

PRIMER NOTE

Characterization and isolation of DNA microsatellite primers for *Arapaima gigas*, an economically important but severely over-exploited fish species of the Amazon basin

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Abstract

Arapaima gigas is a CITES II listed species, but it is also one of the most important food fish of the Amazon basin with a historically very large commercial and subsistence fishery. By the 1980s it became commercially extinct in many areas, and in early 2001 the Brazilian government banned all fishing, although this has not prevented illegal fishing. Therefore we developed 14 variable microsatellite markers for *A. gigas* to gain a knowledge of its population structure, which is needed to implement a sound management policy, and also to provide us with the forensic tools to manage and monitor this over-exploited fish.

Keywords: Amazon, *Arapaima gigas*, conservation genetics, cross-species amplification, microsatellites, pirarucu

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Arapaima gigas is one of the most important food fish species of the Amazon basin. Traditionally, *Arapaima* has formed a significant portion of the diet of Amerindians living near larger bodies of water. Starting as early as the late 18th century, a commercial fishery was developed for *Arapaima* to satisfy demand for bacalhau, a salted and dried cod for which *Arapaima* was found to be an excellent freshwater substitute. Its prominence and commercial importance increased during the colonial era as a cheap protein source and its popularity continues to this day. Until the early 1960s, *Arapaima* appears to have been very abundant near the main Amazonian cities, such as Manaus in the State of Amazonas, Brazil, and Santarém and Belém in the State of Pará, Brazil, which served as centres of export and preparation of bacalhau (Crossa & Petrere 1999). Due to its enormous commercial importance, *Arapaima* became increasingly scarce in the 1970s until in the 1980s, it became commercially extinct near larger Amazonian cities (Goulding 1980). In 1975, *Arapaima* became one of the few fish species listed

by the Convention on International Trade in Endangered Species (CITES) II convention. In early 2001, the Brazilian government banned all fishing for *Arapaima*, although this does not prevent illegal fishing, and sale in markets and restaurants. Knowledge of the population structure of *A. gigas* is needed to implement sound management policies and to provide us with forensic tools to manage and monitor this over-exploited fish. Therefore we developed and report 14 highly variable microsatellite markers for *A. gigas*.

Total genomic DNA was extracted from muscle or liver tissue preserved in 95% ethanol. The tissue was dissolved and digested with a proteinase K/sodium dodecyl sulphate (SDS) solution followed by phenol and chloroform extraction, then, addition of 5 M NaCl followed by 70% ethanol precipitation of DNA product. Microsatellite loci were isolated and identified from a partial genomic library enriched for CA and CT repeats following the protocol of Tenzer *et al.* (1999) modified by Garner *et al.* (2000). Enriched DNA was ligated into an Invitrogen pCR®4 TOPO cloning vector and transformed into chemically-competent *Escherichia coli* cells supplied in the TOPO TA Cloning® kit, following the manufacturer's recommendations. After recovery, the transformed cells were grown

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Table 1 Characteristics of 14 microsatellite loci of 15 *Arapaima gigas* individuals from Santarém, Pará, Brazil

Locus GenBank No.	Repeat motif	Primer (5'–3')	Size range (bp)	No. of alleles	T_a (°C)	H_O	H_E
AgCTm1 AY176172	(GT) ₃ (CT) ₁₂	F: CCTGTTGCTCATCTGTTATC R: ATTGCCTCCTGTTCTTGTCG	302–304	2	55	0.40	0.46
AgCTm3 AY176173	(CT) ₁₅	F: ATCTGTTTGTTGGGTCCTCGAC R: TGTGAGGACAAGCTCCAGAG	293–299	3	55	0.73	0.63
AgCTm4 AY176174	(CT) ₂₂	F: TTTCCCGGACGAGAGAAGCTG R: TGTACCAAAGTGTATGGAGAG	275–283	3	58	0.73	0.66
AgCTm5 AY176175	(CT) ₂₉	F: GCAGCCCCAGTCTTGGGAAGG R: AGCACGGTGAATATCTGTGC	258–280	8	58	0.67	0.87
AgCTm7 AY176176	(CT) ₂₉	F: CCTCTACTTCTCAACCAGC R: CAGCACGGTGAATATCTGTG	277–299	6	58	0.67	0.71
AgCTm8 AY176177	(CT) ₅ AT(CT) ₅ AT(CT) ₄ TA(CA) ₁₃ (CT) ₁₃	F: TGGAATCCATTGTGACAGC R: GACATCTTTTCCAGCTAGCC	272–274	2	58	0.53	0.42
AgCAm2 AY176178	(CA) ₂₂	F: AGCTCTCAGTACTGATGCTG R: CCGATCATCTGTTTGCTCTG	295–323	7	58	0.60	0.85
AgCAm4 AY176179	(CA) ₇	F: CGGGGCTTTTGACTGCAAGC R: GGAAGGGGTCGGCTCAGTG	210–212	2	55	0.00*	0.30
AgCAm13 AY176180	(GTA) ₂ (CA) ₂₇	F: TGAAAATCCTGTGGGACCTG R: CCTAAAAACACATCACACTG	300–334	6	58	0.73	0.80
AgCAm15 AY176181	(CA) ₁₉	F: GGCTTACATCAAGCACTTAA R: TTACTGGGTTGAGTTTGTGAC	226–244	5	58	0.67	0.61
AgCAm16 AY176182	(CA) ₁₉	F: CTCCTGGGCATCATGGGTAG R: TCTGTGTCCTCCAGGCAACAG	247–273	6	58	0.40	0.50
AgCAm18 AY176183	(GA) ₂ (CA) ₉ CTCACT(CA) ₂₀	F: TTACTGAGGGCAATGACACG R: TGATTACTCAGCAGGTCCTG	357–375	4	58	0.53	0.64
AgCAm20 AY176184	(CA) ₁₂	F: GGAATGAGCAGGTTTCCCAG R: CTCCTCTTCTGACATGACG	263–267	3	58	0.80	0.60
AgCAm26 AY176185	(CA) ₁₄	F: ACAGGGACCAGTAAGTGCC R: TGCCATAAGCACCCGGTAGG	213–217	3	58	0.53	0.66

All forward primers were labelled with FAM-6, HEX or TET dye. Repeat motif is derived from a sequenced clone; T_a , locus specific annealing temperature; H_O , observed heterozygosity; H_E , expected heterozygosity; * significant heterozygote deficiency ($P < 0.05$).

overnight on a 1 × Luria-Bertani (LB)/Amp (Maniatis *et al.* 1982) agar plate. Individual colonies were picked and re-grown for 6 h in a 96-well culture plate containing 180 µL of liquid 1 × LB/Amp solution. Polymerase chain reaction (PCR) amplification using M13 forward (–20) and M13 reverse primers was performed directly on the bacterial cultures following the PCR protocol of Invitrogen. PCR products were purified using Quiagen spin-columns, and sequencing reactions were performed with internally-placed T7 and T3 primers using Perkin Elmer's recommended protocol for BigDye® V2.0 sequencing chemistry. Products were visualized on a Perkin Elmer ABI 3100 Genetic Analyser.

High quality microsatellite repeats were found in 45 of 96 clones sequenced. Primers annealing to flanking regions were designed for the 45 positive clones. PCR was performed in 10 µL reaction volumes containing 5.5 µL double-distilled H₂O, 1.0 µL 10 × buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂), 2.0 µL primer mix (0.2 µM each forward and reverse primer), 0.8 µL dNTP mix (200 µM each dNTP), 0.2 U Sigma RedTaq® DNA Polymerase, and about 5 ng DNA. PCRs were run on a Perkin Elmer

GeneAmp PCR 9700. An initial denaturation step (94 °C, 2 min) was followed by 35 cycles of 10 s at 94 °C, 10 s at the locus specific annealing temperature (Table 1), 30 s at 72 °C, and a final extension step for 60 min at 72 °C. Sixteen of the primers consistently produced strong single bands and therefore, we labelled the forward primers with the dyes FAM-6, HEX and TET to allow multiplexing. PCR products generated with labelled primers were visualized on a Perkin Elmer ABI 3100 Genetic Analyser. Allele sizes were scored against an internal GeneScan-500 (Rox) size standard. Individuals were genotyped using GENESCAN Analysis 3.7 and GENOTYPER 3.7 software from Perkin Elmer.

Of the 16 loci analysed, two were monomorphic and 14 were variable (Table 1). Levels of variability detected at these 14 loci were high, with the number of alleles ranging from two to seven. Eleven of the 14 microsatellites had observed heterozygosities greater than 50% (Table 2). Using Arlequin (Schneider *et al.* 2000) we found a significant heterozygote deficiency ($P < 0.05$) for the locus AgCAm4.

Although *A. gigas* has no close relatives, we tested for cross-species amplification of primers' in *Heterotis niloticus*,

Table 2 Cross-species amplification with *Arapaima gigas* microsatellite primers

Species	Locus													
	AgCT m1	AgCT m3	AgCT m4	AgCT m5	AgCT m7	AgCT m8	AgCA m2	AgCA m4	AgCA m13	AgCA m15	AgCA m16	AgCA m18	AgCA m20	AgCA m26
<i>Gnathonemus petersii</i>	++	++	++	-	++	+	++	-	++	-	++	-	++	-
<i>Heterotis niloticus</i>	-	-	-	-	-	-	+	-	-	-	+	-	++	-
<i>Osteoglossum bicirrhosum</i>	-	++	-	-	++	++	-	-	-	-	-	+	++	-
<i>Osteoglossum ferreirai</i>	-	-	-	-	+	-	-	-	-	-	-	-	-	-
<i>Pantodon buchholzi</i>	++	++	-	-	++	+	+	-	++	-	-	++	-	-

++ indicates a very good, and potentially useful amplification; + indicates weaker amplification or multiple bands; - indicates no amplification. Amplifications were done at 50 °C annealing temperature, the rest of the protocol was same as for *A. gigas*.

the African sister species of *A. gigas*, as well as more distantly related osteoglossoform fish including *Osteoglossum bicirrhosum* and *O. ferreirai*, both from the Amazon basin, *Pantodon buchholzi* from West Africa and the mormyrid *Gnathonemus petersi* from Central Africa (Table 2). All reactions were carried out at a 50 °C annealing temperature, with the rest of the amplification protocol the same as that for *A. gigas*. Loci were judged as potentially useful in other species if they amplified as a strong distinct band in the size range near that of *A. gigas*; weaker bands or multiple bands were scored as questionable but of potential use (Table 2). PCR fragments for other species were not sequenced to determine homology or the presence of a repeat element.

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