Molecular Ecology Notes (2005) doi: 10.1111/j.1471-8286.2005.01079.x

PRIMER NOTE

Characterization of microsatellite loci for the detection of temporal genetic shifts within a single cohort of the brown demoiselle, *Neopomacentrus filamentosus*

M. ELIZABETH JONES and PAUL H. BARBER
Boston University, Department of Biology, 5 Cummington Street, Boston, MA 02215, USA

Abstract

***Neopomacentrus filamentosus*, a common damselfish on the Indo–Australian archipelago, undergoes significant shifts in size and mitochondrial genetic structure upon larval settlement and metamorphosis to juvenile stages. We characterized five polymorphic microsatellite loci in order to study temporal genetic shifts within a single generation of *N. filamentosus* sampled first as larval settlers then again as demersal juvenile recruits. All loci were extremely polymorphic and exhibited high levels of heterozygosity. While all loci from the larval samples conformed to Hardy – Weinberg expectations, significant heterozygote deficiencies were seen in two loci in the juvenile samples, likely due to extreme size-selective mortality imposed post-settlement.**

Keywords: damselfish, ecological selection, microsatellites, *Neopomacentrus*, selective neutrality

Received 01 April 2005; revision accepted 13 May 2005

Temporal sampling of the common reef fish, *Neopomacentrus filamentosus*, on the Dampier Archipelago, Western Australia has shown that natural selection favouring larger, faster growing fish causes significant shifts in size structure within a single generation over successive developmental stages (Vigliola & Meekan 2002), and that these phenotypic shifts are coupled with significant shifts in temporal genetic structure of a noncoding mitochodrial DNA locus (Barber *et al*. submitted). Size-selective mortality in other marine fishes has been shown to result in shifts in genetic structure within a protein coding locus linked to growth rate (Planes & Romans 2004). Due to linkage to such loci under selection, genetic diversity at presumably neutral markers, such as microsatellites or noncoding DNA regions, could indirectly be shaped by ecological selection. We developed microsatellite loci to examine temporal shifts in allele frequencies and genetic structure within a single cohort of *N. filamentosus* to determine the effects of size selective mortality on measures of genetic structure in a nuclear genetic marker.

Microsatellite markers were developed in part following the details of 'Microsatellite Easy Isolation 2000' (http://www.uga.edu/srel/DNA_Lab/protocols.htm; subsequently refined in Glenn & Schable 2005) and protocols described by Hamilton *et al*. (1999), Tenzer *et al*. (1999) and Garner *et al*. (2000). Genomic DNA was isolated from 40 mg muscle tissue from a single *N. filamentosus* using a DNeasy Tissue Kit (QIAGEN) and digested simultaneously with *Xmn*I, *Hae*III and *Alu*I (New England Biolabs), yielding blunt-ended DNA fragments ranging from 300 to 600 bp. Restriction fragments were dephosphorylated with Shrimp Alkaline Phosphatase (SAP; USB Corporation), ligated to SNX adaptors (Hamilton *et al*. 1999), nick-repaired and subsequently amplified with SNX-forward. Polymerase chain reaction (PCR) products were probed for microsatellite repeats using a 3′ biotin-labelled $(TAGA)_{28}$ oligo and captured via streptavidin-coated magnetic beads (Dynabeads M-270, Dynal Biotech). Repeat-enriched DNA was recovered by a second round of PCR with SNX-forward, cloned into pGEM-T Easy Vectors (Promega), transformed into One Shot chemically competent *Escherichia coli* (Invitrogen), and grown overnight at 37 °C on Luria–Bertani agar plates supplemented with Ampicillan and X-gal. Ninety-five insert-containing colonies were identified by blue/white screening and amplified via colony-PCR with the plasmid primers T7 (5′-GTAATACGACTCACTATAGGGC-3′) and M13R (5′-GGAAACAGCTATGACCATG-3′). Excess

Correspondence: M. E. Jones, Fax: 508 289 7512; Email: Jonese@bu.edu

Table 1 Summary of details for polymorphic microsatellite loci from 93 larval settlers and 64 juvenile recruits of *Neopomacentrus filamentosus*; 5% nominal significance is considered at $P < 0.005$. T_a, annealing temperature; N_A, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity. GenBank Accession nos for clone sequences are DQ022202–DQ022206

Locus	Primer sequence (5′–3′)	T_a	Repeat sequence	Size range (bp)	N_A	Larvae H_E/H_O	Juveniles H_E/H_O
Nfil-44	F: HEX — AAGAGAAGAGGAAGAGACG R: ATTTGCAAGAACTATGGACA	58	$(TAGA)_{28}$	256–432	37	0.956/0.878	0.951/0.828*
Nfil-50	F: 6-FAM — CTGAGATCTGGGCGTAAGGA R: GGAACCGGGATTAATAAAGT	54	$(TAGA)_{34}$	220–524	56	0.977/0.477	0.982/0.508
Nfil-56	F: HEX — AGGGTCTTGGCTGGCTGTC R: GTGGTGTCGCTTCTTATTTC	58	$(GT)_{23}$	150–224	28	0.946/0.946	0.934/0.875
Nfil-72	F: 6-FAM — CCTTTGAATTGGAGATGAGC R: ACACCCCCGTTGCCTCATAC	54	$(GT)_{18}$	210–336	49	0.960/0.910	0.969/0.952
Nfil-78	F: HEX — AAAATGTCAAATGTCACTG R: CCATCCACAAGTATTAACCT	58	$(TG)_9$	282–324	18	0.861/0.772	0.835/0.688*

*Indicates significant departure from expected Hardy–Weinberg proportions after Bonferonni correction for multiple comparisons.

primers and dNTP's were chemically removed with Exonuclease I and SAP. Reactions were sequenced in one direction with T7 using Big Dye Terminator version 3.1 chemistry and electrophoresed on an ABI 377 Automated DNA Sequencer (Applied Biosystems). Forty-five clones containing tandem repeats were sequenced in the opposite direction with M13R. Forward and reverse strands were assembled, edited, and characterized using SEQUENCHER 4.2 (Gene Codes).

Forward and reverse primers for 15 clones containing perfect repeats were designed using PRIMER 3 (Rozen & Skaletsky 2000) and OLIGO 4.06 (National Biosciences). DNA from seven *N. filamentosus* was extracted by Chelex (Walsh *et al*. 1991) and amplified in 10 μL reactions containing GeneAmp 1× PCR Buffer II (containing 10 mM Tris-HCl, pH 8.3 and 50 mM KCl; Applied Biosystems), 200 μM each dNTP, 2.0 mM $MgCl_2$, 0.5 μM each primer, 0.2 U Ampli*Taq* DNA Polymerase (Applied Biosystems) and either 0.5 or 2.0 μL DNA. Cycling parameters were 94 °C for 2 min, followed by 35 cycles at 94 °C, either 54 or 58 °C, and 72 °C for 30 s each, followed by a 30-min extension at 72 °C. Samples were screened for variation on a 2% NuSieve GTG agarose gel stained with ethidium bromide. Of 15 loci, three polymorphic $(GT)_n$ and two polymorphic $(TAGA)_n$ repeats were identified and the forward primer from each of these pairs was fluorescently labelled with HEX or 6-FAM (Table 1) for detection on an ABI 377.

DNA from 93 larval settlers and 64 3-month old juvenile recruits from a single cohort of *N. filamentosus* was extracted by Chelex and amplified with fluorescently labelled primers using cycling parameters described above. PCR products were run on an ABI 377 with an MRK400 ROX-labelled size standard (Gel). Allele sizes were scored using GENESCAN 3.1.2 (Applied Biosystems) and STRAND 2.2 (UC Davis' Veterinary Genetics Laboratory; http://www.vgl.ucdavis.edu/informatics/STRand/). Expected and observed heterozygosities were compared by exact tests using a Markov chain (1000 dememorization steps, 100 000 steps in chain) in ARLEQUIN 2.0 (Guo & Thompson 1992; Schneider *et al*. 2000). Pairwise genetic disequilibrium between all pairs of loci was calculated in FSTAT 2.932 (Goudet 1995).

Loci were extremely polymorphic, ranging from 18 to 56 alelles (Table 1). No pair of loci was found to exhibit significant genetic disequilibrium (data not shown). Observed heterozygosity was high, ranging from 0.477 to 0.946 in larval and 0.508 to 0.952 in juvenile samples. Heterozygosity increased over temporal sampling in two loci, but decreased in three, and significant heterozygote deficiencies were seen in the juvenile samples in two of these loci (Nfil-44 and Nfil-78, after Bonferonni correction). Heterozygote deficiencies are often a sign of null alleles. However, since deficiencies were only observed among juvenile recruits and not larval settlers, null alleles are unlikely to account for this pattern. Instead, heterozygote deficiencies in the juveniles likely result from pronounced genetic drift following extreme size-selective mortality in which up to 80% of settlers may be removed by selection (Planes & Romans 2004), resulting in directional genetic shifts in loci linked to growth rate, and stochastic genetic shifts in loci that are not. The results indicate that these markers will be useful in the study of temporal genetic structure in future studies of *N. filamentosus*.

Acknowledgements

We thank Laurent Vigliola and Mark Meekan for *Neopomacentrus filamentosus* samples, and Gabi Gerlach and Devin Drown for technical and data analysis advice.

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