 doi: 10.1046/j.1471-8286 .2003.00375.x

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

IZENI P. FARIAS,*† TOMAS HRBEK,†§ HENNER BRINKMANN,† IRACILDA SAMPAIO‡ and AXEL MEYER†

*Universidade do Amazonas, Departamento de Biologia, ICB, Manaus, Amazonas, Brazil, †University of Konstanz, Faculty of Biology, Konstanz, Germany, ‡Universidade Federal do Pará, Campus de Bragança, Pará, Brazil, §Washington University School of Medicine, Department of Anatomy and Neurobiology, St. Louis, MO, USA

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

Received 18 September 2002; revision accepted 20 November 2002

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*.

Correspondence: Izeni P. Farias, Universidade do Amazonas, Departamento de Biologia, ICB, Manaus, Amazonas, Brazil. Fax: +55 (92) 631-8313, E-mail: izeni@argo.com.br

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

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	$(GT)_3(CT)_{12}$	F: CCTGTTGCTCATCTGTTATC	302–304	2	55	0.40	0.46
AY176172		R: ATTGCCTCCTGTTCTTGTCG					
AgCTm3	$(CT)_{15}$	F: ATCTGTTTGTGGGTCTCGAC	293–299	3	55	0.73	0.63
AY176173		R: TGTGAGGACAAGCTCCAGAG					
AgCTm4	$(CT)_{22}$	F: TTTCCCGGACGAGAGAACTG	275–283	3	58	0.73	0.66
AY176174		R: TGTACCAAAGTGATGGAGAG					
AgCTm5	$(CT)_{29}$	F: GCAGCCCCAGTCTTGGAAGG	258–280	8	58	0.67	0.87
AY176175		R: AGCACGGTGAATATCTGTGC					
AgCTm7	$(CT)_{29}$	F: CCTCTACTTCCTCAACCAGC	277–299	6	58	0.67	0.71
AY176176		R: CAGCACGGTGAATATCTGTG					
AgCTm8	$(CT)_5AT(CT)_5AT(CT)_4$	F: TGGAACTCCATTGTGACAGC	272–274	2	58	0.53	0.42
AY176177	$TA(CA)_{13}(CT)_{13}$	R: GACATCTTTTCCAGCTAGCC					
AgCAm2	$(CA)_{22}$	F: AGCTCTCAGTACTGATGCTG	295–323	7	58	0.60	0.85
AY176178		R: CCGATCATCTGTTTGCTCTG					
AgCAm4	$(CA)_7$	F: CGGGGCTTTTGACTGCAAGC	210–212	2	55	0.00*	0.30
AY176179		R: GGAAAGGGGTCGGCTCAGTG					
AgCAm13	$(GTA)_2(CA)_{27}$	F: TGAAAATCCTGTGGGACCTG	300–334	6	58	0.73	0.80
AY176180		R: CCTAAAAACACATCACACTG					
AgCAm15	$(CA)_{19}$	F: GGCCTACATCAAGCACTTAA	226–244	5	58	0.67	0.61
AY176181		R: TTACTGGGTTGAGTTTTGAC					
AgCAm16	$(CA)_{19}$	F: CTCCTGGGCATCATGGGTAG	247–273	6	58	0.40	0.50
AY176182		R: TCTGTGTCTCCAGGCAACAG					
AgCAm18	$(GA)_2(CA)_9CTCACT(CA)_{20}$	F: TTACTGAGGGCAATGACACG	357–375	4	58	0.53	0.64
AY176183		R: TGATTACTCAGCAGGTCCTG					
AgCAm20	$(CA)_{12}$	F: GGAATGAGCAGGTTTCCCAG	263–267	3	58	0.80	0.60
AY176184		R: CTCCCTCTTCTGACATGACG					
AgCAm26	$(CA)_{14}$	F: ACAGGGACCAGTAAGTGGCC	213–217	3	58	0.53	0.66
AY176185		R: TGCCATAAGCACCGGGTAGG					

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 H_2O, 1.0 μL 10× buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 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

	Locus													
Species	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
Gnathonemus petersii	+ +	+ +	+ +	–	+ +	+	+ +	–	+ +	–	+ +	–	+ +	–
Heterotis niloticus	–	–	–	–	–	–	+	–	–	–	+	–	+ +	–
Osteoglossum bicirrhosum	–	+ +	–	–	+ +	+ +	–	–	–	–	–	+	+ +	–
Osteoglossum ferreirai	–	–	–	–	+	–	–	–	–	–	–	–	–	–
Pantodon buchholzi	+ +	+ +	–	–	+ +	+	+	–	+ +	–	–	+ +	–	–

+ + 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, *Pantadon 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.

Acknowledgements

The authors thank Barbara Gautschi and Trent Garner (ETH Zürich) for technical assistance and Marcelo Crossa (IPAM Santarém) for collecting the *Arapaima gigas* samples. Sébastien Lavoué provided samples of *Heterotis niloticus*. This research was supported by Universidade do Amazonas (to I.P.F.), the National Science Foundation (INT-0002213 to T.H.), EMBRAPA (PRODETAB)/CNPq and Universidade Federal do Pará (to I.S.), and the University of Konstanz and the Deutsche Forschungsgemeinschaft (to A.M.). Permission to collect tissue samples has been granted by IBAMA.

References

Crossa M, Petrere M Jr (1999) Morphometric relationships and indirect determination of the length frequency structure of the pirarucu *Arapaima gigas* Cuvier. *Fisheries Management and Ecology*, **6**, 173–186.

Garner TWJ, Gautschi B, Röthlisberger S, Reyer H-U (2000) A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Molecular Ecology*, **9**, 2173–2175.

Goulding M (1980) *Fishes and the Forest*. University of California Press, Los Angeles.

Maniatis T, Fritsch EF, Sambrook J (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schneider S, Roessli D, Excoffier L (2000) Arlequin ver 2000: A software for population genetic data analysis. University of Geneva, Genetics and Biometry Laboratory.

Tenzer I, Sd I, Morgante M, Gessler C (1999) Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. *Phytopathology*, **89**, 748–753.