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Rapid and inexpensive analysis of genetic variability in *Arapaima gigas* by PCR multiplex panel of eight microsatellites

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ABSTRACT. The aim of the present study was the development of a multiplex genotyping panel of eight microsatellite markers of *Arapaima gigas*, previously described. Specific primer pairs were developed, each one of them marked with either FAM-6, HEX or NED. The amplification conditions using the new primers were standardized for a single reaction. The results obtained demonstrate high heterozygosity (average of 0.69) in a Lower Amazon population. The multiplex system described can thus be considered a fast, efficient and inexpensive method for the investigation of genetic variability in *Arapaima* populations.

Key words: *Arapaima gigas*; Pirarucu; Microsatellites; Multiplex polymerase chain reaction; Conservation genetics; Lower Amazon

INTRODUCTION

Arapaima gigas is the largest tropical fresh water fish, reaching up to 3 m in length and 200 kg (Saint-Paul, 1986). *Arapaima* is distributed mainly in the flooded regions of the Amazon Basin, locally known as pirarucu, and in the upper Essequibo River in Guyana (Lüling, 1964).

Traditionally, *Arapaima* has been of great importance in the diet of Amerindians living on the Amazon region rivers. From the early 18th century on, fishing *Arapaima* became commercially important for the Amazon Basin people. This economic importance has lasted from the colonial times to the present days. Due to overfishing, *A. gigas* is one of the few fish species listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora II. Despite its economic importance, little is known about its genetic structure and its population distribution in the Amazon (Farias et al., 2003; Hrbek et al., 2005).

Estimating the genetic diversity of natural populations is one of the most important forms of evaluating a species' viability. For most fish species, this kind of investigation has been centered on the analysis of the variability of microsatellite markers (Perez-Enriquez et al., 1999).

Fourteen different microsatellites of *Arapaima* have been described (Farias et al., 2003). However, the individual genotyping of several microsatellite markers is costly and time consuming. A way of reducing costs and optimizing analysis is the use of multiplex microsatellite polymerase chain reaction (PCR), as it amplifies several microsatellites in a single PCR and allows the electrophoresis of all amplified fragments in a single capillary or lane (Neff et al., 2000).

In the present study, we describe the development of a multiplex panel of eight microsatellite markers of *Arapaima*. We used this multiplex genotyping system to verify the level of genetic variability in the natural population of *Arapaima* in the Lower Amazon.

MATERIAL AND METHODS

A total of 32 samples of *Arapaima* were caught in Sauaçu Lake in the Juruti municipality in the Lower Amazon, Pará State, Brazil. An amount of 10 g of muscle tissue was collected from each individual, preserved in 95% ethanol, and stored at 4°C. Total genomic DNA was extracted from tissue digested in a proteinase K/sodium dodecyl sulfate solution, and DNA purification was by the standard phenol/chloroform method, followed by precipitation with isopropanol (Sambrook et al., 2001).

From the 14 microsatellites isolated by Farias et al. (2003), eight markers were selected (AgCAm2, AgCAm13, AgCAm15, AgCAm16, AgCAm18, AgCTm3, AgCTm5, AgCTm7) based on the highest level of polymorphism of each microsatellite. For each marker, new primer pairs were developed using the program Primer3 (Rozen and Skaletsky, 2000). Each new forward primer was marked with one of the following fluorescent dyes: FAM-6, HEX or NED (Table 1). All primers had the same annealing temperature and all markers with the same fluorescent dye amplified fragments were of different sizes to avoid allele overlapping.

Multiplex PCR was carried out in 20-µL reaction volumes containing 5.5 µL double-

Table 1. Multiplex panel used to genotype 32 *Arapaima gigas* individuals from Sauaçu Lake in the Juruti municipality, Lower Amazon region, Pará State, Brazil.

Locus GenBank Accession No.	Repeat motif	Primer (5′-3′)	Dye	Size range (bp)	n_A	H_O	H_E
AgCAm16 AY176183	$(CA)_{19}$	F: GCAAGCAGTAAAGAAAGGACAGA R: AACGGACCCACTTCTCTATGTG	HEX	91-105	10	0.78	0.83
AgCAm15 AY176181	$(CA)_{19}$	F: GGCATCAAATGTGACTGCTACT R: GAACAAGGCTAGATTGCAAAGTT	FAM-6	128-144	4	0.59	0.61
AgCAm13 AY176180	$(GTA)_2(CA)_{27}$	F: TGTTTCCAACTGTGTCCAACA R: TGCTGTAAACACCAGTTACCTCA	HEX	135-169	10	0.71	0.75
AgCTm3 AY176173	$(CT)_{15}$	F: CAAAAGGATTGCCTGTTAGGG R: CCAGGTATGAGTTTTGCAAGGT	NED	138-146	5	0.78	0.75
AgCTm5 AY176175	$(CT)_{28}$	F: AAGGATCGATACATCAAGAGCTTC R: CTCCCTCTGTGGTGCTACAAT	NED	175-191	5	0.56	0.65
AgCAm18 AY176183	$(GA)_2(CA)_9CTC$ AAACT$(CA)_{20}$	F: CTCAACTGTGATGCTGGGAAT R: CTTTGGCCCAGGATAAACAA	FAM-6	175-193	6	0.59	0.60
AgCAm2 AY176178	$(CA)_{22}$	F: AGGAGATGCAAACACCCTGTA R: CCGATCATCTGTTTGCTCTGT	HEX	217-247	13	0.90	0.84
AgCTm7 AY176176	$(CT)_{29}$	F: AAGGATCGATACATCAAGAGCTTC R: ACGGTGAATATCTGTGCTTGG	FAM-6	233-259	11	0.65	0.76

n_A = number of alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity.

distilled H_2O, 2.0 µL 10X buffer (100 mM Tris-HCl, 500 mM KCl), 0.7 µL $MgCl_2$ (15 mM), 9.6 µL primer mix (0.3 µM of each forward and reverse primer), 1.0 µL dNTP (200 µM of each dNTP), 0.2 µL of Platinum™ *Taq* DNA polymerase (Invitrogen) (5 U/µL), 1 µL of DNA (10 ng). PCRs were run on a Perkin Elmer GeneAmp 9700 with initial denaturation at 94°C for 11 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and final a extension at 72°C for 30 min. Products were visualized in Applied Biosystems 3130 Genetic Analyzer. Allele sizes were stipulated using the size standard GeneScan 500 (Rox) (Applied Biosystems). Consistent allele designation and typing quality were assured by simultaneous electrophoresis analysis of an allele sample of known size. Samples were genotyped using the software GeneMapper 3.7 (Applied Biosystems).

The primers developed were validated by direct sequencing of each microsatellite of homozygous individuals. Individual PCRs were carried out for each system without the use of marked primer. Sequencing reactions were performed with BigDye® v3.0 (Applied Biosystems) kit. The sequencing products were visualized in the Applied Biosystems 3130 Genetic Analyzer.

We analyzed the genetic variability using the observed and expected heterozygosity and Hardy-Weinberg equilibrium deviations. We also estimated the allele number per locus and compared the proportion of locus pairs in linkage disequilibrium. All tests were carried out using the software Arlequin 3.01 (Excoffier et al., 2005) with the Bonferroni correction.

RESULTS

The observed heterozygosity found in the *Arapaima* population of the Lower Amazon showed an average of 0.69, with the allele number per locus ranging from four (AgCTm5) to thir-

teen (AgCAm2), an average of 8.0 alleles. None of the loci displayed significant Hardy-Weiberg equilibrium deviations. There were no locus pairs with significant linkage disequilibrium.

DISCUSSION

The observed heterozygosity value found was high and similar to that reported by Farias et al. (2003) in the analysis of 14 microsatellites of *Arapaima* in 15 individuals caught in Santarém City, Pará State, Brazil. This high genetic variability found in our analysis also corroborates the findings of Hrbek et al. (2005) for two mtDNA segments in several *Arapaima* populations in the Amazon Basin.

The multiplex panel of eight microsatellites developed in this study was shown to be capable of quantifying the genetic variability of the *Arapaima* population investigated. The results indicate that it can be used as a fast and inexpensive method with high discriminating power to evaluate the variability of *Arapaima* populations.

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