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TECHNICAL NOTE

Characterization of 18 polymorphic microsatellite loci from *Bathymodiolus manusensis* (Bivalvia, Mytilidae) from deep-sea hydrothermal vents

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Abstract Species in the genus *Bathymodiolus* are mytilid mussels found at deep-sea hydrothermal vents and cold seeps. Next-generation sequencing techniques were employed to identify eighteen unlinked polymorphic microsatellite loci for *Bathymodiolus manusensis* from Manus Basin in the western Pacific. Allele frequencies for eight loci conform to Hardy–Weinberg expectations and observed heterozygosities ranged from 0.04 to 0.90 (mean $H_O = 0.45$, SD = 0.25). Ten of eighteen loci cross-amplified in *Bathymodiolus heckerae* from Atlantic seeps. Microsatellites developed for *Bathymodiolus manusensis* are being deployed to study connectivity among populations of this species colonizing geographically discrete back-arc basin vent systems.

Keywords Mytilid mussel · Back-arc basin · Chemoautotrophic · *Bathymodiolus* · Hydrothermal vent · Microsatellite

Bathymodiolin mussels (Mytilidae) are a species-rich group found globally at multiple deep-sea habitats, including hydrothermal vents and cold seeps (Van Dover et al. 2002). Species in the genus *Bathymodiolus* derive nutrients from chemoautotrophic bacterial endosymbionts (Won et al. 2003). Previous studies have focused on phylogenetics and phylogeography of this genus (Miyazaki et al. 2004; Faure et al. 2009; Kyuno et al. 2009); limited information is available on fine-scale population structure for this genus. Next-generation sequencing techniques were used to identify 18 polymorphic microsatellite markers from *Bathymodiolus manusensis* (Hashimoto and Furuta 2007) samples collected from Manus Basin in the western Pacific.

Bathymodiolus manusensis DNA was extracted using Proteinase K digestion followed by phenol:chloroform extraction. Total non-enriched genomic DNA was sequenced on a Roche 454 GS-FLX instrument at the Institute for Genome Science and Policy at Duke University using GS-FLX Titanium reagents (Roche, Branford CT). The sequencing run included four independent Multiplex Identifiers (MIDs). A single MID from *Bathymodiolus manusensis* resulted in 28,824 sequences greater than 200 bp in length and were used for subsequent analyses to ensure enough flanking sequence was present for primer design. Repetitive elements were located using Msatcommander (Faircloth 2008) and flanking primers designed with Primer3 software (Rozen and Skaletsky 2000). Forward primers were designed with a T3-tag (5′-ATTAACCCTCACTAAAGGGA-3′) to allow labeling of PCR products with a fluorescently labeled universal T3-primer (Schuelke 2000).

Genomic DNA templates were amplified with 10 µl polymerase chain reactions (PCRs) as follows: 1 µl DNA, 1 µl 10× PCR Buffer (200 mM Tris, pH 8.8; 500 mM KCl; 0.1% Triton X-100, 0.2 mg/ml BSA), 0.8 µl 25 mM $MgCl_2$, 0.8 µl 2.5 mM dNTP's, 0.2 µl 10 µM Forward primer, 0.8 µl 10 µM Reverse primer, 0.8 µl 10 µM labeled (FAM, NED, PET, or VIC) T3 primer (Eurofins: Huntsville, AL), and 0.1 µl Taq polymerase (1 unit, Bioline: Taunton, MA). Reactions were run under the following conditions: 95°C 4 min; 25 cycles of 94°C 15 s, 62°C 15 s, 72°C 30 s; 8 cycles of 94°C 15 s, 53°C 15 s,

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Table 1 Polymorphic microsatellite loci from *Bathymodiolus manusensis*

Locus name	Repeat motif	Primer sequence	Size range	N_A	H_{obs}	H_{exp}	P	Cross amplification
Bm01	$(AC)_6$	F: CCACACTGTTAGAACAATTCCAAA	290–296	3	0.07	0.17	0.000*	0/4
		R: CTGAAATTGGCGTTGAAACA						
Bm06	$(AC)_6$	F: GGGCAAACCGAACATAGAAA	221–228	4	0.22	0.44	0.000*	3/4
		R: TTGCCTTTGTTGTTGACTCAG						
Bm16	$(AT)_6$	F: GCAAAAATCAAATTTTCAGTCCA	223–246	5	0.07	0.17	0.000*	0/4
		R: GTAATGGCCTGGAGGTGTTC						
Bm17	$(AT)_6$	F: CAGGTGATTGTTTGTTTTGTTGA	261–265	3	0.23	0.23	0.240	0/4
		R: GCCAACAAAGATTTCCCACA						
Bm22	$(AAAC)_6$	F: CCAATCAATGTTCCGGTTTC	236–291	16	0.90	0.89	0.816	4/4
		R: GGGTGGTGGTGTGAACACTT						
Bm23	$(GTTT)_7$	F: AAACATCATAAACCATATGCCAAC	237–273	6	0.67	0.63	0.455	2/4
		R: ATCTTGATCAGGGCAAGGTG						
Bm48	$(AAT)_4$	F: CCCATTCAATTTTACCCCACT	202–204	3	0.04	0.48	0.000*	0/4
		R: AGCCAAGAAAAGCTAGTGCAG						
Bm52	$(GTTT)_5$	F: TCGGGACTAAGGGTATTGGA	229–279	10	0.37	0.84	0.000*	0/4
		R: GCGGCCATTCTAAATAAGCA						
Bm53	$(GAGT)_5$	F: TCTGGTCTTTTGAGTGGAAGC	245–275	6	0.63	0.61	0.164	0/4
		R: CGCGGACTGTTGTTTCAGT						
Bm55	$(ACAG)_4$	F: CAAGAAAGTGTGACGGATGG	186–252	17	0.48	0.70	0.000*	2/4
		R: TCGGTATAAAATCGGCTGAAA						
Bm62	$(GTTT)_4$	F: CCGTGTTGCTCATCTTTTCA	207–318	11	0.54	0.85	0.000*	0/4
		R: GGGCGAACAGAGCGTTATTA						
Bm63	$(GTTT)_4$	F: GGGAAGAGGGGTGTTTGTTT	318–349	10	0.62	0.70	0.090	0/4
		R: TGGCATTCATGGAGATACACA						
Bm64	$(AATC)_6$	F: GCTGAAATACTGCTAATGTTGGTG	229–252	7	0.32	0.55	0.000*	3/4
		R: GCCTTACAAGGTAACCACTTCTG						
Bm70	$(GAGT)_{10}$	F: ACTAGCTTGCGACAGGCATT	215–260	12	0.47	0.86	0.000*	3/4
		R: CCAACTCAGCGTGTTGGTC						
Bm72	$(AATC)_{19}$	F: AAACCCCGTTCTCGTTATGA	214–318	22	0.49	0.91	0.000*	1/4
		R: CGAGCATGCTGACCATTACA						
Bm76	$(GTTT)_6$	F: TGTTTTGCCGGACATCATATT	187–206	7	0.64	0.63	0.372	2/4
		R: CCCACGTTTCTCATACATTTCA						
Bm81	$(GATT)_6$	F: TGAAATGGTATGGAATGAATGG	197–245	16	0.75	0.83	0.063	3/4
		R: CATTGTCCCCTTTGAAATCG						
Bm83	$(GAT)_9$	F: TTGGGTTCAAGTTCTTCATTTG	208–233	10	0.54	0.61	0.015	4/4
		R: TGTCATGTTCAATGTGTTTTGC						

N_A represents number of alleles observed; H_{obs} and H_{exp} are observed and expected frequencies of heterozygotes respectively; P is probability of significant deviations between observed and expected heterozygosities (* denote significant deviations after Bonferroni correction; $P < 0.05$); cross amplification shows number of positive amplifications/total using *Bathymodiolus heckerae* genomic DNA as a template

and 72°C 30 s; final extension 72°C for 10 min. PCR products were diluted 1:5 and 2 µl added to 0.05 µl Orange DNA Size Standard size standard (MCLab, San Francisco CA) and 4.95 µl water, denatured at 95°C for 10 min. Size-fragment analysis was conducted on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City CA). Chromatograms were scored using Genemarker v1.8 (SoftGenetics LLC: State College, PA). Sequences for the markers described (Table 1) are deposited in Genbank (Accessions HM755992–HM756009).

Deviations from Hardy–Weinberg Equilibrium (HWE) were calculated using Arlequin v3.1 (Excoffier et al., 2005). Heterozygote excess, heterozygote deficiency and linkage disequilibrium were tested with Genepop version 4.0.10 (Rousset 2008) and corrected for multiple comparisons using the sequential Bonferroni approach (Rice

1989). Presence of null alleles, stutter, and large allele dropout were assessed using MicroChecker (1000 randomizations; van Oosterhout et al. 2004). The software LOSITAN (25,000 simulations; Antao et al. 2008) was used to detect loci potentially under selection.

Ninety-six primer pairs were screened for robust amplification using DNA from eight individual *Bathymodiolus manusensis*. Primers showing strong polymorphic products were used to amplify another 96 individuals. Results from eighteen loci scored on a minimum of 63 individuals are presented in Table 1. Eight loci conformed to Hardy–Weinberg expectations, while the remaining ten showed significant homozygote excess ($P < 0.05$), and remained significant after sequential Bonferroni corrections ($k = 18$). MicroChecker indicated the presence of null alleles at these ten loci, and three loci (Bm06, Bm64, and Bm70) showed evidence of scoring errors due to stutter. There was no evidence for linkage disequilibrium among loci, nor did LOSITAN indicate selection at any locus. All markers were tested for cross amplification in another bathymodiolin mussel from the Atlantic Ocean (*Bathymodiolus heckerae*) using standard reaction conditions; 10 loci showed positive cross-amplification (Table 1).

Microsatellite markers have been developed for five other invertebrates from deep-sea chemosynthetic-based ecosystems (*Branchipolynoe seepensis*: Daguin and Jollivet 2005; *Bathymodiolus childressi*: Carney et al. 2006; *Riftia pachyptila*: Fusaro et al. 2008; *Ifremeria nautilei*: Thaler et al. 2010; *Chorocaris* sp2: Zelnio et al. 2010). *Bathymodiolus manusensis* microsatellite markers will be deployed on populations in the western Pacific to explore connectivity within Manus Basin. The data generated will establish a baseline of genetic diversity to assess ecosystem changes caused by both natural and non-natural disruptive events over time.

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