

## PERMANENT GENETIC RESOURCES

# Isolation and characterization of microsatellite markers for the ornamental discus fish *Symphysodon discus* and cross-species amplification in other Heroini cichlid species

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## Abstract

The discus fishes (*Symphysodon* spp.) are economically important ornamental species. Thirteen microsatellite markers were developed from a CT<sub>12</sub>- and CA<sub>12</sub>-enriched whole genomic DNA library of *Symphysodon discus*. Allelic variability was tested on 44 individuals of two species (*S. discus* and *S. aequifasciatus*). Allelic richness ranged from two to 11 alleles per locus and observed heterozygosities from 0.083 to 0.998. All loci were at Hardy–Weinberg equilibrium, and no pair of loci showed linkage disequilibrium within a species. Cross-species amplification was also successfully performed in the Neotropical cichlids *Uaru amphiacanthoides*, *Hoplarchus psittacus*, *Hypselecara coryphaenoides*, *Pterophyllum* sp., *Mesonauta* sp. and *Heros* sp.

**Keywords:** Amazonia, Cichlidae, discus, microsatellites, ornamental fish, *Symphysodon*

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The economically important fishes of the genus *Symphysodon* Heckel, 1840 originate from the Amazonian basin. They are grouped into five forms: 'Heckel' from the Negro and Trombetas River basins 'pineapple' from the Abacaxis River, 'green' from the upper Amazon, 'blue' from the central Amazon and 'brown' from the lower Amazon. Based on meristic and colour-pattern data, 'Heckel' 'pineapple' correspond to *Symphysodon discus* Heckel, 1840 and remaining forms to *Symphysodon aequifasciatus* Pellegrin, 1904. Morphological and molecular analyses suggest a close phylogenetic relationship of *Symphysodon* with *Uaru*, *Heros*, *Mesonauta* and *Pterophyllum* (Farias *et al.* 2000); all species belong to the Heroini tribe of Neotropical cichlids (Kullander 2003).

Since no microsatellite DNA markers are available for *Symphysodon* species or any other South American cichlids, we report the isolation and characterization of 13 microsatellite markers for the discus fish. Loci were isolated using

an enrichment technique (Farias *et al.* 2003). Briefly, genomic DNA from *Symphysodon discus* was extracted, completely digested with the restriction enzyme *Mbo*I (Fermentas), a 300–1000-bp size fraction was gel extracted, and fragments were ligated into an oligonucleotide adaptor and polymerase chain reaction (PCR) enriched using the inward extending adaptor primer. PCR products were denatured, and then hybridized to biotin-labelled CT<sub>12</sub> and CA<sub>12</sub> probes linked to DynaBeads (Invitrogen) via a biotin–streptavidin bond. Hybridized DNA–probe complex was washed in successively more stringent standard saline citrate solutions, and finally the DNA was eluted off of the probe using a TE buffer. The enriched DNA was ligated into an Invitrogen pCR 2.1 TOPO cloning vector and transformed into competent *Escherichia coli* cells (TOPO TA Cloning kit). The transformed cells were grown overnight on 1× Luria-Bertani agar plates supplemented with 100 µg/mL of ampicillin (Sambrook & Russell 2001). Individual colonies were picked, and regrown in liquid Luria-Bertani/amp medium for 16 h in a 96-well culture plate. PCR amplification using M13 (–20) forward and M13 reverse primers was

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**Table 1** Characterization of 13 microsatellite loci for the discus fish *Symphysodon discus*

Locus	Repeat motif	Primer sequence (5'-3')	T <sub>a</sub> (°C)	Size range (bp)	N/A	H <sub>O</sub>	H <sub>E</sub>	P	GenBank Accession no.
Sd04	(CA) <sub>16</sub>	F-CCCTATGCAGAAGGAGGTGA R-TTGAGCTCACACGGAGAATG	65	135–139	23/3	0.173	0.280	0.15	EU109267
Sd05	(CT) <sub>6</sub> cc(CT) <sub>10</sub>	F-TAAGAGTTCCCCCAATCAGC R-TTGTGACAGTGGAGGAGCA	65	281–293	23/5	0.565	0.592	0.75	EU109268
Sd08	(GA) <sub>12</sub>	F-TGAGAGGCTCCTTCAGCTTT R-CAGCAGCCACTTCACTGGTA	65	312–314	24/3	0.083	0.122	1.00	EU109269
Sd10	(CA) <sub>9</sub>	F-CGTGGTTCTTACTGGCTTGC R-GTAGGTCTGCCCACTACTGT	65	170–174	23/3	0.086	0.127	1.00	EU109270
Sd11	(GAATA) <sub>8</sub>	F-GACAGCTGCAGACAGTCTTTTT R-CCAATCTCATTGTACACCTCCA	55	161–200	10/4	0.500	0.568	0.18	EU109271
Sd12	(AT) <sub>4</sub> (GA) <sub>6</sub>	F-AAAATCAGCGTGTGGGTTTC R-CTGTGAGTCAGCCTGTCCAA	55	306–312	24/3	0.250	0.264	1.00	EU109272
Sd14	(GT) <sub>5</sub> (AG) <sub>27</sub>	F-CAGCAGGCCTTGTTCAGAA R-CAGAGTAGTACAGAGTGCCATCTCA	55	357–365	24/4	0.250	0.233	1.00	EU109273
Sd15	(GAGAA) <sub>12</sub>	F-TCCCGAGATGTTTAATGCTG R-CAAACATTTCTGAAATTCAACC	55	163–213	24/11	0.958	0.873	0.73	EU109274
Sd22	(CT) <sub>2</sub> (GT) <sub>7</sub>	F-ACCAATCCAAGCCTGTCACT R-GTCGACTTCCCGGCATTAG	55	238–242	24/3	0.250	0.266	1.00	EU109275
Sd23	(CA) <sub>9</sub>	F-CAGACTCCAAGCTGTGCTTT R-GACTTGTCTAGCAGCTCAGGAC	55	344–364	22/4	0.136	0.175	1.00	EU109276
Sd25	(GT) <sub>7</sub>	F-CTGGTTCTGCACATTTGCTTG R-CCCGAATCAAGATGATGCTC	55	181–191	24/3	0.083*	0.264	0.01*	EU109277
Sd27	(GT) <sub>13</sub>	F-GGAAGCACTAGTACACAATT R-CCTTGGCACCCCTTTCATTA	55	144–146	24/2	0.125	0.158	1.00	EU109278
Sd30	(CT) <sub>10</sub> (CA) <sub>12</sub> (CT) <sub>15</sub> (CACT) <sub>5</sub> (CA) <sub>20</sub>	F-CTGCGTTTCACGTCTTCGTA R-CCCCAGATGTACTGCCAGAT	55	200–216	24/9	0.625	0.730	0.28	EU109279

T<sub>a</sub>, PCR annealing temperature; N, the number of successfully genotyped individuals in the 24 individuals sampled for each locus; A, number of alleles; H<sub>O</sub>, observed heterozygosity; H<sub>E</sub>, expected heterozygosity; \*significant only before Bonferroni correction.

performed directly on the bacterial cultures (Sambrook & Russell 2001). PCR products were purified using GFX spin-columns (GE Healthcare), and sequenced with T7 and SP6 primers using ET terminator sequencing chemistry (GE Healthcare). Sequences were determined on a MegaBACE 1000 (GE Healthcare).

Eight hundred and sixty-four clones were sequenced and 41 flanking primers pairs were designed using the PRIMER 3 software (Rozen & Skaletsky 2000). Each forward primer had an M13 sequence tail added to its 5' end to allow for dynamic fluorescent labelling following the protocol of Schuelke (2000). PCR was performed in 10-μL reaction volumes with 50 mM KCl, 10 mM Tris-HCl, 1.75 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.2 μM reverse primer, 0.1 μM forward primer, 0.1 μM FAM-labelled M13 label primer, 0.05 U/μL LGC Biotecnologia *Taq* DNA Polymerase, and about 5 ng of genomic DNA. PCRs were run in a Thermo Hybaid thermocycler and had two main steps: an initial denaturation step (94 °C, 1 min) followed by 25 cycles of 50 s at 94 °C, 50 s at primer-specific annealing temperature (Table 1), 1 min at 72 °C; followed by 20 cycles of 40 s at 94 °C, 35 s at 50 °C, 40 s at 72 °C, and an extension step for 20 min at

72 °C. PCR fragments were resolved in MegaBACE1000 and analysed in the FRAGMENT PROFILER version 1.2 software using the ET-400 ROX size standard (GE Healthcare).

We tested the loci using 24 *S. discus* collected from the vicinity of Barcelos on the Negro River, Amazonas, Brazil. Out of the 41 primer pairs designed, 20 were optimized, while the remaining 21 either did not amplify or stutters made accurate interpretation of product length difficult. Of the 20 optimized loci, seven were monomorphic and the remaining 13 had two to 11 alleles per locus (Table 1). Expected heterozygosities were calculated and tested against observed heterozygosities by  $\chi^2$ . Observed and expected heterozygosities ranged from 0.083 to 0.998 and from 0.122 to 0.827, respectively. No locus showed significant deviation from Hardy-Weinberg equilibrium, and no loci were at linkage disequilibrium after Bonferroni correction. Calculations were performed in the program ARLEQUIN 3.1.1 (Excoffier *et al.* 2005). The presence of potential null alleles in the locus Sd25 was inferred using the program MICRO-CHECKER 2.3 (van Oosterhout *et al.* 2004).

The 13 optimized loci were also tested in 20 *S. aequifasciatus* from Manacapuru, Amazonas, Brazil using the same PCR

**Table 2** Characteristics of the 13 microsatellite loci cross-amplified in *Symphysodon aequifasciatus* from Manacapuru, Amazonas, Brazil

Locus	N/A	Product size range	H <sub>O</sub>	H <sub>E</sub>
Sd04	19/4	125–137	0.684	0.624
Sd05	20/3	287–293	0.400	0.377
Sd08	19/3	318–330	0.263	0.286
Sd10	20/1	176	—	—
Sd11	20/10	165–210	0.900	0.892
Sd12	20/3	298–214	0.100	0.146
Sd14	18/4	353–361	0.167	0.213
Sd15	19/16	143–238	0.895	0.935
Sd22	19/2	240–242	0.211	0.193
Sd23	20/4	356–366	0.200	0.236
Sd25	19/3	181–191	0.105	0.154
Sd27	19/3	126–152	0.211*	0.422
Sd30	18/14	234–278	0.833	0.903

N, the number of successfully genotyped individuals in the 20 individuals sampled for each locus; A, number of alleles; H<sub>O</sub>, observed heterozygosity; H<sub>E</sub>, expected heterozygosity; \*significant only before Bonferroni correction

conditions as for *S. discus*. All loci amplified and were polymorphic with the exception of the locus Sd10 which was monomorphic (Table 2). We further tested these 13 loci in the heroine species *Uaru amphiacanthoides*, *Hoplarchus psittacus*, *Hypselecara coryphaenoides*, *Pterophyllum* sp., *Mesonauta* sp. and *Heros* sp., and nearly all locus/species combinations worked (Table 3).

In conclusion, we identified 13 polymorphic microsatellite loci which provide useful genetic markers for the study of genetic structuring and species boundaries in the genus *Symphysodon*, as well as for conservation genetic studies. Additionally, these microsatellite markers also appear useful for similar studies in other Heroini species.

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**Table 3** Cross-species amplification results of 13 microsatellite loci characterized in *Symphysodon discus* and tested for their utility in six other species of the tribe Heroini. Loci were judged as potentially useful in other species if they amplified as a strong distinct band in the size range near that observed in *S. discus*; weaker bands of correct expected size were scored as of potential use

Species (N)	Locus												
	Sd04	Sd05	Sd08	Sd10	Sd11	Sd12	Sd14	Sd15	Sd22	Sd23	Sd25	Sd27	Sd30
<i>Uaru amphiacanthoides</i> (2)	++	++	++	+	++	++	++	++	+	++	–	++	++
<i>Hoplarchus psittacus</i> (2)	++	++	++	+	++	+	–	++	++	++	++	++	++
<i>Hypselecara coryphaenoides</i> (2)	++	+	++	+	++	–	++	++	++	–	–	++	++
<i>Heros</i> sp. (1)	++	++	++	++	++	++	++	++	++	–	–	++	++
<i>Pterophyllum</i> sp. (2)	++	+	++	–	++	–	–	++	+	++	–	++	++
<i>Mesonauta</i> sp. (2)	++	+	++	++	++	++	++	++	++	++	++	++	+

N indicates numbers of individuals used; ++ indicates successful amplification; + indicates weaker amplification; – indicates no amplification or inconsistent amplification. Amplifications were carried out at 50 °C annealing temperature; the rest of the protocol was same as for *Symphysodon discus*.