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TECHNICAL NOTE

Isolation and characterization of microsatellite markers for the marine black nerite *Nerita atramentosa*: tools for assessment and design of marine protected areas

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Abstract Black nerite snails are ecologically important herbivores of the intertidal zone of Australia's temperate marine coast. A set of 13 microsatellites was developed for the black nerite *Nerita atramentosa* using 1/8 of a shot-gun pyrosequencing reaction. From 46,971 sequences containing putative microsatellite motifs, 18 were selected for primer design. Thirteen microsatellite loci were successfully genotyped using three multiplex reactions in 45 specimens of black nerite from two localities. The number of alleles per locus varied from four to 23 (mean = 12.4) and the observed heterozygosity ranged from 0.40 to 0.96 (mean = 0.72). Only one locus showed significant deviation from Hardy–Weinberg equilibrium probably due to null alleles. No linkage disequilibrium between pairs of loci was detected. These polymorphic markers represent useful tools for connectivity and seascape genetic studies of Australian black nerites. These studies should provide valuable information for the design and assessment of marine protected areas in temperate waters of Australia.

Keywords MPA · Seascape genetics · Genetic connectivity · Australia · Mollusk

Development of efficient networks of marine protected areas (MPAs) is a major goal of conservation and management programs of marine ecosystems. The efficiency of MPAs as management tool on a scale larger than the reserve boundaries depends mainly on the dispersal of individuals from MPAs to surrounding habitats (Gaines et al. 2010). For an optimal design of MPAs, it is relevant to identify patterns on population connectivity between the reserve and adjacent areas. Molecular approaches provide efficient ways to estimate genetic connectivity but often require the use of hypervariable co-dominant markers, such as microsatellites (Saenz-Agudelo et al. 2009). Hence, the development and analysis of microsatellite markers in widespread marine species would provide and important tool for assessing connectivity and informing the design and assessment of a system of MPAs.

Australian black nerites (genus *Nerita*) are one of the dominant herbivores in the intertidal zone of the Australian temperate coast (Spencer et al. 2007). Recent studies indicate that two black nerites occur in temperate mainland Australia. *Nerita atramentosa*, a species described from Western Australia, is found on the south coast as far east as Wilsons Promontory in Victoria; *N. melanotragus* is the dominant species of black nerite in southeastern Australia, from southern Queensland to Wilsons Promontory (Waters et al. 2005; Spencer et al. 2007). *N. atramentosa* lays eggs during summer, they hatch after 2 weeks and the larvae stay in the water column for approximately 5–6 months before settling in winter (Underwood 1975). Their wide distribution and long dispersal capability make Australian black nerite an attractive system to assess ecological and historical factors that could be influencing population connectivity along the temperate coast of Australia.

Microsatellite loci for Australian black nerite were obtained using a next generation DNA sequencing (NGS) technology. A total of 10 μg of genomic DNA was extracted from muscle tissue of a black nerite specimen and sent to the Australian Genome Research Facility (http://www.agrf.com.au) for DNA sequencing. That was conducted on 1/8 of a 70 9 75 PicoTiterPlate using the Roche GS FLX (454) system as described elsewhere (Margulies et al. 2005).

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The software MSATCOMMANER (Faircloth 2008) was used to screen contigs for microsatellites using the default settings. MICROFAMILY (MeglÉCz 2007) and PRIMER 3 (Rozen and Skaletsky 1999) were used to select contigs and design primer sets as described in Carvalho and Beheregaray (2011).

A total of 217,455 reads were obtained, from which 46,971 sequences contained putative microsatellite motifs. MICROFAMILY recovered 121 unique loci, from which the best 18 loci were chosen for polymerase chain reaction (PCR) trials. Amplifications and PCR conditions followed Beheregaray et al. (2004) using a 63–55°C touchdown. From initial PCR optimizations with the 18 markers, 13 amplified consistently and were selected for further analyses. For the microsatellite loci characterization, 24 individuals from Peak Bay, South Australia (34°29′12″S, 136°04′02″E) and 21 individuals from Albany, Western Australia (35°01′48″S, 117°53′02″) were genotyped.

The PCRs were performed separately, products were combined in three runs namely PlexA, PlexB and PlexC (Table 1), and amplified peaks were detected on an ABI 3130 Sequencer (Applied Biosystems). The resulting profiles were examined to determine alleles using GENEMAPPER 4.0 (Applied Biosystems). MICROCHECKER (Van Oosterhout et al. 2004) was used to assess scoring errors and null alleles in the amplified genotypes. Number of

Table 1 Characteristics of the 13 microsatellite loci isolated from *Nerita atramentosa*

Locus	Primer sequences[a]	M13 label	Repeat motif	N_A	Size range (bp)	H–W	H_O/H_E
PlexA							
Neat01	F: AATACACCACGATAACATCC	PET	(CTGT)10	7	190–214	0.4438	0.600/0.596
	R: CTCATCTCCTCCATCACC						
Neat02	F: GGATTTCCAGTTTGCTTC	VIC	(CCTGA)11	16	247–407	**0.000***	0.611/0.915
	R: TTCTTCAGACCTTACTAGCC						
Neat04	F: CATGCAGTAGCAGTAGTAGC	6-FAM	(GGT)10	5	225–237	0.1178	0.400/0.429
	R: TTTTAGTATGACGAATGGAC						
Neat10	F: AGAGATCATTTTCTGCTCC	VIC	(AC)13	4	205–217	0.9096	0.605/0.568
	R: TGCATTTATGTGTTCATCC						
Neat19	F: GTCAAAGTCATACCACGC	NED	(AGGT)10	11	196–236	0.1302	0.822/0.863
	R: TCTTGCTCTCCTATTTATCC						
PlexB							
Neat05	F: CAGTTCAGACAAAGGAAGG	PET	(CA)14	10	200–220	0.666	0.800/0.780
	R: GAATTGAGGGAGTTGGAG						
Neat07	F: ATGACTGTGCAATAGTTGG	6-FAM	(ACAG)21	11	320–384	0.9965	0.666/0.543
	R: GTCTCTCTGTCCTTTACCG						
Neat14	F: TTCAAGATTCACAACTTCAG	VIC	(GAT)12	12	204–237	0.3163	0.622/0.601
	R: TGACTTACTTACTTACTGCCC						
Neat16	F: TAATGGAACTACTTTCCTGC	NED	(AGGT)12	23	196–296	0.5122	0.956/0.910
	R: CTCCTCCTACACCAATACAC						
PlexC							
Neat03	F: CTTGTGAAGGTAGATTGAGG	NED	(TG)11	7	180–192	0.5198	0.733/0.743
	R: AGCAAACAATGGAGACAG						
Neat09	F: ATACTTGGTCTCTTCGGTC	PET	(AC)18	17	218–254	0.0596	0.822/0.921
	R: AAATAGGCATAAATGTGCTC						
Neat12	F: TTTTTATTTCTACCTCGCAC	6-FAM	(CTT)18	22	220–286	0.0285[§]	0.861/0.935
	R: ACTTCTTTCTTTCTTTTTCG						
Neat18	F: TCTTAACCTTTTGCATACTG	VIC	(AGG)14	16	230–278	0.0111[§]	0.860/0.929
	R: TTAGATGAATGATGGATGAG						

Number of alleles (N_A), range of allelic size, Hardy–Weinberg P values (H–W), observed (H_O) and expected (H_E) heterozygosity, all based on 45 specimens

* Significant after Bonferroni correction

§ Not significant after Bonferroni correction

[a] Forward primers were tagged with a 5′M13 universal sequence (5′TGTAAAACGACGGCC) and label using four different fluorescence labels

alleles (N_A), expected (H_E) and observed (H_O) heterozygosity, linkage disequilibrium and Hardy–Weinberg proportions were estimated using GENEPOP V4 (Rousset 2008). Sequential Bonferroni corrections were applied when conducting multiple statistical tests (Rice 1989).

All 13 loci were polymorphic, with an average of 12.4 alleles per locus (ranging from 4 to 23 alleles) and H_O ranging from 0.40 to 0.96 (mean = 0.72). After sequential Bonferroni correction, evidence for null alleles was observed only for Neat02, but with no evidence of scoring errors. No deviations from Hardy–Weinberg equilibrium were detected, except for Neat02 and there was no evidence of linkage disequilibrium between pairs of loci. Cross-amplification was tested for all the loci in *N. melanotragus* using five samples from New South Wales, Australia. Amplification succeed for six loci and polymorphism was detected on five of them. The microsatellite markers reported here represent useful tools for connectivity and seascape genetic studies of Australian black nerites. These studies should provide valuable information for the design and assessment of MPAs in temperate waters of Australia.

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