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Isolation and characterization of microsatellite loci in the Neotropical fish *Astyanax altiparanae* (Teleostei: Characiformes) and cross-species amplification

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Introduction

We isolated and characterized 11 polymorphic microsatellite loci from the Neotropical fish *Astyanax altiparanae*, considered of economic interest, whose stocks have been seriously endangered by the introduction of predatory fishes. The analyses in a population of 33 specimens detected a large number of alleles (ranging from 4 to 11) and high levels of heterozygosity (0.64–0.88) at these loci, indicating their usefulness in population genetic studies. Cross-species amplification was successful only in species of *Astyanax*, 43% of which were polymorphic.

The Characiformes constitute one of the dominant and more diverse orders among tropical fishes, with more than 1800 species, among which the family Characidae is the most diverse, with species spread throughout the Neotropical region. However, the interrelationships among the Characidae are poorly known (Reis *et al*. 2003) and remain under discussion (Javonillo *et al*. 2010; Mirande 2010).

The genus *Astyanax* (Characiformes, Characidae) comprises 163 described species (Froese and Paulay 2010), and its systematics are very complex and several studies have currently shown that the genus needs to be more thoroughly characterized. *A. altiparanae*, encountered along the south and southeast Brazilian rivers, was formerly included in the complex '*A. bimaculatus*' (Garutti and Britski 2000), which is widely distributed in South America. *A. altiparanae* is of great economic interest, also being utilized as bait in sport fishing and in aquaculture programmes (Garutti and Britski 2000; Porto-Foresti *et al*. 2010). However, the stocks of this species are seriously endangered by introduced predatory fishes, such as tucunaré (*Cichla* spp.) and corvina (*Plagioscion squamossissimus*) (Agostinho *et al*. 2007).

Many molecular markers have been frequently used for the *Astyanax* species (Prioli *et al*. 2002; Leuzzi *et al*. 2004; Peres *et al*. 2005). However, there are no microsatellite data available for this group. These markers can be valuable tools to investigate genetic variability, with applications to conservation and population genetics (Oliveira *et al*. 2006). Their use in stock characterization of *A. altiparanae* may have practical implications for fisheries, fish farming, and conservation biology. We describe the isolation and characterization of 11 novel microsatellite loci from *A. altiparanae*, and their cross-amplification for potential utility in studies of additional species.

Materials and methods

A microsatellite-enriched genomic library was obtained for *A. altiparanae* following the protocol described by Billotte *et al*. (1999). Genomic DNA was extracted using the commercial Wizard Genomic DNA Purification kit (Promega, São Paulo, Brazil). The total DNA was digested with *Rsa*I and enriched in $(AC)_n$ and $(AG)_n$ repeats. Enriched fragments were amplified by polymerase chain reaction (PCR) and then linked into a pGEM vector (Promega) and transformed into competent XL1-blue *Escherichia coli* cells. Positive colonies were tested by PCR to confirm the presence

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of inserts. Selected recombinant colonies were sequenced using the primers T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and the BigDye Terminator kit (Applied Biosystems, São Paulo, Brazil), and electrophoresed on an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, USA). Flanking primers were designed with Primer3 software (Rozen and Skaletsky 2000).

Results and discussion

We isolated and sequenced a total of 48 positive colonies, resulting in 25 good quality flanking sequences. The selected sequences were used to characterize a sample of 33 *A. altiparanae* specimens, collected in the Batalha river (22°6′40.92″S, 49°16′5.81″W), Brazil, and tested in five individuals of the species *Salminus brasiliensis*, *Brycon amazonicus*, *Brycon hilarii*, *A. fasciatus*, *A. bockmanni*, *A. paranae*, *A. abramis*, *A. schubarti*, *A. ribeirae* and *A. jacuhiensis*. PCR was performed in 20 μL reaction volume containing approximately 10.9 μL H_2O miliQ, 2.75 μL PCR buffer 10×, 1.25 μL $MgCl_2$ 50 mM, 1.5 μL dNTP 1.25 mM (Invitrogen, São Paulo, Brazil), 1 μL of each primer 10 μM, 0.1 μL *Taq* DNA polymerase (Invitrogen) 5U/μL and 1.5 μL of genomic DNA. The conditions for amplification were 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at the annealing temperature (see table 1), 5 s at 72°C, and a final extension time of 5 min at 72°C. The amplification products were separated in 6% denaturing polyacrylamide gel and visualized by silver nitrate staining, photographed, and analysed using the Kodak Digital Science program Eastman Kodak Company, Rochester, USA. Allele scoring was done using the 10-bp DNA Ladder (Invitrogen, São Paulo, Brazil) as size standard.

Among the 25 tested primer pairs, 11 loci were highly polymorphic (GenBank accession numbers JQ246359 to JQ246369). The allele number varied from 4 (Asty12) to 11 (Asty21) by locus; the value of expected heterozygosity varied from 0.64 (Asty12) to 0.88 (Asty13), and three loci showed deviation from the Hardy–Weinberg equilibrium (HWE) ($P < 0.01$) (table 1). They were calculated using GENALEX v6.1 software (Peakall and Smouse 2007). Pairwise tests for linkage disequilibrium among loci were calculated using GENEPOP 3.3 package (Raymond and Rousset 1995), and were nonsignificant. Micro-Checker (Van Oosterhout *et al*. 2004) was used to verify possible causes of HWE departures, and the analysis showed no evidence of stuttering, allelic dropout, or null alleles as a possible cause of HWE departures.

Cross-species amplification was investigated in 10 additional species of the same family (table 2). All 11 primers analysed revealed a high level (89%) of cross-amplification in species of *Astyanax*, 43% of which were polymorphic. On the other hand, with noncongeneric species (*Salminus brasiliensis*, *Brycon amazonicus* and *B. hilarii*), the cross-amplification did not show positive results. Barbará *et al*. (2007) revealed that the transferability for fish species can be around 70% in congeners, lowering to 60% among

Table 1. Description of microsatellite loci and primer sequences in *Astyanax altiparanae.*

	Primer sequence (5′-3′)	Repeat motif	Lenght (bp)	T_a(°C)	n	A	H_o	H_e	F_{is}
Asty 4	GGTCACTGGAGGACAGATGTT GGCATGTGCTTGAATGGA	$(AC)_{17}$	200	53	31	5	0.852	0.754	−0,129
Asty 11	TAAATCTATAAAGTCACCAT TTTGTTTTTCTGCCGCTGTTT	$(AC)_{12}$	151	50	32	9	0.630	0.837	0,248
Asty 12	AGACACAATCAGCCGCCGAAATG ATCCCCTCTCCACAACCCAACACA	$(GT)_8$	163	58	25	4	0.742	0.638	−0,162
Asty 13	AAATGGGTGCAAGCAACG TGCCTGTCTGTAAGCATGTG	$(GT)_8$	160	58	25	10	0.563	0.877	0,359*
Asty 15	CAACTTTTACTTAAAACCTGC ATGGGTCTTTACTGCTGAATGTAT	$(AC)_{17} – (CT)_6$	212	50	33	6	0.500	0.773	0,353*
Asty 16	AAAGTAAAGGGCATCTGTGGAGAA AGAGGGCATCATTGTACATTTTTG	$(AC)_{10}$	165	52	32	8	0.769	0.860	0,106
Asty 21	TTTATGGGGACCGTGAGATGTGC CAGGGGCAGCGGTGATACCT	$(CA)_9$	150	57	24	11	0.500	0.852	0,413*
Asty 23	TCAATGGAACCTATGGACAAC GTGGGAAGTAGCCTAATAAATA	$(CA)_{12}$	160	52	30	6	0.600	0.743	0,193
Asty 24	AGACCAAACACTAGGGCTCAG TTCGTCAATCTTCTTTCACTCTT	$(GT)_9$	139	52	32	7	0.677	0.800	0,153
Asty 26	CCCATTGATCCTGCCTCTAA CAGTCCTGACACAGAGAT	$(GT)_8$	190	58	25	8	0.654	0.856	0,236
Asty 27	GCATTGTTCAGGTTGGGTCT AAACGTGGTGAGAGGGAGTG	$(GT)_8$	150	58	25	9	0.667	0.809	0,176

Intrapopulational analysis: T_a, annealing temperature; n, number of individuals; A, allele number; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{is}, endogamy index. *Loci that displayed significant deviations from Hardy–Weinberg equilibrium ($P < 0.01$).

Table 2. Cross-amplification of the 11 polymorphic loci in seven species of *Astyanax* and three others species of Characidae.

Species	Primer - Asty										
	4	11	12	13	15	16	21	23	24	26	27
Astyanax paranae	P	M	P	M	P	M	P	M	P	M	M
A. bockmanni	–	P	P	–	P	M	P	P	P	P	M
A. fasciatus	–	P	P	P	P	P	P	P	P	P	M
A. jacuhiensis	P	P	P	P	P	P	P	P	P	P	P
A. abramis	M	P	M	P	–	M	M	P	P	P	M
A. schubarti	M	P	P	–	–	M	M	P	M	M	M
A. ribeirae	M	P	P	P	–	M	P	P	P	P	M
S. brasiliensis	M	–	M	–	M	M	M	M	M	M	–
B. amazonicus	M	–	M	M	M	M	M	M	M	M	M
B. hilarii	M	–	M	M	M	M	M	M	M	M	M

P, polymorphic; M, monomorphic; –, no amplification.

genera of the same family, which showed that the success of transferability of microsatellite loci were directly linked to phylogenetic relationship between the groups tested.

Astyanax spp. represent an excellent model group for studies of evolutionary mechanisms (Langecker *et al*. 1991; Jeffery 2001) because they are naturally distributed into structured groups (Garutti and Britski 2000), favouring vicariant processes responsible for the great diversity of Neotropical fishes (Castro 1999). Additionally, several *Astyanax* spp. are distributed in species complexes, such as *bimaculatus*, *fasciatus* and *scabripinnis* (Moreira-Filho and Bertolo 1991; Fernandes and Martins-Santos 2004; Artoni *et al*. 2006), which are abundant in rivers and other aquatic habitats throughout the Neotropical region (Reis *et al*. 2003). Consequently, the results obtained here reinforce the applicability of the microsatellites for parentage population genetic and studies in this heterogeneous group of fishes.

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