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PRIMER NOTE

Microsatellite loci for the mayfly *Baetis rhodani* (Baetidae, Ephemeroptera)

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Abstract

The first microsatellite primers are described for the mayfly family Baetidae (order Ephemeroptera). Seven polymorphic loci were isolated and characterized for the species *Baetis rhodani* from two enriched genomic libraries. A total of 183 individuals were genotyped using these loci. Allelic diversity was high for all loci, and observed heterozygosities ranged between 0.382 and 0.772. A heterozygote deficiency was detected in some loci, suggesting the presence of 'null' alleles.

Keywords: Baetidae, Ephemeroptera, freshwater insect, mayfly, microsatellites

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Many studies have successfully applied microsatellites to investigate the population structure of a wide range of species. However the abundance and structure of microsatellites in the genome varies considerably among taxa. This is reflected in the frequency of publications and also number of loci developed. For example approximately 26–75 loci have been published for Hymenoptera, whereas 2–15 loci have been developed for Lepidoptera (Neve & Meglecz 2000). Obtaining microsatellites has been problematic in certain groups of organisms among plants, several invertebrates and avian groups.

To our knowledge only three published papers have characterized microsatellites for aquatic invertebrate groups (Cooper *et al*. 1996; Gibbs *et al*. 1998; Wilcock *et al*. 2001). None of these have been for the family Baetidae (order Ephemeroptera), despite a large number of biology and population studies within this family, in particular for *Baetis rhodani*. We describe the development of seven polymorphic microsatellites for this species, an acid-sensitive mayfly that is widespread and abundant across Europe.

Two genomic DNA libraries were developed following a standard microsatellite enrichment protocol (Hammond *et al*. 1998). Four to 30 μg of genomic DNA was digested with the 4 base-cutting MboI restriction enzyme. Size-selected DNA between 300 and 800 bp was ligated to the linker oligonucleotides SAULA (5′-GCG-GTA-CCC-GGG-AAG-CTT-GG-3′) and SAULB (5′-GAT-CCC-AAG-CTT-CCC-GGG-TAC-CGC-3′). Between one and three rounds of capture were performed with the biotinylated microsatellite probes CA_{22}, GA_{22}, and AAT_{15}. Recombinant colonies were screened for the presence of microsatellites using a polymerase chain reaction (PCR) based method with the repeat-specific primers 5′-$(GA)_{10}$-3′, 5′-$(CA)_{10}$-3′, and 5′-$(AAT)_7$-3′ (Lunt *et al*. 1999).

Inserts from the positive colonies were either amplified and purified from the PCR product (GeneClean® kit), or purified from plasmid DNA (CONCERT™ Rapid Miniprep). Purified products were sequenced in the forward and reverse direction with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) using a 9700 thermal cycler (Perkin-Elmer). The fragments were separated on an ABI 377 Automated Sequencer (PE Applied Biosystems).

A total of 211 positive colonies were identified and sequenced from the 971 clones with inserts (recombinants). Of these, 147 contained microsatellites, an enrichment success of 73.5%. Overall both libraries were equally successful in terms of enrichment, although differences were observed between the rounds of capture. With three rounds a high degree of enrichment was observed, although this was associated with reduced insert size and flanking regions that were insufficient in length for primer design (unpublished data).

Microsatellite sequences were selected if the repeat was a minimum of 10 base pairs in length and not interrupted, and the flanking region between the microsatellite repeat and vector sequences was sufficient in length to design primers (≥ 30 base pairs). Primer pairs were designed for 14 microsatellite sequences using MACVECTOR 7 (Oxford

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Table 1 The characteristics of seven microsatellite loci isolated from *Baetis rhodani*. T_a is the optimal annealing temperature; H_O is the observed heterozygosity; and H_E is the expected heterozygosity

Locus name	Repeat	Primer Sequence (5′–3′)	GenBank accession no.	T_a (°C)	Primer (μM)	Allele size range (bp)	No. of alleles	H_O	H_E
Brh-1	$(CA)_{11}$	*F GTGCGTTACATACGAATAAGTG	AY081168	61	0.25	221–245	13	0.470	0.796
		R CGTGTTAGAGAAAATGAGCC			0.25				
Brh-2	$(CT)_{18}$	F ATTGCCGACAAAACTCGC	AY081169	61	0.10	148–182	14	0.537	0.708
		*R CGTCTCATTGATATACCCTGC			0.10				
Brh-3	$(CA)_{12}$	F ATGCAGTGAATGAGCCGC	AY081170	57	0.20	197–237	15	0.382	0.749
		*R CACTTTGAGCCAATCTTAATAGAGC			0.20				
Brh-4	$(GA)_{21}$	F TAGCCCCATTTGCCTTTG	AY081171	61	0.20	165–219	19	0.772	0.797
		*R GGTTTTATGATGTGTTCGGTTG			0.20				
Brh-5	$(TC)_{15}$	*F AAACGGTCTCTCTCTGTCTG	AY081172	61	0.10	201–235	17	0.756	0.845
		R CTGTCAAAACGAACAAACC			0.10				
Brh-6	$(CT)_{26}$	*F GGATGGATGGATGGATGC	AY081173	57	0.15	107–131	13	0.562	0.787
		R CAAAGTTGGTCAAAGGCG			0.15				
Brh-7	$(CA)_{15}$	F TGTGTGTAACAAGAAAACGC	AY081174	57	0.30	158–224	34	0.630	0.926
		*R GATTCGTCCCTTATGTATTAGC			0.30				

* indicates the fluorolabelled primer.

Molecular Group). These were tested on DNA extracted from decapitated individuals of *B. rhodani* larvae using the PUREGENE™ DNA Isolation kit for Cell and Tissue (Gentra Systems), and amplified in 10 μL PCR reaction volumes containing 40–100 ng template DNA, 0.15 units of *Taq* DNA Polymerase with 1 × *Taq* buffer containing 20 mM Tris-HCl (pH 8.2) and 50 mM KCl, 2 mM $MgCl_2$, 300 μM total dNTP concentration (Gibco Life Technologies), 1 μg BSA (Promega), and 0.1–0.30 μM of each primer (MWG Biotech). An initial denaturation step of 3 min at 94 °C was followed by 35 cycles of 30 s at 94 °C, an annealing step of 45 s (Table 1), 1 minute at 72 °C, and a final extension step at 72 °C for 10 min using a Robocycler Gradient 96 (Stratagene).

The optimization of PCR conditions was unsuccessful for seven of the primer pairs, which displayed either consistently poor amplification of alleles, or the presence of nonspecific products within the allele size range. The other seven loci were amplified in three optimized multiplex PCR systems (*Brh*-1 & 2, *Brh*-4 & 5, and *Brh*-3 & 6) and one singleplex system (*Brh*-7). The microsatellites were scored using an ABI 377 Automated Sequencer with GENESCAN software. Fragment sizes were determined using a TAMRA 350 internal size standard (PE Applied Biosystems). All genotypes were checked for allelic drop-out, by diluting the DNA template and then genotyping each individual at least twice.

The 183 individuals analysed were taken from eight streams located in the Wye, Usk and Tywi river catchments covering mid to South Wales. The observed heterozygosity (H_O) and expected heterozygosity (H_E) values were calculated for each locus using GENETIX version 4.02 (University of Montpellier II) (Table 1). The number of alleles per locus ranged from 13 to 34, and observed heterozygosity values were between 0.382 and 0.772. These values were low compared to the expected for all loci except *Brh*-4 and *Brh*-5, suggesting the presence of 'null' alleles due to priming site substitutions. Alternatively the deviation could be due to population fragmentation, as the samples originate from different streams. The population structure and dispersal of *B. rhodani* is currently being studied using these loci.

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