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PRIMER NOTE

Microsatellite DNA markers for analysis of population structure in the sea urchin *Centrostephanus rodgersii*

SAM C. BANKS, MAXINE P. PIGGOTT, JANE E. WILLIAMSON and LUCIANO B. BEHEREGARAY
Molecular Ecology Group for Marine Research (MEGMAR), Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

Abstract

We describe the development of 13 variable microsatellites developed to investigate population structure and dispersal in the sea urchin *Centrostephanus rodgersii*. This species is the dominant grazing herbivore in southeast Australian coastal waters and has the ability to modify benthic community structure. The microsatellites we identified showed a range of allele numbers (4–21) and expected heterozygosity (0.32–0.91) in two sampled populations. Contrary to previous findings in free-spawning marine invertebrates, genotype proportions in neither population deviated significantly from Hardy–Weinberg expectations.

Keywords: *Centrostephanus rodgersii*, enrichment, larval dispersal, microsatellites, sea urchin

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The diadematid sea urchin *Centrostephanus rodgersii* is the dominant grazing herbivore on rocky reefs along the coast of southeastern Australia (Andrew & Byrne 2001). Little is known about larval dispersal and population structure in echinoids, and marine invertebrates generally, so we developed microsatellite markers to investigate genetic structure in *C. rodgersii*. Estimates of the scale of larval dispersal will aid in understanding the southward spread of this species that has coincided with increasing sea temperatures. Data on *C. rodgersii* will be combined with population genetic data sets from other codistributed taxa (Schwartz *et al*. 2005; Banks *et al*. 2006) to investigate the influence of ocean currents and coastal geography on the population structure of marine species in southeastern Australasia.

We collected *C. rodgersii* individuals near Eden (37°01′S, 149°56′E) and extracted genomic DNA from gonad tissue from a single individual stored in 100% ethanol using a modified salting-out procedure (Sunnucks & Hales 1996). We used an enrichment technique (Fischer & Bachmann 1998; Saltonstall 2003) to isolate microsatellites. Briefly, 5 μg of DNA from a single individual was digested with the restriction enzymes *Rsa*I and *Hae*III (Boehringer) and the digested fragments were ligated to two oligo adaptors. The digested DNA was annealed to dGA_{10} and dGT_{10} biotinylated probes and selectively purified using streptavidin magnetic particles (Promega). The microsatellite-enriched eluates were amplified in a polymerase chain reaction (PCR) using one of the oligo adaptors as a primer and the PCR product was used to repeat the enrichment protocol from the probe annealing step. The enriched DNA was purified using an UltraClean 15 DNA purification kit (MoBio Laboratories), ligated into a pcR 2.1-TOPO vector (Invitrogen) and transformed into TOP10 cells (Invitrogen). We PCR-amplified the plasmid DNA directly from the colonies using M13 forward (–20) and reverse (–40) primers and sequenced the purified products on an ABI 377 automated DNA sequencer (PE Applied Biosystems) using dye terminator chemistry.

From the first library, we obtained few sequences containing long dinucleotide repeats and many clones had identical sequences. Despite using the single-stranded conformation polymorphism (SSCP) technique to avoid sequencing identical clones (Piggott *et al*. 2006), additional libraries were required to obtain sufficient variable microsatellites for population analyses. We repeated the enrichment protocol until we obtained microsatellite-enriched DNA that showed no evidence of preferential enrichment of a small number of sequences (Fig. 1). Cloning from the first enrichments (A and B in Fig. 1) yielded few unique sequences. Following SSCP analysis and sequencing of 227 and 136 clones from libraries A and B, we designed primers for only three and six repeat sequences from these

Correspondence: Sam Banks, Fax: 61 (2) 9850 8245; E-mail: sbanks@bio.mq.edu.au

libraries, respectively. In contrast, of 35 clones sequenced from the third library (C), only four were redundant and we were able to design primers for 18 repeat sequences. Primers for the 27 microsatellite loci were designed using the PRIMER 3 program (Rozen & Skaletsky 1997).

We assessed the variability of each microsatellite locus in 30 individuals sampled from Jervis Bay (35°7′S, 150°45′E) and 30 individuals from Cape Howe (37°30′S, 149°59′E). The loci were amplified in 10 μL radiolabelled PCRs containing ~50–100 ng of template DNA, 4 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Promega), 200 μM of dCTP, dGTP, and dTTP, 20 μM of dATP, 1.5–2.5 mM $MgCl_2$ (see Table 1), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 0.04 μL [α-^{33}P] dATP at 1000 Ci/mmol. PCR amplifications were performed in an MJ Research PTC-100 Thermal Cycler and were initiated at a melting temperature of 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, annealing for 30 s and extension at 72 °C for 45 s, with a final 10-min extension step at 72 °C. The annealing temperatures followed a 'touchdown'

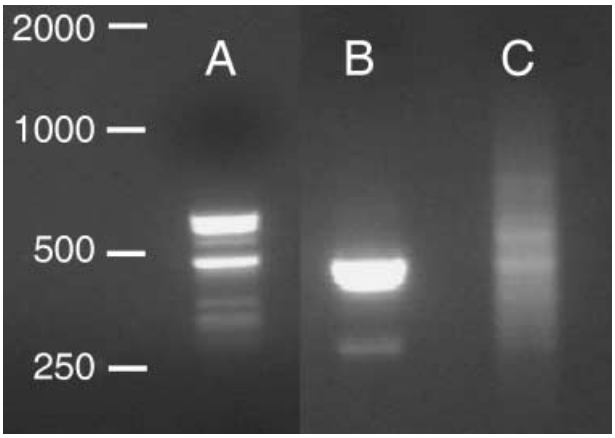

Fig. 1 Microsatellite-enriched DNA from *Centrostephanus rodgersii* ran on a 2% agarose gel next to a 2000-base pair size marker. A, B and C are the results of enrichment from different DNA samples using the restriction enzymes *Hae*III and *Rsa*I (A and C) and *Hae*III, *Pvu*II and *Pst*I (B) and dGA_{10} and dGT_{10} probes. Strong distinct bands suggest preferential enrichment of particular sequences. The third enrichment (C) yielded the most productive microsatellite library.

Table 1 Primer sequences and characteristics of 13 *Centrostephanus rodgersii* microsatellites, including PCR annealing temperature (T_a) and magnesium chloride concentration. The primer names indicate which round of enrichment/cloning each locus was isolated from. Loci labelled CRO1 were from enrichment A in Fig. 1, while the CRO2 and CRO3 loci were from enrichments B and C, respectively. Sample size (N) Number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosities are based on average values across two samples of 30 individuals each from Jervis Bay and Cape Howe, southeastern Australia

Locus	Primer sequences (5′–3′)	Repeat structure	[Mg^{2+}] (mM)	T_a (°C)	N	N_a	Size range (bp)	H_O	H_E	GenBank Accession no.
CRO1-6	AGTTTCACGGATCCCGTATG GCCCACAAGAAATCATCAAAAC	$CA_{8(INT)}$	3	62–56	30	5.5	139–166	0.391	0.335	DQ666354
CRO2-7	CATCTGTGACCATGCAGGAG GCCACGGTGTGATAGTGATG	$GA_{6(INT)}$	3	62–56	30	5.5	196–210	0.729	0.600	DQ666355
CRO2-9	CGTCGCGTGTATACAATGTC TATCGACGGCTACACTGCAC	$GA_{35\ (INT)}$	3	62–56	30	21	267–310	0.899	0.907	DQ666356
CRO2-10	GCGAGATCTACAATCCAATGTC GACAACCGATGACTTATTGTCC	CA_4G_{16}	3	62–56	30	6	160–167	0.810	0.799	DQ666357
CR03-2	CATGCCCCCTAAACAATACG GTGAACACGACCCTGTGTTG	GA_{11}	2	56–50	30	8	217–239	0.838	0.812	DQ666358
CRO3-4	ACGCACACACGCATTTATTC CACAGCCCATCCTCAAATG	GA_{12}	2.5	55–47	30	4	125–132	0.671	0.540	DQ666359
CRO3-10	CTTCAGCCACTTCCGAGTTC AAGGCATTCACTGAACAAAGTG	CA_{10}	2.5	55–47	30	10.5	175–209	0.777	0.771	DQ666360
CRO3-11	AATTGCGTTCATGCTTAGCC AAAGTCCTTCTGGCATAACACC	$GA_{42(INT)}$	3	55–47	30	15	209–247	0.940	0.900	DQ666361
CRO3-12A*	GCGTATATGTGTATGCGTTGC TTGTCCTCCTTTGTTCGATG	CA_{15}	2.5	55–47	30	7	101–127	0.402	0.393	DQ666362
CRO3-12B*	TCTTTTGGAAGTCATCGGTTG TGGAGAAGCAAATTTTAAAAAGC	$CA_{50(INT)}$	2.5	55–47	30	13	167–199	0.797	0.782	DQ666362
CRO3-22	AATCATGGCTAGGTGCTTGC ATGAACGCACCCATAGACG	$CA_{7(INT)}$	2.5	55–47	30	3	143–147	0.409	0.322	DQ666363
CRO3-58	TTGTTATCGCAGCTTTCAGC TATCGCACAGCGGTAAAATC	CA_{39}	2.5	55–47	30	8	209–218	0.688	0.722	DQ666364
CRO3-61	GCATGTTCAGTGGGTTATATGG GCTTTACGCGATGGACTACC	GA_{34}	2.5	55–47	30	6	125–143	0.616	0.602	DQ666365

*The repeat sequences of loci CRO3-12A and CRO3-12B are only 168 base pairs apart.

protocol, decreasing in 2 °C increments per cycle to the final annealing temperature (Table 1). The PCR products were separated by 6% polyacrylamide gel electrophoresis, visualized by autoradiography and sized by comparison to an A- and T-terminating M13 control DNA sequencing reaction size marker.

We identified 13 polymorphic microsatellite markers (Table 1). The total number of alleles identified ranged from four to 22 per locus, and the expected proportion of heterozygotes ranged from 0.322 to 0.907. Unusually for a free-spawning marine invertebrate, genotype proportions did not differ from Hardy–Weinberg expectations at any locus or population as tested in GENEPOP version 3.4 (Raymond & Rousset 1995). Using GENEPOP version 3.4 (Raymond & Rousset 1995), we did not detect significant linkage disequilibrium between any locus pairs, including CRO3-12A and CRO3-12B, in which the repeat sequences were only separated by 168 base pairs. Although these are not independent markers, both primer sets are presented here as they showed different levels of variability. The range in repeat sequence length and diversity of the microsatellites described here will allow us to investigate patterns of population structure in *C. rodgersii* at a range of spatial scales.

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