (Arroio Grande, Brazil – 32°10'S 53°00W). Chascomús is considered an oligohaline (<5gL^{-1}) lake, with average

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temperature of 14°C, average pH of 8.6 and high concentration of suspended materials (Capítulo and Freire, 1995; Berasain *et al*., 2005). In 1925, atherine fish ("pejerrey") incubators were installed in the city of Chascomús, Argentina, furnishing fertilized eggs and larvae for introduction in public and private waters. However, these programs were precarious with little support and were limited to embryo allotment or newly ecloded larvae and without supervision of water courses (Somoza *et al*., 2008). The Chasqueiro dam, built at the end of the 1970s, has a channel system approximately 50km long and physical infrastructure contributing for regional agricultural production (ALM, 1994). The region presents a sub-tropical climate, average water temperature varying from 11 to 27°C, pH of 6.8 to 7.4 and alkalinity from 30 to 45 mgL^{-1} (ALM, 1994). The presence of "pejerrey" is a consequence from the introduction made with the purpose of increasing the local fishery production, since this species does not occur naturally in artificial reservoirs (Piedras, unpublished).

Table 1. Clones of microsatellites of "pejerrey" *Odontesthes bonariensis*

Locus	Accession	Repeat motif	Primer sequences 5´- 3´	T_a (°C)	Allele (pb)
Obo19TUF	AB375413.1	$(CA)_{16}$	F: TACTCAGCCTACCCTAATGCG R: TTGTGTGTTTGTGTGTGGAGG	55ºC	215-237
Obo21TUF	AB375414	$(GT)_{30}$	F: TGTGGGTTGTAATCTCCTGCC R: TTGTGTGTTTGTGTGTGGAGG	56ºC	160-200
Obo64TUF	AB375424.1	$(GT)_{20}$	F: GGAAGTGACCAGATAGGCAGC R: TGCTGCCATTTCCTTCCTG	55ºC	132-152
Obo71TUF	AB375426.1	$(CA)_{40}$	F: TCCTCCCTTTCTGTCTGTTTCC R: TTAGGACACCAGAGCCCAAAG	56ºC	170-208
Obo77TUF	AB375428.1	$(CA)_{28}$	F: TCAGAGACGGATACACACTTGGC R: TCTACCAAATCGGGGGAAGG	58ºC	158-186
Obo79TUF	AB375429.1	$(CA)_{16}$	F: GCGGTGACAGGACAACTATTTC R: GGGATGTTTTCAGTGGTGTCAG	56ºC	94-100

T_a, annealing temperature.

Genomic DNA was extracted from the caudal flipper (200-300 mg), stored in 95% ethanol and kept at -20°C, using a modified salt protocol (Barrero *et al*., 2008). Amplifications of PCR were made in a final volume of 25µL, containing 20ng of genomic DNA, 5pmol of each *locus* specific primers, 100µM of dNTPs, 1X *buffer* of Taq reaction buffer and 0.25U of DNA Taq polymerase (Fermentas, Burlington, Canada). Amplification conditions were 5min at 94°C, followed by 35 cycles of 45s at 94°C, 45s according to the temperature of each *locus* and 45s at 72°C, with a final extension time of 8 min at 72°C. The PCR products were verified in agarose gel at 1% stained with GelGreen™ (Biotium, Hayward, USA). All products were submitted to gel electrophoresis in Spreadex EL 600 Wide Mini S-2x25™ (Elchrom Scientific, Zurich, Switzerland). Gels were performed at 60V, for 4.5 min and afterwards elevated to 120V and 254mA for 360 min, with constant temperature of 20°C. The alleles were obtained using the scale Marker M3™ (Elchrom Scientific, Zurich, Switzerland). The confirmation of allele sizes was done through selection of samples with different alleles, submitted to genotyping in automatic sequential ABI 377 Genetic Analyzer (Applied Biosystems, Foster City, USA) and analyzed with software Genotyper 2.0® (Applied Byosystems, Foster City, USA). The number of alleles of each *locus*, expected (He) and observed (Ho) heterozygosity, index of endogamy (Fis), Hardy-Weinberg equilibrium test of probability (HWE) and genetic differentiation (Fst) were obtained using the GENOPOP version 4.0 software (Rousset, 2008).

From the total of six *loci*, five demonstrated efficiency for genetic analysis in the two populations of *O. bonariensis*. Seventeen microsatellites were amplified by Koshimizu *et al*. (2009) among 25 individuals of "pejerrey" collected from natural water bodies in Argentina (Laguna Chascomús, n=9). The *locus* 0bo64TUF showed low amplification in the samples from Argentina and no amplification in Brazilian samples. The number of alleles varied from 4 to 15 and for all samples a total of 49 alleles was obtained, with positive values of 8.16 alleles per

locus. Similar results were obtained by Koshimizu *et al*. (2009), who reported a number of alleles per *locus* in "pejerrey" ranging from 3 to 18 (mean of 10). Beheregeray and Sunnucks (2000) successfully amplified in species *O. bonariensis* and *O. humensis* twelve microsatellite *loci* developed for the species *O. argentinensis. O. perugiae* showed a number of alleles per locus ranging from 2 to 11. The index of endogamy with positive values suggests a deficit of heterozygote for all *loci* analyzed in the two populations. For the Brazilian population, only the *locus* 0bo77TUF appears to be in equilibrium of Hardy-Weinberg while in the Argentine population the *loci* 0bo19tuf, 0bo64TUF and 0bo79TUF are in Hardy-Weinberg; equilibrium (Table 2). Koshimizu *et al*. (2009) reported significant deviations from Hardy-Weinberg equilibrium detected in Obo19TUF. No significant linkage disequilibrium was evidenced between *loci*. The more accentuated deficit of heterozygote in the population of Chasqueiro (Brazil) can be explained by the foundation effect at the time of the animal introduction in the dam (Hartl and Clark, 1997). Probably, few spawns were used and there was no common source control. Because of a few founders, exists the possibility of accentuated break of genetic variability of the new populations, when compared to the original population. There also exists a high probability of endogamy occurrence.

Table 2. Genetic analysis of six *loci* microsatellites in the two populations of "pejerrey" *Odontesthes bonariensis*

Locus	N	A	Ho	He	Fis	HWE
Obo19TUF						
Brasil	40	4	0.25	0.534	0.5352	0.0071*
Argentina	40	3	0.525	0.617	0.1513	0.1925
Obo21TUF						
Brasil	39	10	0.615	0.829	0.2796	0.0112*
Argentina	40	13	0.85	0.90	0.0572	0.0156*
Obo64TUF						
Brasil	-	-	-	-	-	-
Argentina	21	4	0.523	0.659	0.2101	0.1938
Obo71TUF						
Brasil	36	7	0.5	0.773	0.3565	0.0093*
Argentina	40	9	0.675	0.869	0.2263	0.0278*
Obo77TUF						
Brasil	30	5	0.7	0.7909	0.1168	0.1518
Argentina	39	9	0.692	0.8078	0.1126	0.0184*
Obo79TUF						
Brasil	40	4	0.625	0.7573	0.1688	0.0433*
Argentina	38	4	0.605	0.725	0.1681	0.0700

*The *loci* with significant deviation from HWE (P<0.05). N, sample size; A, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; Fis, inbreeding coefficient; HWE, Hardy-Weinberg equilibrium.

The value of Fst between the two populations was 0.1303, which supports that the populations show moderate differentiation (P<0.05), caused by geographical separation, through the obstruction of gene flow (Liu *et al*., 2009).

The loss of the genetic variability decreases the capacity that a population has to adapt to different environmental conditions, being of extreme importance the selective reproduction. However, the genetic variation is difficult to estimate without polymorphic genetic markers. The high polymorphism, analyzed in five microsatellite *loci*, provided an efficient tool to the study of genetic variation of *O. bonariensis.*

The significant genetic differentiation in the analyzed populations can supply the basis for future programs of genetic improvements, through the combination of material from divergent populations to develop lines or accomplishment of a selection program. These markers will be used in the orientation of crossings to form a base-population for the genetic improvement that is in progress.

Keywords: fish, genetic variability, polymorphism

RESUMO

Foram identificadas a divergência e a variabilidade genética, por meio do polimorfismo de seis marcadores microssatélites, de duas populações de Odontesthes bonariensis, utilizadas em manejos de cultivo e com potencial para fornecimento de reprodutores para programas de melhoramento genético. Do total de seis loci, cinco demonstraram eficiência para análise genética nas duas populações de O. bonariensis. A diferenciação genética significante nas populações analisadas pode fornecer a base para futuros programas de melhoramentos genéticos, através da combinação de material das populações divergentes para o desenvolvimento de linhagens ou execução de um programa de seleção.

Palavras-chave: peixe, variabilidade genética, polimorfismo

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