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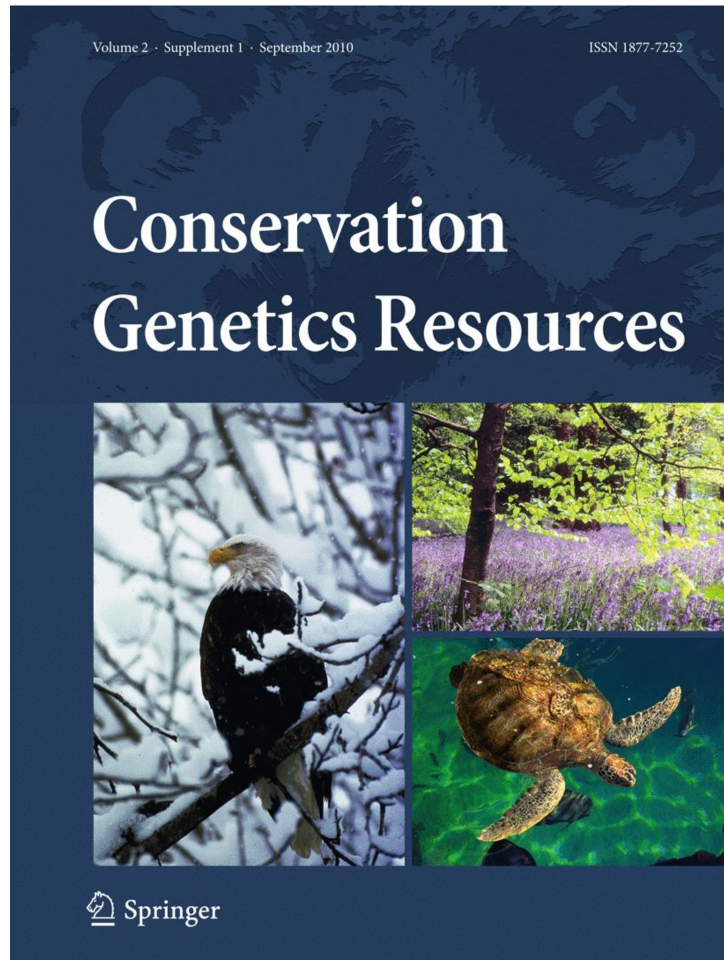

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TECHNICAL NOTE

Isolation of 15 new polymorphic microsatellite markers from the blue-spine unicornfish *Naso unicornis*

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Abstract The blue-spine unicornfish *Naso unicornis* is a widely distributed reef herbivore that is highly prized in tropical Indo-Pacific fisheries. Appropriate management for *N. unicornis* and other exploited reef fishes requires detailed knowledge of larval migrant exchange between isolated adult meta-populations and an understanding of recruitment patterns at both local and larger geographic scales. To this end, we have developed 15 microsatellite loci to evaluate levels larval connectivity and detect genetic patterns relevant to demographic processes in this species. Microsatellites were isolated from total genomic DNA using biotinylated probes and magnetic bead capture. We screened these loci against 90 individuals sampled from Guam in the tropical West Pacific. Loci contained 5–23 alleles (mean = 15.7) and had a mean observed and expected heterozygosity of 0.66 and 0.82, respectively. One locus, which did not conform to the expectations of Hardy–Weinberg equilibrium, is probably under selection. Four others are probably confounded by the presence of null alleles.

Keywords *Naso unicornis* · Reef fishes · Fisheries · Indo-Pacific · Micronesia

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Introduction

Microsatellite loci are useful molecular markers that are employed in a number of highly sensitive population level analyses such as assignment tests, parentage analysis, mixture and admixture analysis (Manel et al. 2005). In recent years these markers have become increasingly sought after for studying coral reef fish species, which often have large geographic ranges and complex patterns of population connectivity at varying spatial scales (Purcell et al. 2006; Thacker et al. 2007; Hepburn et al. 2009; Planes et al. 2009). However, development of these loci are expensive, time consuming and, compared to the diversity of coral reef fish taxa, the number of microsatellite markers available to reef fish biologists are few. Here we present 15 new polymorphic microsatellite loci for the blue-spine unicornfish *Naso unicornis* isolated from total genomic DNA using the enrichment protocol of Glenn and Schable (2005).

Naso unicornis is a large (~70 cm max length) herbivorous coral reef fish that grazes on large thallate macroalgae (Choat et al. 2002). Because reef fish herbivory increases the resilience of coral reefs to climate change (Hughes et al. 2007), this species is believed to have a functional impact on reef ecosystems proportionate to its density and biomass. Yet, *N. unicornis* is also a heavily targeted species by fisheries in many parts of the tropical Indo-Pacific. Commercial fisheries on Pohnpei (Micronesia), for example, harvest approximately 1,521 kg of reef fishes per day, of which *N. unicornis* and the closely related *Naso lituratus* are a substantial component of the total landing, approaching 28% (Rhodes et al. 2008). Therefore, understanding the population dynamics of the blue-spine unicornfish is critical from both a fisheries and conservation management perspective.

A recent phylogeographic study, using mitochondrial markers, revealed that the isolated adult meta-populations of *N. unicornis* are genetically uninterrupted between the Indian and Pacific oceans, indicating that the migratory pelagic larvae of this species are highly dispersive (Horne et al. 2008). A tendency for long distance dispersal leading to the external replenishment of local populations would potentially allow unicornfish stocks to quickly recover in areas where they have been heavily fished. However, mitochondrial population connectivity does not equate to recent migrant exchange (Hellberg 2007). High-resolution microsatellite analyses are necessary to assess the extent to which larval recruitment from external sources is demographically significant.

Methods and results

We extracted total genomic DNA from fin clips of three *N. unicornis* adults collected from the Seychelles, Lizard Island (Great Barrier Reef) and Tonga. Tissue was digested with proteinase K and DNA extracted using a salt-chloroform method (Sambrook et al. 1989). Total genomic DNA was digested into 300–1,000 bp fragments using the restriction enzymes Rsa I and Hae III separately. Double stranded SNX linkers were ligated to both sides of the blunt ended fragments using T4 DNA ligase, in accordance to the protocol of Glenn and Schable (2005).

Linker ligated DNA fragments were PCR amplified using the super SNX-24 primer and subsequently annealed to four different combinations of biotinylated oligo probes of di, tri and tetra-nucleotide motifs [$(AG)_{16}+(AC)_{16}$, $(AAG)_8$, $(AAAC)_6+(AATT)_8$, $(AAGG)_6+(AGCG)_4$]. Reaction mixtures consisted of 25 µl 2× hyb solution (Glenn and Schable 2005), 10 µl mixed oligos (1 µM each), 10 µl linker ligated DNA, 5 µl nuclease free H_2O for a total volume of 50 µl. Thermocycler temperatures for the annealing of probes were as follows: 95°C for 5 min, 70°C for 5 s followed by 99 five-second incremental step downs of 0.2°C and 50°C for 10 min followed by 20 five-second incremental step downs of 0.5°C.

50 µl of Streptavidin bonded magnetic beads (Dynabeads, Invitrogen Dynal, Olso, Norway) were washed twice in 250 µl of TE buffer, twice in 1× hyb solution and suspended in a final volume of 150 µl 1× hyb solution. The hybridized DNA-oligo fragments were added to the magnetic bead solution and captured with a magnetic particle separator, while the supernatant and miscellaneous DNA was discarded.

Microsatellite enriched DNA was again PCR amplified using the super SNX-24 primer and ligated into the pCR®2.1-TOPO vector (Invitrogen) as per the manufacturer's instructions. Cloned inserts were sent to the Australian Genome Research Facility at the University of Queensland, Brisbane for bacterial transformation and sequencing with universal M13 primers.

The resulting DNA sequences were screened, microsatellite loci identified and primers designed using the program Msatcommander (Faircloth 2008). A total of 71 loci were detected and the 38 best (those with the longest number of repeats) were chosen for further screening. Loci were amplified in a small number of individuals using standard PCR parameters. Polymorphism was tested by direct sequencing, which revealed high sequence variation, within loci and in the flanking regions. Additionally, length variation was not consistently uniform across loci, with many alleles differing in length by single base pairs due to small indels in the flanking sequences. In general, the microsatellite loci of *N. unicornis* could be described as imperfect or compound, as many had different repetitive elements adjacent to each other or were bisected by non-repeating base pairs. Though not optimal for microsatellite analysis, the excessive amount of polymorphism observed in the nuclear genome of *N. unicornis* is consistent with mitochondrial studies which show abnormally high genetic diversity in this and other *Naso* species (e.g. *Naso vlamingii* Klanten et al. 2007; *Naso brevirostrus* Horne et al. 2008).

The 15 cleanest and most polymorphic loci were screened against 90 adult *N. unicornis* (114–485 mm FL) collected from Guam in the tropical West Pacific. Sample DNA was PCR amplified using forward primers that were flurolabeled with either HEX, TET or FAM. PCR products of each of the three dyes were combined into 96-well plates, purified using a standard ethanol ammonium acetate clean-up and read using Amersham MegaBACE instrumentation at the James Cook University Genetics Analysis Facility. The program Genepop 4.0 (Rousset 2008) was used to assess allelic diversity, estimates of heterozygosity for each locus, to test for departures from Hardy–Weinberg equilibrium (Table 1) and to test linkage disequilibrium. Mean observed and expected heterozgosities were 0.66 and 0.82, respectively. Data from five loci (Nuni02, Nuni04, Nuni06, Nuni13, Nuni15) departed significantly from Hardy–Weinberg equilibrium. Often such departures are due to the presence of null alleles. The program Micro-Checker (van Oosterhout et al. 2004) indicated that Nuni02, Nuni06, Nuni13 and Nuni15 are confounded by null alleles but that Nuni04 is not and may be either under selection or otherwise linked to a gene region that is.

Tests of linkage disequilibrium indicate that locus Nuni02 may be linked to Nuni13 and Nuni10. However, additional samples from other locations need to be screened to confirm linkage between these loci.

Table 1 Description of 15 microsatellite loci for the blue-spine unicorn fish (*Naso unicornis*)

Locus	Repeat motif	Primer sequence	T_a (°C)	N_a	Size range (bp)	H_O	H_E
Nuni01	$(CA)_{12}$	F: 5′ ACGCACAGTTGAGGGAGAG	60	10	167–185	0.77	0.79
		R: 5′ AAGGACAAAGTGTGAGGGG					
Nuni02	$(CA)_{16}$	F: 5′ CTCTGGGTATGCTTAATGGGC	60	11	166–189	0.40	0.82***
		R: 5′ CCAGCCTTGTTGTTCACCG					
Nuni03	$(CA)_{20}$	F: 5′ GATGAGGCTACACACGCTG	60	21	151–196	0.90	0.93
		R: 5′ AGGAGTTTCACTTCCTCCCAC					
Nuni04	$(CA)_{20}$	F: 5′ GAACACACGGGCTGCTG	58	19	140–185	0.83	0.89**
		R: 5′ CCATGTATTTGGAGAGTAGTAGTGC					
Nuni05	$(CA)_{23}$	F: 5′ CCCCTTCCTGTGGCTGTAG	60	17	181–214	0.89	0.88
		R: 5′ CCTGGTTTGCACTTGGAGC					
Nuni06	$(CA)_{22}$	F: 5′ AGTGTCGCTCCTTCAGTGC	61	21	171–215	0.61	0.92**
		R: 5′ CGCAGGTGAACGGCATATC					
Nuni07	$(GA)_{34}$	F: 5′ GATTCAGGCACGCCACAC	60	23	212–246	0.86	0.91
		R: 5′ TGTTTGTGCAGCTTGGGAG					
Nuni08	$(GTT)_{11}$	F: 5′ CGCATTTTGTTTCCACTGCC	60	12	158–179	0.62	0.75
		R: 5′ AGGATCCGCTGGTTACCTC					
Nuni09	$(TAGA)_{13}$	F: 5′ TCCCAGTTATCACCGCCTG	60	17	173–249	0.84	0.88
		R: 5′ TCCAATACACCTGTTCTGCC					
Nuni10	$(CATT)_{11}$	F: 5′ TGCTCCTACTCGACTCATTTCC	60	21	205–248	0.82	0.88
		R: 5′ CTGGAGTTTGTGAGTTGTCGG					
Nuni11	$(CTT)_9$,$(GTT)_2$	F: 5′ TGCTAACTGCCAAGGACCC	60	5	161–174	0.43	0.51
		R: 5′ TGAACCTGAAAACGAGGAGC					
Nuni12	$(CA)_{10}$,$(ACGC)_{13}$	F: 5′ TCACAGAGTGTGTATGATTGTCTG	60	16	202–250	0.88	0.88
		R: 5′ CCCTGCTGGTCATTGTGTTG					
Nuni13	$(GGTT)_6$, TTT, $(GTT)_5$	F: 5′ TAGTTCCTCAGCACAGCCC	60	19	204–247	0.71	0.86**
		R: 5′ TCCTAATCTCAATGCACTGGC					
Nuni14	$(ATT)_6$, $(GTT)_{10}$	F: 5′ TGTTTCGCTGCCATCAGAG	60	15	234–262	0.72	0.81
		R: 5′ TCGACACAATGAAGTGCCAG					
Nuni15	$(CTT)_2$, TTTCTC, $(CTT)_5$	F: 5′ TCCTCTCCACTGGCATCTG	60	9	210–229	0.42	0.59***
		R: 5′ GCCTCCATGCAGACATTAGC					

Screening results from 90 adult *N. unicornis* from Guam

Genbank accession numbers: XXX-XXX

T_a, PCR primer annealing temperature in degrees Celsius; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

** Significant departure from Hardy–Weinberg equilibrium $P < 0.01$, *** $P < 0.001$

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