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PRIMER NOTE

# A set of microsatellite DNA markers for the one-lined pencilfish *Nannostomus unifasciatus*, an Amazonian flooded forest fish

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**Abstract**

**The one-lined pencilfish *Nannostomus unifasciatus* is a small fish from the flooded forests of the Amazon basin. Pencilfish are popular in aquaria and are used as ornamental fishery resource by riverine communities from central Amazonia. Here we report a set of nine microsatellite loci for *N. unifasciatus*. Number of alleles and heterozygosity per locus in a sample of 30 pencilfish ranged from 4 to 19 and from 0.39 to 0.86, respectively. These markers will prove useful to investigate population genetic structure, identify conservation units, and to conduct phylogeographical reconstructions in pencilfish from the flooded forests of Amazonia.**

*Keywords*: Amazon rain forest, conservation genetics, Lebiasinidae, microsatellites, *Nannostomus unifasciatus*, phylogeography

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Despite its enormous importance as a source of biodiversity, little is known about the evolutionary processes that generate diversification in Amazonia. This is particularly true for the extremely diverse and understudied Amazonian fish fauna. We are using genetic and biogeographical information to investigate spatial and temporal patterns of evolutionary diversification in small fish species inhabiting the forests of the Rio Negro floodplain (RNF), in central Amazonia (e.g. Beheregaray *et al*. 2004). One of our target taxa is the one-lined pencilfish *Nannostomus unifasciatus* (family Lebiasinidae), which belongs to a group of around 14 small species found in flooded forests, rivers and streams of the Amazon basin (Weitzman 1978). Pencilfish are popular in aquaria and are used as fishery resource by riverine communities from the middle RNF, one of the world's major fishing grounds for ornamental fish (Chao *et al*. 2001). The RNF's ornamental fishery represents an important source of income for the local people and is believed to have inhibited their move to nonsustainable and more environmentally damaging economic activities (Chao *et al*. 2001). In this study we report a set of microsatellite loci for *N. unifasciatus* that will prove useful to investigate population structure and identify conservation units in pencilfish populations target by RNF's ornamental fishery. These microsatellites also represent powerful DNA markers for phylogeographical reconstruction of recent divergences and to resolve taxonomic uncertainties in *Nannostomus*.

We isolated microsatellites using a modification of an enrichment technique (Fischer & Bachmann 1998) described in Saltonstall (2003). Genomic DNA was digested with *Rsa*I and *Hae*III and fragments ligated to two oligo adaptors. Biotinylated oligo probes ($dGA_{10}$ and $dGT_{10}$) were hybridized to the digested DNA and selectively retained using streptavidin magnetic particles (Promega). Polymerase chain reactions (PCRs) were performed on the microsatellite-enriched eluate using one of the oligo adaptors as a primer. The product from the first PCR was used as template to repeat the enrichment process. The enriched library was

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**Table 1** Characteristics of nine microsatellite loci isolated from *Nannostomus unifasciatus*. Number of alleles ($N_a$), observed ($H_O$) and expected ($H_E$) heterozygosities are based on a sample of 30 individuals from middle Rio Negro, Amazonia

| Locus | Primer sequences (5′–3′) | Repeat structure | $Mg^{2+}$ (mM) | $N_a$ | Size range (bp) | $H_O/H_E$ | GenBank Accession no. |
|---|---|---|---|---|---|---|---|
| Nu 6 | F: CACCCGGTTCAAGTCACACG<br>R: GGAATTTCCAAATGAAAGGA | $(CA)_{12}$ | 2.5 | 9 | 131–177 | 0.60/0.63 | AY496028 |
| Nu 11 | F: GGCTCAGATCAATTCCACAG<br>R: AACCGAAGAAACATTATAGGTGG | $(CA)_9$ | 2.5 | 6 | 191–201 | 0.36/0.40 | AY496029 |
| Nu 25 | F: GTGCATGTCTGAGCATCTGG<br>R: CCCCAAACCCACCAGTTATC | $(CA)_{13}$ | 2 | 11 | 125–153 | 0.66/0.70 | AY496030 |
| Nu 26 | F: GGATGAGTTCCTCTTGAGACCG<br>R: CCCACACAGTCTCTCAGCAT | $(CA)_7$ | 2 | 11 | 110–134 | 0.69/0.72 | AY496031 |
| Nu 28 | F: TTGTGGTTGGAACTTGGATG<br>R: CCAGGGGATACATGCACTC | $(CA)_{13}$ | 2.5 | 16 | 162–218 | 0.74/0.77 | AY496032 |
| Nu 29 | F: GCGGCCAGAATGATCTACAGG<br>R: ACTAAACCCACCAGCAAGCA | $(CA)_{23}$ | 2 | 19 | 158–212 | 0.83/0.86 | AY496033 |
| Nu 39 | F: CTCCAGCTTAGGGCTTATGC<br>R: TCACCAACTCCTCTGGCAG | $(CA)_8$ | 2 | 4 | 205–237 | 0.64/0.67 | AY496035 |
| Nu 44 | F: CACAAACAAACAGTGGCTTTAATC<br>R: TCTTGCAGAACCAATTTTGTG | $(CA)_{11}$ | 2.5 | 5 | 179–201 | 0.33/0.39 | AY496036 |
| Nu 48 | F: TTGCGTCCTTCTTGTTTGTG<br>R: CTGTGGAGGGTGCAATTATG | $(CA)_{15}$ (interrupt) | 2.5 | 6 | 213–241 | 0.67/0.66 | AY500366 |

purified using a gene clean kit (Qbiogene), ligated into pcR 2.1-TOPO vector (Invitrogen) and transformed into TOP10 cells. Plasmid DNA was purified and sequenced on an ABI 377 automated DNA sequencer (PE Applied Biosystems) using dye terminator chemistry. Fifty putative positive clones were sequenced and microsatellite primers developed using PRIMER 3 (Rozen & Skaletsky 1997). Both long and short repeated loci were chosen (Table 1) because markers with different repeat numbers are expected to inform at different evolutionary timescales (e.g. Beheregaray & Sunnucks 2000).

Microsatellite loci were amplified by PCR using a 10 μL radiolabelled reaction containing ~50–100 ng of DNA, 12 pmol of each primer, 0.5 U of *Taq* DNA polymerase, 200 μM of dCTP, dGTP and dTTP, 20 μM of dATP, 2–2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 0.05 μL [α-$^{33}$P]dATPat 1000 Ci/mmol overlaid with mineral oil. PCRs were performed in a MJ Research thermocycler and consisted of 94 °C for 3 min, followed by a 'touchdown' (32 cycles at 94 °C/20 s, annealing/45 s and 72 °C/60 s), and a final step of 72 °C for 4 min. The annealing temperature of the touchdown PCRs decreased two degrees per cycle until stabilizing at the fifth cycle (from 63 °C to 55 °C). PCR products were separated by 6% polyacrylamide gel electrophoresis and visualized by autoradiography. The software GENEPOP 3.3 (Raymond & Rousset 1995) was used to estimate expected ($H_E$) and observed ($H_O$) heterozygosities and to test for linkage disequilibrium.

Nine loci successfully amplified and were screened for variation in a sample of 30 one-lined pencilfish collected from Rios Tea, Cuiuni and Igarapé Arixaná (middle Rio Negro, Brazil). Loci revealed high genetic variation: number of alleles per locus ranged from 4 to 19 and expected heterozygosities from 0.39 to 0.86 (Table 1). Most loci were at Hardy–Weinberg equilibrium in all populations. However, Nu11 and Nu44 showed homozygote excesses possibly related to null alleles (for Nu44 several individuals failed to amplify in three independent PCRs). No evidence for linkage disequilibrium was detected. Amplification of more than one allele in a sample of two brown pencilfishes (*N. eques*) was also achieved for all loci (except for Nu44) without additional optimizations, suggesting that these markers can be potentially useful across the genus *Nannostomus*.

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## References

Beheregaray LB, Sunnucks P (2000) Microsatellite loci isolated from *Odontesthes argentinensis* and the *O. perugiae* species group and their use in other South American silverside fish. *Molecular Ecology*, **9**, 629–631.

Beheregaray LB, Möller LM, Schwartz TS, Chao NL, Caccone A (2004) Microsatellite markers for the cardinal tetra *Paracheirodon*

*axelrodi*, an economically important fish from central Amazonia. *Molecular Ecology Notes*, **4** (in press).

Chao NL, Petry P, Prang G, Sonneschien L, Tlusty M (2001) *Conservation and Management of Ornamental Fish Resources of the Rio Negro Basin, Amazonia, Brazil (Project Piaba)*. Universidade do Amazonas Press, Manaus, Amazonas.

Fischer D, Bachmann K (1998) Microsatellite enrichment in organisms with large genomes (*Allium cepa* L.). *Biotechniques*, **24**, 796–802.

Raymond M, Rousset F (1995) Population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rozen S, Skaletsky HJ (1997) *PRIMER 3*. Whitehead Institute for Biomedical Research. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

Saltonstall K (2003) Microsatellite variation within and among North American lineages of *Phragmites australis*. *Molecular Ecology*, **12**, 1689–1702.

Weitzman SH (1978) Three new species of fishes of the genus *Nannostomus* from the Brazilian states of Pará and Amazonas (Teleostei: Lebiasinidae). *Smithsonian Contributions to Zoology*, **263**, 1–14.