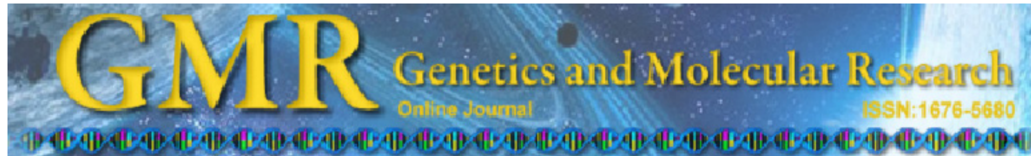

Short Communication

Isolation and characterization of novel microsatellite markers in commercial selected golden Malaysian arowana fish, *Scleropages formosus* (Osteoglossidae)

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ABSTRACT. Malaysian arowana (dragonfish; *Scleropages formosus*) is an ancient osteoglossid fish from southeast Asia. Due to the high demand of the ornamental fish trade and because of habitat loss, the species is close to extinction. We isolated and characterized 10 polymorphic microsatellites of this species, using 5'-anchored PCR. The number of alleles at the 10 microsatellite loci ranged from 2 to 28, with a mean of 7.8/locus. The observed heterozygosity ranged from 0.03 to 0.93 (mean: 0.39), whereas the expected heterozygosity ranged from 0.03 to 0.94 (mean: 0.46). Seven microsatellites deviated from Hardy-Weinberg equilibrium, and three conformed to Hardy-

Weinberg equilibrium and were in linkage equilibrium. These 10 novel microsatellites should facilitate studies of genetic diversity and population structure of arowana to help plan actions for the conservation of the indigenous Malaysian arowana.

Key words: *Scleropages formosus*; RAMs; Microsatellites; Genetic variation; Conservation

The golden arowana, *Scleropages formosus*, is also known as dragonfish, Asian bonytongue or kelisa in Malaysia, and it belongs to the family Osteoglossidae from southeast Asia (Kottelat et al., 1993) and one of the ancestral teleost clades, where it is a primitive fish from the Jurassic era. It is one of the most expensive ornamental fish species in the world. Because of its high value in the ornamental fish industry (Suleiman, 1999), the golden arowana has been excessively captured and traded in Malaysia. The population is facing a sharp decline due to hunting because of commercial interest as an aquarium fish that forces the wild population to be caught in abundance. This is especially true for Malaysian golden arowanas and red arowanas, which have reached a stage of near extinction since 1980 (Dawes et al., 1999). Therefore, they have been listed as an endangered species by the IUCN (International Union for the Conservation of Nature) Red List (2006), and international trade in these fishes is controlled under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as an Appendix I protected fish. However, there is no recent development of conservation status by IUCN and inadequate background knowledge on the population structure and diversity of the wild arowana populations. Recent study by Yue et al. (2006) also isolated microsatellites from red arowana. Polymorphic DNA markers are expected to be a highly useful tool for understanding genetic information of the fish. In this study, 10 novel microsatellites were isolated and characterized to facilitate genetic studies of the commercially selected golden arowana fish.

The microsatellite sequences were isolated using a library-enrichment anchored polymerase chain reaction (PCR) primer protocol based on a previously described procedure (Fisher et al., 1996; Kumar et al., 2002; Bhassu et al., 2008). 5'-Anchor PCR was used to amplify the regions located between two microsatellites in genomic DNA of golden arowana. Genomic DNA was extracted from scales using a GF-1 commercial genomic DNA extraction kit (Vivantis Technologies Sdn. Bhd, Malaysia) following the manufacturer protocol. Five degenerate primers were used to amplify the arowana genome: BP5 (5'- $(N)_3$YY$(BM)_3$B$(AG)_6$ -3'), BP8 (5'- $(K)_2(YH)_3$Y$(GTT)_5$ -3'), BP10 (5'- $(K)_2$DRDRD$(TC)_{10}$ -3'), VJ1 (5'- (N)7KKVRVRV(CT)10 -3') and VJ2 (5'- NNNKKRVRVCT$(CCT)_4$C -3'), where K = G/T, V = G/C/A, R = G/A, N = A/C/G/T, H = A/C/T, Y = T/C, B = C/G/T, D = A/G/T, M = A/C, and S = C/G. PCR was carried out in a total volume of 10 µL containing ~20 ng genomic DNA, 1X PCR buffer, 0.25 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 µM of each primer, 3 to 5 mM $MgCl_2$, 1.5 U Taq polymerase (Promega) and deionized water. Amplification was performed in a MultiGene Thermal Cycler (Applied Biosystems) with an initial 3-min predenaturation at 96°C, followed by 35 cycles of denaturation at 94°C for 10 s, an appropriate annealing temperature for 10 s and an extension at 72°C for 30 s. A final extension step at 72°C for 7 min was included. PCR products were then cloned into a yTA cloning vector (Yeastern Biotech Co., Ltd.) according to manufacturer instructions with minor modification. Ten recombinant clones from each

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primer were randomly selected for plasmid extraction, followed by DNA sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on the ABI PRISM 377 DNA Sequencer. The sequences were screened using the Vector Screen software in NCBI. Results from the BLAST of the clone sequence show that more than 50% were connected to the fish model *Danio rerio*, zebrafish. The clone sequences of all degenerate primer were submitted to GenBank. The primers flanking the microsatellite repeat regions were designed using the PRIMER3 program (Rozen and Skaletsky, 1997) with product lengths ranging from 150 to 300 bp.

These primers were then used to screen polymorphisms in 30 wild arowana individuals from a natural population. One scale was removed and stored in 70% ethanol and kept at -80°C. The PCR amplifications were performed in a 10-µL reaction mixture containing 20 ng genomic DNA, 1X PCR buffer, 0.25 mM of each dNTP, 0.5 µM of each forward and reverse primer, 2.5-5.0 mM $MgCl_2$, 1.5 U Taq polymerase (Promega) and deionized water. PCR profiles involved: initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, annealing temperature (Table 1) for 30 s, and extension at 72°C for 30 s with a final extension at 72°C for 7 min in a MultiGene Thermal Cycler (Applied Biosystems). PCR products were run on a 4% (w/v) metaphor agarose gel at 250 V for 2 h and 30 min. The 10 polymorphic primer pairs identified were sent for labeling to double confirm the polymorphism. The forward sequence of all the polymorphic primers was labeled with 5' FAM fluorescent dye and determined with GeneScan™ 500 LIZ size standard and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Of the 52 primer sets tested, 10 of them showed reliable polymorphism (Table 1). Among the 10 markers, 4 were dinucleotide microsatellites (SFO_BP10_A39, SFO_BP10_A43, SFO_VJ1_A7A, and SFO_VJ1_A13), three were mononucleotide repeats (SFO_BP10_A32, SFO_VJ2_A129A, SFO_VJ2_A135) and single tetra (SFO_VJ2_A133), penta (SFO_BP8_A13) and hexa (SFO_BP8_A13A) microsatellite repeats were present. The characteristics of the 10 polymorphic loci are summarized in Table 1. The average allele number of these markers was 7.8/locus, with a range of 2 to 28 per locus. The observed heterozygosity ranged from 0.03 to 0.93 with an average of 0.39, whereas the expected heterozygosity averaged 0.46, ranging from 0.03 to 0.94. Significant deviations from Hardy-Weinberg expectations were found at loci SFO_BP8_A13, SFO_BP8_A13A, SFO_BP10_A32, SFO_BP10_A39, SFO_VJ2_A129A, SFO_VJ2_A135, and SFO_VJ1_A7A, suggesting heterozygote deficiency ($P < 0.05$). These deviations may have resulted from a small population size associated with a bottleneck population or small founder population. Three loci (SFO_BP10_A43, SFO_VJ2_A133 and SFO_VJ1_A13) conformed to Hardy-Weinberg equilibrium (Table 1) and showed linkage between all these loci. We used the Micro-Checker software (Van Oosterhout et al., 2004), and there was no large allele drop out and no scoring error present in our data. However, two null alleles (SFO_BP10_A43 and SFO_VJ2_A133) were detected. All these parameters and tests were computed using the Genepop version 3.4 software (Raymond and Rousset, 1995). These newly developed single locus microsatellites for *Scleropages formosus* will indeed assist the documentation for stock enhancement programs that will be initiated to conserve its existing population found in the natural habitat. This study also aims to document scientific data, which could facilitate the captive breeding program for conservation by acquiring knowledge on the genetic diversity of both the wild and captive population.

Table 1. Ten novel microsatellite markers from the golden arowana fish (*Scleropages formosus*).

Locus	GenBank accession No.	Primer sequences (5'-3')	Repeat motif	Ta (°C)	No. of alleles	Product size (bp)	He	Ho	HWE (P)
SFO_BP8_A13	GQ213987	F: TACTCAGTTCGGGTGAAG R: TCACACACTTTGGGACCT	$(CGTTT)_2$	57	2	245	0.36384	0.46667	0.28348
SFO_BP8_A13A	GQ213987	F: GTGAAGCTATTGTTGAAGAC R: GGTAACAGTTCACATTCATC	$(CTGTTT)_2$	49	2	256	0.38136	0.50000	0.15738
SFO_BP10_A32	GQ853109	F: CTCTCTCTTCTAATGTGGTG R: TTCTAGCTCTTCTCTGTTTG	$(G)_{10}$	57	2	242	0.09661	0.10000	1.00000
SFO_BP10_A39	GQ853110	F: GTGTGACGTTTCATACTTTC R: CTGACTTTTTCCCACTTTAC	$(TG)_6$	57	2	190	0.20960	0.23333	1.00000
SFO_BP10_A43	GQ853112	F: TACCTCTCCAGTATGGTAAA R: TCTCTCTCTCTCACACACAC	$(TG)_{14}$	54	6	262	0.63503	0.33333	0.00000
SFO_VJ2_A129A	GQ853127	F: TGAAGTGACTCTAACTTGCT R: CCCATAACTTCACCATACTA	$(A)_{12}$	58	28	229	0.94068	0.93333	1.00000
SFO_VJ2_A133	GQ853129	F: GTTCTCATTTGATTCACTCC R: GGTTACATCCAGACTATGTG	$(TCTG)_5$	58	19	197	0.93559	0.43333	0.00000
SFO_VJ2_A135	GQ853131	F: CCTGAACCAGATCCTAAA R: CACTACACAAGGTGAATTTG	$(A)_{17}$	58	2	155	0.09661	0.10000	1.00000
SFO_VJ1_A7A	GQ853116	F: GGCTCTTTATGGAAACATAG R: CTCTCTCTCACACAAACACT	$(TG)_9$	58	2	202	0.03333	0.03333	1.00000
SFO_VJ1_A13	GQ853119	F: TTCCTAGCACTTTAGTGTGT R: GATCTTTAACTGCATCCTC	$(GT)_{22}$	58	13	264	0.85424	0.76667	0.00000

Ta = annealing temperature; He = expected heterozygosity; Ho = observed heterozygosity; HWE = Hardy-Weinberg equilibrium.

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