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Novel polymorphic microsatellites for studying genetic diversity of red Asian arowanas

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Abstract

Asian arowana (*Scleropages formosus*) is a highly endangered fish species listed in Appendix 1 of CITES since 1980. Fourteen novel polymorphic microsatellites were isolated from a CA-enriched partial genomic DNA library, and were used for studying genetic diversity of 41 red arowanas from the wild population. Surprisingly the average allele number of the 14 polymorphic microsatellites was as high as 12.4/locus. The average observed heterozygosity was 0.78 ranging from 0.51 to 0.95, and the gene diversity was quite high (0.78). All these data suggest that high level of genetic diversity existed in the red Asian arowana population.

The Asian Arowana (*Scleropages formosus*), commonly known as the Dragonfish, belong to an ancient family of fishes, the Osteoglossidae, which literally means bony-tongue. There are basically four different natural occurring color varieties for Asian Arowanas, namely Cross Back Golden from West Malaysia, Super Red from Indonesia's West Kalimantan, Red Tail Golden from Indonesia Pekanbaru and the Green variety, which can be found in rivers of Malaysia, Indonesia, Myanmar and Thailand (Goh and Chua 1999). Due to its popularity and great demand, Asian Arowanas have been fiercely hunted in its native habitat for profits, causing declination of the population of these fish in the wild. This is especially so for Malaysian Golden Arowanas and Red Arowanas, which have reached a stage of near extinction since 1980 (Dawes et al. 1999). Arowanas have been classified as an endangered species, threatened with extinction in CITES appendix I since 1980 (Dawes et al. 1999). However, there has been no systematic survey work or genetic assessments on the wild population. In this note we describe the isolation and characterization of 14 new polymorphic microsatellites for studying genetic diversity of red Asian arowana.

Fin clips of 41 wild-caught adult (>5 years) red Asian arowanas, which were originated from Indonesia's West Kalimantan were collected in 1998 and 1999, and kept in absolute ethanol. DNA was extracted from fin clips and purified by using a method developed previously (Yue and Orban 2001). Genomic DNA was extracted from a red Asian arowana individual using a traditional method (Miller et al. 1988). A partial genomic DNA enriched with CA-repeats was constructed as described (Fischer and Bachmann 1998) with some major modifications. The CA-repeats were enriched by a method described by Fischer and Bachmann (1998). After enrichment with CA-repeats, DNA enriched with CA-repeats was amplified in 25 μl reaction using the 21-mer adapter as primer under the condition in Yue et al. (2000). PCR products of 25 μl were electrophoresed on a 1% low-melt agarose gel, fragments between 0.4 and 1.25 kb were cut from the gel, cleaned and

concentrated with home-made glass-milk (Yue and Orban 2001). About 50 ng of concentrated DNA was directly ligated to 25 ng pGEM-T vector (Promega) according to the manufacturer's protocol. The ligated products were transformed to XL-10 gold super-competent cells (Strategene), and plated upon LB-agar containing 50 mg/l ampicillin, 60 mg/l IPTG and 40 mg/l X-gal. White colonies were picked into 96-well PCR plates containing 100 μl LB buffer/well, and cultured in a 37 °C incubator for 12 h. Colony PCR was conducted using M13 and M13 reverse primers as described (Yue et al. 2000). PCR products of 5 μl were treated with 2 μl Exo I-SAP mixture containing 1 × SAP buffer (USB), 0.2 U Exo I (USB) and 0.5 U SAP (USB) at 37 °C for 30 min and 85 °C for 15 min to remove the remaining M13 and M13 reverse primers. One microliter of Exo I-SAP treated PCR product was sequenced in both 5′ and 3′ directions using Bigdye (Applied Biosystems), M13/M13r primers and ABI3730xl sequencer (Applied Biosystems). Forward and reverse sequences of each clone were assembled using Sequencher (GenCodes). Primers were designed in the regions flanking repeats using software PrimerSelect (DNAStar). One primer of each pair was labeled with either FAM or Hex fluorescent dye (Genset).

For genotyping of each microsatellite, PCR was performed in 25 μl reaction containing 1× PCR buffer (Finnzymes) with 1.5 mM $MgCl_2$, 0.2 μM of each primer, 200 μM dNTPs, 30 ng genomic DNA

Table 1. Characterization of microsatellites and genetic diversity in 41 red Asian arowanas

Locus (GenBank no.)	Repeat motif	Primers (5′-3′)	T_a (°C)	Size range (bp)	Allele no.	H_o	H_e
D101 (AY894662)	$(CA)_{16}$	CTGAATTTGATGGAAGTCTGTGT AGTTGGTTTAAAGTGGCTGGAG	55	153–199	11	0.78	0.81
D102 (AY894663)	$(AC)_{27}$	GTTATCAGCCCTCACACCT TTCCTCCCACAGTCCAAAGA	55	153–167	7	0.71	0.61
D104 (AY894664)	$(CT)_{17}ACCA(TC)_{12}$	AGGCCACACCTCCTCCCTCAGAC CAGCCTCCTCCTGGGAACACTGTC	55	228–254	13	0.85	0.83
D105 (AY894665)	$(CA)_{18}$	GATGCCGGTGTTATCAGTGAC TTGGATGAGGGAGAAACAAATAAT	55	237–265	13	0.76	0.85
D106 (AY894666)	$(GT)_{16}$	ATATTGCGCTTTGATTCATAAACCG GCAGGGCGTGCTCTCCACT	55	191–239	18	0.90	0.88
D107 (AY894667)	$(AC)_{10}$	CTGCTGCTGCTGGGAGCGTAGC AGCTGTGGAAAGAGCTGGCCTTCA	55	160–170	6	0.73	0.68
D108 (AY894668)	$(AC)_9$	ATCTGCAAAAGAAGTGCCACAGC CACTAAACCGGTGTCCAATCCTG	55	215–271	17	0.71	0.89
D109 (AY894669)	$(CA)_{19}$	ACCTCCGCGCTTTACTTTTTGAGC CGGAGCGCCCTGCCTGCTA	55	194–226	14	0.54	0.90
D111 (AY894670)	$(TG)_{22}$	TTCCTCCCACACTCCAAAGAC TATCATTCCACAAACCTCATTCAC	55	158–162	2	1.00	0.51
D114 (AY894671)	$(GT)_{17}(AT)_2(GT)_{15}$	CCATGACCCCGCTTGGAAC AGACCACCGGATGTAGAGAAGTGC	55	156–210	19	0.86	0.90
D115 (AY894672)	$(GT)_{21}$	GTCGCTCCAGCTCTGCTACTTT GTGAGGCTGGGTTCGTCCTG	50	202–270	24	0.83	0.95
D116 (AY894673)	$(AC)_{19}$	CAGCCTCCCTCCCACTCTCCTT TTCCCCTGCCCCTATCAACATCT	50	107–125	8	0.46	0.62
D117 (AY894674)	$(CA)_{28}$	CGATCCCTGCTCAGTCTGTGT AAAGGGTCACTCAGCCATACATC	55	152–174	5	1.00	0.55
D118 (AY894675)	$(GT)_7$	ACTATGATCCGCATCGGGAGAT AGGGGAAAAGGGTCTAAGATGATG	55	104–104	1	0.00	0.00
D119 (AY894676)	$(CA)_{28}$	TGGGGGCTTTCGTTGCAATAACAC TCCCCGGTCAGGATGGACGAT	55	173–213	17	0.76	0.92

T_a: annealing temperature; H_o: observed heterozygosity; H_e: expected heterozygosity.

and 1.0 U of DyNAzyme DNA polymerase (Finnzymes) on PTC-100 PCR machines as described (Yue et al. 2000). PCR products of 1 μl were electrophoresed on a sequencer ABI3730xl (Applied Biosystems) following the manufacturer's protocol. The size of alleles were analyzed against the Rox500 size standard (Applied Biosystems) using Genemapper v3.5 (Applied Biosystems). The number of alleles, observed and expected heterozygosity, fixation index (*f*), Hardy–Weinberg equilibrium and linkage disequilibrium were analyzed as described previously (Yue et al. 2004).

Forty-eight clones were sequenced, 25 contained microsatellite sequences, but only 17 sequences were unique. Out of the 17 sequences, 15 had enough flanking regions for primer design. All 41 red Asian arowanas were genotyped with the 15 microsatellites. All markers except D118, were polymorphic (Table 1). The allele number of the polymorphic loci ranged from 2 to 24 with an average of 12.4/locus, which is higher than that of the 32 captive red arowanas analyzed previously (Yue et al. 2004). The most polymorphic locus was D115 showing 24 alleles with a size range between 202 and 270 bp, whereas locus D111 was the least polymorphic, displaying two alleles (158 and 162 bp). The average observed and expected heterozygosity were 0.78 and 0.78, respectively. As compared with other endangered fish species, such as bull trout (Spruell et al. 2003) and paddlefish (Heist et al. 2002), the allele number in the red Asian arowanas is bigger, and gene diversity is higher. Six (D102, D108, D109, D111, D116 and D117) of the 14 polymorphic loci showed significant ($P < 0.05$) deviations from Hardy–Weinberg equilibrium. Among the 14 loci, no pair of loci showed linkage. All these data suggest that high genetic diversity existed in the red Asian arowana population. However, in recent years only few red arowanas could be found in West Kalimantan of Indonesia, thus the substantial reduction of the arowanas in wild might have happened in the past few years. Due to difficulties in acquiring tissue samples of current wild individuals, information about genetic diversity of the current wild population is still very limited. It is necessary to conduct further quantitative assessment of the wild population, and to get tissue samples from current wild populations. The 14 polymorphic microsatellites described here in combination with previously published ones (Yue et al. 2000) can be used for further studies on the genetic diversity and population structure of wild populations for conservation purposes.

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