

Isolation and characterization of nuclear-encoded microsatellite DNA primers for the African bonytongue, *Heterotis niloticus*

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Abstract We report the development of nine polymorphic nuclear-encoded microsatellite DNA loci for the African bonytongue (*Heterotis niloticus*), the only species of the osteoglossiform family Arapaimidae distributed in the Eastern Hemisphere. We describe the process to isolate these loci, and the primers and conditions for amplifying them using the polymerase chain reaction. We tested these primers with a sample of 40 individuals from two natural populations in Benin, West Africa. The number of alleles for the nine microsatellites ranged from 2 to 9, observed heterozygosity from 0.125 to 0.882, and expected heterozygosity from 0.156 to 0.810. No significant deviations from Hardy–Weinberg equilibrium or linkage disequilibrium were observed, and there is no indication of null alleles.

Keywords Conservation genetics · Genetic diversity · Artisanal fishery

The African bonytongue, *Heterotis niloticus*, is a member of the family Arapaimidae within the order Osteoglossiformes, the most primitive subdivision of the Teleostei.

This freshwater fish ranges across the Nile-Sudan region of Africa, including drainages within the basins of the Gambia, Senegal, Niger, Volta and Nile rivers, as well as Lake Chad and coastal drainages in Togo and Benin (Nelson 2006). This species supports important artisanal fisheries throughout its range. In some regions of West Africa, however, heavy exploitation may be unsustainable (Adite et al. 2006). Therefore, research on the genetic structure and genetic diversity of African bonytongue populations is needed to inform fisheries management and conservation (Allendorf and Luikart 2007). Herein, we report the development of polymorphic microsatellite loci for this species, which will help to advance conservation genetics for this phylogenetic relict and economically important fish species.

Muscle samples were collected from several locations in the Ouémé and Niger rivers in Benin and preserved in 70% ethanol. We extracted DNA of four individuals using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer instructions and overnight Proteinase-K digestions to ensure complete digestion of tissue samples. We combined the DNA from the four individuals and used the protocol described in Renshaw et al. (2009) to isolate and characterize microsatellites. The only variant was that during the hybridization period, mixtures were heated to 95°C for 10 min and then kept at 58°C [(CA)₁₃ hybridization] or 47°C [(CAT)₈ and (GAT)₈ hybridizations] for one and half hours. Two 96-well tissue culture plates containing microsatellites were sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida (<http://www.biotech.ufl.edu/>) for sequencing with universal M13 primers.

A total of 55 candidate loci containing microsatellite motifs were identified. Primers flanking these microsatellite regions were designed using Primer3 (<http://frodo.wi.mit.edu>).

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Table 1 Nine polymorphic microsatellites for the African bonytongue, *Heterotis niloticus*

Loci	Primer sequence (5'-3')	GenBank	Repeat	T _A (°C) ^a	Size range
<i>Hni94</i>	F-GCCACCGTTTGAGTGAATTT ^{-HEX} R-TCTGAGAACACGGTAGGATGC	JF279686	(GT) ₁₃	55.0°	166–174
<i>Hni67</i>	F-CGCCTGTAAGCAATGAATGA ^{-6-FAM} R-TGCCATGGATGTCACAAGTT	JF279687	(CA) ₁₆	55.0°	307–315
<i>Hni19</i>	F-GGCTGTGCAATTTGACATGA ^{-HEX} R-GTGCCTACAGCCATTTACCC	JF279688	(GT) ₁₇	55.8°	119–123
<i>Hni61</i>	F-AGCTTGCATCTGGAAGCATT ^{-6-FAM} R-GTATCCACCTGGACCCACAG	JF279689	(CA) ₁₂	55.0°	327–352
<i>Hni62</i>	F-CCTGGCTTTAGAGGCAGTTG ^{-6-FAM} R-CAGCTGTAGCAGGTTTGCTG	JF279690	(CA) ₁₁	55.0°	276–294
<i>Hni14</i>	F-CAACCACTGCCATAGGAGGT ^{-HEX} R-GAACCAAGTCGAGCCATCAT	JF279691	(GT) ₂₇	55.8°	125–152
<i>Hni52</i>	F-AAGGGGACCCTTGCAGTAGT ^{-HEX} R-GATGCAACATGCACTGCTCT	JF279692	(CA) ₁₆	55.0°	267–279
<i>Hni47</i>	F-AACACATTACGCCCTTGGAG ^{-6-FAM} R-CAGCCCTGTGTACACTTCCA	JF279693	(CA) ₁₆	55.8°	270–279
<i>Hni37</i>	F-CCACTTCAGAAAAAGCCATTG ^{-HEX} R-CAGGACCATAACACAACACCTG	JF279694	(CA) ₂₇	52.1°	234–239

^a T_A annealing temperature, 6-FAM and HEX fluorescent tags

Table 2 Microsatellite allelic diversity, Hardy–Weinberg and inbreeding coefficient (F_{IS}) for two natural populations of the African bonytongue, *Heterotis niloticus*

Loci	SOK							LCO						
	N	N _A /N _P	AR	Ho	He	P _{HW}	F _{IS}	N	N _A /N _P	AR	Ho	He	P _{HW}	F _{IS}
<i>Hni94</i>	18	4/0	3.342	0.389	0.4769	0.107	0.212	21	5/1	3.864	0.524	0.553	0.464	0.078
<i>Hni67</i>	17	3/0	2.198	0.177	0.164	1.000	-0.043	19	4/1	2.764	0.263	0.240	1.000	-0.071
<i>Hni19</i>	19	3/0	2.901	0.421	0.554	0.175	0.265	21	3/0	2.842	0.476	0.418	0.830	-0.114
<i>Hni61</i>	18	3/2	2.143	0.167	0.156	1.000	-0.041	19	1/0	1.000	1.000	0.000	N/a	N/a
<i>Hni62</i>	16	2/0	1.978	0.313	0.264	1.000	-0.154	15	3/1	2.896	0.467	0.424	0.715	-0.065
<i>Hni14</i>	10	8/0	7.470	0.800	0.810	0.194	0.065	10	8/0	7.470	0.800	0.810	0.174	0.065
<i>Hni52</i>	17	7/1	5.785	0.882	0.789	0.775	-0.088	13	6/0	3.203	0.923	0.760	0.954	-0.176
<i>Hni47</i>	19	8/2	5.652	0.790	0.731	0.755	-0.053	21	6/1	4.625	0.810	0.744	0.688	-0.064
<i>Hni37</i>	8	2/0	2.000	0.125	0.492	0.054	0.774	11	2/0	2.000	0.455	0.351	1.000	-0.25

SOK So river, LCO Lake Codo, N number of individuals assayed, N_A/N_P number of alleles/number of private alleles, AR allelic richness, Ho observed heterozygosity, He expected heterozygosity, P_{HW} probability of deviation from Hardy–Weinberg expectations, F_{IS} inbreeding coefficient, n/a only one allele amplified and P_{HW} and F_{IS} cannot be assessed

edu/) and purchased from Integrated DNA Technologies (IDT). We tested for amplification by screening individuals using 15 µl PCR reactions containing 50–100 ng DNA, 1 × PCR buffer, 0.5 U Taq DNA polymerase (Genscript), 0.5 µM of each forward and reverse primer (IDT), 200 µM of each dNTP(Genscript), and 2 mM MgCl₂ (New England Biolabs). Our PCR amplifications in a MyCycler (Biorad) consisted of an initial denaturation at 95°C for 2 min, followed by 38 cycles of denaturation at 95°C for 30 s, annealing at 52.0–59.0°C for 50 s, extension at 72°C for 50 s, and a final extension at 72°C for 10 min. We visualized

PCR products under UV light after electrophoresis on a 1.5% agarose gel stained with Ethidium Bromide.

We obtained PCR products for 19 microsatellites. For these, we purchased fluorescently tagged forward primers with either 6-Fam or Hex from IDT. Nine primer combinations consistently amplified PCR products. Information on these primers is summarized in Table 1. To determine levels of variation for each locus, we extracted DNA from 40 individuals, 19 from the So River and 21 from the Lake Codo populations in Benin, and PCR-amplified the nine loci using fluorescently labeled primers under conditions

outlined above. We ran PCR products on an ABI 377 automated sequencer using Genescan[®]-400 HD Rox Size Standard (Applied Biosystems) for sizing. We carried out allele sizing and calling using Applied Biosystem's Genescan[®] 3.1.2 and Genotyper[®] version 2.5 software. We estimated the number of alleles, expected and observed heterozygosities, and F_{IS} with the software program FSTAT (Goudet 1995). We tested for significant departures from Hardy–Weinberg equilibrium (HWE) and genotypic equilibrium for each pair of microsatellites markers using Fisher's exact test, as implemented in GENEPOP (<http://genepop.curtin.edu.au/>; Raymond and Rousset 1995; Rousset 2008). We checked for the presence of null alleles using MICRO-CHECKER (Van Oosterhout et al. 2004).

The number of alleles identified for the nine polymorphic loci ranged from two (Hni37) to nine (Hni47); gene diversity (expected heterozygosity) ranged from 0.156 to 0.810, while observed heterozygosity ranged from 0.125 to 0.882 (Table 2). No significant deviations from HWE expectations were encountered after Bonferroni corrections (Rice 1989). No pairwise comparison of microsatellites deviated significantly from genotypic equilibrium, suggesting that none of these microsatellites are linked in these populations. MICRO-CHECKER did not suggest null alleles were present. The nine microsatellites developed in this study will be useful for ecology and conservation genetic studies of *Heterotis niloticus*, which will help in conservation and management of this species.

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