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TECHNICAL NOTE

Isolation of novel microsatellite markers for tambaqui (*Colossoma macropomum*, Cuvier 1818), an important freshwater fish of the Amazon

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Abstract *Colossoma macropomum* is an endemic species from Amazon basin. It is widely commercialized as food, becoming one of the main items in Amazonian fisheries. Despite its economic importance, genetic information of small captivity populations is not currently available. The present contribution describes 14 new microsatellite loci used to analyze 30 individuals of *C. macropomum*. The number of alleles for each locus ranged from 4 to 24. The observed (*Ho*) and expected (H_E) heterozygosity values ranged from 0.318 to 1.000 and 0.729 to 0.949, respectively. Out of 14 polymorphic loci, nine did not deviate from Hardy–Weinberg Equilibrium after Bonferroni correction. These new microsatellite loci will contribute towards the genetic of small artificial populations, as well as pedigree control of fish farms of *C. macropomum*.

Keywords *Colossoma macropomum* · Genetic diversity · Captivity · Polymorphism · Microsatellite markers

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The tambaqui, *Colossoma macropomum* (Cuvier, 1818), is one of the most economic important species in the region. Even though the production of this species in captivity has increased in the past few years, there are strong indications, such as the reduction in the landing of the fish in Amazonian markets and the continued reduction in the size of the fish captured, that the natural populations of tambaqui are suffering from overexploitation as reported by Isaac and Ruffino (1996) more than 10 years ago and reinforced by Araújo-Lima and Ruffino (2004) *apud* Santos et al. (2007). Despite its economic importance, genetic information of small captivity populations is not currently available. Since Santos et al. (2009) and Hamoy et al. (2010) described microsatellite loci from natural population, we have developed and characterized novel markers from wild and captivity populations to help the management and control of fish artificial production.

Microsatellite loci were developed from genomic enriched library following the protocol described by Billotte et al. (1999). The protocol used to total genomic DNA extracted was described by Sambrook et al. (1989). The purified total DNA was digested with Rsa I and enriched in $(CT)_8$ and $(GT)_8$ repeats. Enriched fragments were amplified by Polymerase Chain Reaction (PCR), linked into a pGem-T Easy vector (Promega) and then transformed into competent XL1-Blue *Escherichia coli* cells. The positive clones were selected using the β-galactosidase gene and grown overnight in an HM/FM medium with ampicillin. Plasmid DNA was purified and 96 positive clones were sequenced using T7 and SP6 primers as well as the v3.1 Big Dye terminator kit (PerkinElmer Applied Biosystems) with an ABI 377 automated DNA sequencer (Applied Biosystems). A total of 16 *primers* were designed using the program Oligo Explorer v 1.2 (Gene Link, Inc.) and a M13 sequence tail was added in the 5′ end of each forward

Table 1 Characteristics of the 14 *C. macropomum* microsatellite loci

SSR locus	GenBank accession no.	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	A	Allele size (pb)	H_E	H_O	f	PIC	P value* HWE
TB1	HM190138	F:^{FAM}ACACTTTCACATCCATCTAC R: CCTCTGTTTTTCCTCTTT	$(CT)_{13}$	45	16	90–122	0.861	0.636	0.265	0.887	0.000*
TB2	HM190139	F:^{FAM}TGTTTGTTGACTTTCTGCCC R: CAGTTTCACCCAGTTCTCCC	$(AC)_{19}$	65	8	102–124	0.827	0.318	0.621	0.781	0.000*
TB3	HM190140	F:^{FAM}ACTGTAAATGGCTGACCTCG R: GATGGCAGCACTACAAGACT	$(AC)_{18}$	65	12	122–156	0.894	0.444	0.507	0.865	0.000*
TB4	HM190141	F:^{FAM}GAAGTCCTCACAAGTAATG R: TGGTTTAAAATGATCAGC	$(GT)_{13}CT(GT)_4$	57	5	200–216	0.742	0.412	0.452	0.669	0.002*
TB6	HM190142	F:^{FAM}TAGACAGAACAGGAATGCTT R: AGAGAGATAGAGCGAGAGGT	$(AC)_{10}AT(AC)_{13}$	56	6	238–260	0.787	0.571	0.277	0.737	0.008^{NS}
TB7	HM190143	F:^{FAM}AAACTAAACGCAAATGTCTG R: CTTCAGTTAGCCATTACCTC	$(AC)_{16}$	65	8	220–252	0.859	0.727	0.157	0.820	0.075^{NS}
TB9	HM190144	F:^{FAM}ACACACACAAAGACAAACAC R: GCTCACACATAAACAACTG	$(CA)_{16}$	50	4	166–180	0.729	0.400	0.458	0.661	0.001*
TB10	HM190145	F:^{FAM}TGAGACACTGCCACAAACT R: CATTTTTGGATTTGGCTCTA	$(TG)_6TT(TG)_{11}$	60–45[a]	6	112–124	0.826	0.667	0.196	0.783	0.033^{NS}
TB13	JN020280	F:^{FAM} GATTTTTCCCTTTGTATTTTATG R: CTTTATAGATCCTCCCCAGTTC	$(TG)_{15}$	50	16	271–301	0.909	1.000	−0.102	0.885	1.000^{NS}
TB14	JN020281	F:^{FAM} CACCAGCTCCCCGCAGACA R: AAAGCCAGCCCAGACCATTATCC	$(TG)_{28}$	65	24	162–209	0.949	0.792	0.169	0.926	0.004^{NS}
TB18	JN020282	F:^{FAM} TTAATGAACTACACAACCCACCTG R: TGTTTATATTGTTGAAGCCGAGAG	$(TC)_{17}GT(CT)_3AT(CT)_5$ $AT(CT)_3$	61	12	173–199	0.920	0.929	−0.009	0.896	0.663^{NS}
TB20	JN020283	F:^{FAM} TTCTCTCTCTTCCACACGCTCT R: ACCTCCCTGACGCAAACC	$(CT)_{20}$	61	21	130–199	0.928	0.880	0.054	0.904	0.255^{NS}
TB21	JN020284	F:^{FAM} GATGATGAGATGAGTATGTGC R: TGTAGCATTCTCCTGTCTTC	$(GA)_{27}$	61	18	164–203	0.922	0.963	−0.046	0.897	0.900^{NS}
TB22	JN020285	F:^{FAM} TGCTTGTCTACTTTGTTAC R: CCATGTCTTGACTTTGTA	$(CA)_{10}$	61	11	133–183	0.776	0.654	0.160	0.734	0.076^{NS}

Locus name, Genbank accession number, primer sequences (F: forward primer, R: reverse primer), repeat motif from a sequenced clone, annealing temperature (T_a), number of alleles (A), product size range in base pairs, expected (H_E) and observed heterozygosity (H_O), fixation index (f), polymorphism information content (PIC)

P values for the HWE test, significance threshold adjusted using Bonferroni correction (P-HWE): (P: 5% $\leq$ 0.0036)

NS not significant, * significant, [a] touchdown PCR (Don et al. 1991) with temperatures ranging from 60 to 45°C

primer in order to permit the fluorescent labeling protocol suggested by Schuelke (2000).

These loci were characterized in 30 individuals of *C. macropomum* collected in lakes along Solimões River between the cities of Coari and Manaus (PIATAM Project). The microsatellite fragments were amplified by Polymerase Chain Reaction (PCR) in 10 µl containing 60 ng of genomic DNA template, each forward and M13 Label primer (FAM) at 0.4 µM, reverse primer at 0.8 µM, PCR Master Mix (2×) (Fermentas)—0.05 units/µl Taq DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP. PCR was carried out with two steps: denaturation (94°C, 1 min) followed by 25 cycles of 20 s at 94°C, 20 s at 56°C (specific annealing temperature), 30 s at 68°C; the second step consisted of 20 cycles with the following time and temperature profile: 20 s at 94°C, 20 s at 53°C, 30 s at 68°C, and a final extension at 72°C for 3 min. Amplifying products were checked by electrophoresis on 1% agarose gels containing 0.1 mg ethidium bromide/ml in 1× TBE buffer (pH 8.0). The products from each PCR (1.0 µl) were analyzed on an ABI 3130XL Sequencer and sized using the GeneScan-500 LIZ internal size standard and scored using GeneMapper version 4.0 (Applied Biosystems) software.

Out of 16 microsatellite loci developed for *C. macropomum*, 14 were polymorphic and two monomorphic. The number of alleles to estimate the observed (H_O) and expected (H_E) heterozygosity and fixation index (f) were calculated using GDA v1.1 (Lewis and Zaykin 2000). The test for conformity to Hardy–Weinberg expectation (HWE) and Linkage Disequilibrium (LD) between all pairs of loci was calculated using Fstat v2.9.3.2 (Goudet 2001). The polymorphism information content (PIC) was calculated using CERVUS v3.0.3 (Kalinowski et al. 2007). Significance levels were adjusted to the number of simultaneous tests using sequential Bonferroni correction (Rice 1989). The number of alleles per locus ranged from 4 to 24, with an average of 11.92 alleles per locus (Table 1). Polymorphism information content (PIC) ranged from 0.661 to 0.926. Observed heterozygosity (H_O) and expected heterozygosity (H_E) ranged from 0.318 to 1.000 (average 0.671) and 0.729 to 0.949 (average 0.852), respectively. Five loci (*TB1*, *TB2*, *TB3*, *TB4*, and *TB9*) showed significant deviation from the Hardy–Weinberg Equilibrium (HWE) after Bonferroni correction (P: (5%) < 0.0031). Values of f ranged from -0.009 to 0.621 with average of 0.219. The locus (*TB1*, *TB2*, *TB3*, *TB4*, and *TB9*) were significant for the presence of a null allele, as tested using the Micro-checker software (van Oosterhout et al. 2004). No significant linkage disequilibrium (LD) was detected among all loci, as analyzed using the Fstat. These results may account for no randomly mates in the analyzed populations that might be small in the sampled area. These 14 novel polymorphic microsatellite loci are highly variable and potentially useful tools for genetic studies of natural and cultured populations of *C. macropomum*.

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