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PRIMER NOTE

Trinucleotide microsatellite loci for the peppered moth (*Biston betularia*)

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Abstract

Fourteen polymorphic trinucleotide microsatellites were isolated in the peppered moth, *Biston betularia*, using an enrichment protocol. Moderate to high allelic diversity (three to 22 alleles per locus) was found in a sample from northern England. We are currently using these markers to study the population structure of *B. betularia* in northern England, where a cline for melanism still exists.

Keywords: *Biston betularia*, Lepidoptera, microsatellite

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The rapid changes in melanic colour morph frequencies through the 19th and 20th centuries have established the peppered moth, *Biston betularia*, as a classic example of natural selection in action (Cook 2000). The motivation for developing polymorphic microsatellites for this species was to study the spatial genetic structure in the north of England, where the cline in melanic morph frequencies has been most intensively monitored. Description of the pattern of gene flow will be valuable in explaining the spatial and temporal variation in melanic morph frequencies in terms of gene flow and selection.

Peppered moth genomic DNA was extracted following Saccheri & Bruford (1993). DNA (10 μg) from a single *B. carbonaria* female (from Caldy, Wirral) was digested with *Sau*3AI (Boehringer-Mannheim) and ligated to phosphorylated linkers (S61, 5′-GGCCAGAGACCCCAAGCTTCG-3′ annealed to S62, 5′-PO_4-GATCCGAAGCTTGGGGTCTCTGGCC-3′; Refseth *et al*. 1997). DNA fragments between 500 and 1000 bp were excised from a 2% NuSieve GTG gel (FMC Bioproducts) and purified using a QIAquick gel extraction kit (Qiagen). For enrichment we used 1 mg of M2-80 streptavadin-coated magnetic beads (Dynal) incubated with 200 pmol of 3′-biotin-labelled oligonucleotide (MWG Biotech). We enriched for two different oligonucleotide pools: (i) CAA_8/GAA_8 and (ii) CAG_8/GTG_8. After several differential stringency washes, the enriched DNA was recovered, made double stranded and amplified by polymerase chain reaction (PCR). The DNA fragments were purified using QIAquick PCR purification kit (Qiagen), ligated into pGEM®-T vector (Promega) and transformed into JM109 *Escherichia coli* competent cells (Promega). Full details of the enrichment protocol are provided by Bloor *et al*. (2001). All recombinant clones, identified using blue/white screening, were gridded onto Hybond nylon membranes and positive clones were identified by hybridization with [γ^{33}P]-ATP-labelled oligonucleotides (Sambrook *et al*. 1989). Plasmids were isolated using standard protocols and then cycle sequenced using Big Dye™ chemistry (PE Applied Biosystems) and electrophoresed on an ABI 377. Primers flanking the repeat regions were designed using OLIGO version 6 (Rychlik 2000) or PRIMER 3 (Rozen & Skaletsky 1998).

Loci were initially tested for polymorphism with radioactively labelled primers in a sample of 16 peppered moths caught along a transect between North Wales and Yorkshire. The 12 most polymorphic and easily scorable loci were then studied in a much larger sample of 375. The PCR was performed on a Techne Flexigene thermocycler in 5-μL reaction volumes using ReddyMix PCR Mix (ABgene). The standard PCR cycle was: (i) 3 min at 95 °C; (ii) five cycles of 30 s at 94 °C, 30 s at T_a (°C) and 30 s at 72 °C; (iii) 30 cycles of 30 s at 92 °C, 30 s at T_a (°C) and 30 s at 72 °C; and (iv) 72 °C for 5 min, where T_a is the optimized annealing temperature for each locus (Table 1). Each reaction contained 75 mM Tris-HCl, 20 mM $(NH)_4SO_4$, 0.01% (v/v) Tween 20, 0.2 mM each dNTP, 1.5–3.5 mM $MgCl_2$ (see Table 1), 0.2 μM of each primer, 0.25 U of *Taq* polymerase (ABgene), and approximately 10 ng template DNA. Forward primers were 5′ labelled with Beckman-Coulter fluorescent

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Table 1 Core repeat sequences, primer sequences and summary of polymorphism statistics for 14 microsatellite loci isolated from the peppered moth, *Biston betularia*

Locus Accession no.	Core sequence	Primer sequence (5′–3′)	Size range (bp)	n	H_O H_E	N_a	T_a (°C)	$MgCl_2$ (mM)
Biston 01	$(GTT)_8$	CGTAATTAACCACCCAATAGAGC	100–134	375	0.45	4	57	2.5
AY190965		CGCGAAGGAGTTTTAAGTAGA			0.42			
Biston 02	$(GTT)_6(GTT)_3..(GTT)_3..(GTT)_2$	GCGAGGCATCCGATAGTGAAG	208–260	375	0.55	13	60	1.5
AY190966		CCAACTCGGCTGATGATAACTGA			0.54			
Biston 03	$AAC..(AAC)_6..AAC..(AAC)_4$	TGTCGCCAAGCACCTAAC	243–257	16	0.31	3	61	3.5
AY190967		GTCTCAATTTCAGCGATGACCT			0.56			
Biston 04	$(CAA)_6..(CAA)_5..CAA..(CAA)_2..CAA$	TAAGAGAAGGTGTGGTCGAAGG	269–284	375	0.59	6	60	3.5
AY190968		GAATGGCTTTGGGAATGGTAAC			0.64			
Biston 05	$(GTT)_7GTGTTGTAGCTGTATTGT(GTT)_2$	TTGACTAGCTAATTGTCGC	244–255	8	0.25	3	57	2.5
AY485266		GGTCATACACATAGCCTGTCAAC			0.56			
Biston 06	$(GTT)_{17}(GT)_3GTT$	GCAGTCTTAAATGTATTGCC	99–141	375	0.37	13	57	2.5
AY190970		CACAGTCAGAGACCACGTTTCA			0.36			
Biston 07	$(GAT)_3(GAC)_3(GAT)_4..(GGT)_{12}..(GGT)_3$	AAAGCACTTTACATGGTGGACG	198–231	375	0.46	9	57	1.5
AY190971		GCCTTCGCCAACTTTAAC			0.43			
Biston 08	$(CAA)_6TA(CAA)_6TACAACA(CAA)_2$	GGAATGCAACTGGCTCTCAAGT	185–200	375	0.43	6	60	1.5
AY190972		CGACTGCCTAAAGATTAGCG			0.55			
Biston 09	$(CAA)_3..(ACA)_2..(CAA)_5..(CAA)_5..(CAA)_2$	GGGAGAGGCCTATGTAAACTAAT	239–283	375	0.41	10	60	3.5
AY190973		TTTTCGGAAGCACCTTGTAAAG			0.44			
Biston 10	$CAACA(CAAA)_3CAATCAAAT$	CCAGTAGTTTCGGAGCGTATTCA	112–131	375	0.18	5	61	3.5
AY190974		TGTACAGACACGGCTCGTTTAC			0.18			
Biston 11	$(CAA)_3..(CAA)_7..(CAA)_2(CAAA)_2(CAA)_4..(CAA)_4$	CCGCTTCATGGTAAATAAAG	199–226	375	0.37	10	57	1.5
AY190975		GCGTGATGGTAATGCGATTC			0.38			
Biston 12	$(GTT)_{14}$	CTAAAAGCGAAACTGCGAAC	151–191	375	0.77	22	60	2.5
AY226153		AAGCCCCTAGAAGTTCAATCC			0.82			
Biston 13	$(GTT)_{17}(GT)_3GTT$	AAGTCGGTGAAGTCGGGATAG	286–324	375	0.33	16	60	2.5
AY226154		TTCAGCGCCATAAGTCATC			0.44			
Biston 14	$(GTT)_2..(GTT)_3..(GTT)_5$	CGATGATAGCTCAGTCACACC	193–214	375	0.60	8	60	3.5
AY226155		GTGGTAAAGCACACCTAGCC			0.67			

n, sample size; N_a, number of alleles; T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity.

dyes (D2, D3 and D4). The PCR products were separated by capillary electrophoresis, with a 400-bp size standard (Beckman-Coulter), through a denaturing acrylamide gel matrix on an automated sequencer (Ceq8000; Beckman-Coulter). Alleles were sized using the Ceq8000 fragment analysis software.

Fourteen polymorphic microsatellite loci were isolated in *B. betularia* (Table 1). As is often the case, many of the core repeat sequences (Table 1) consisted of mixtures of repeats or were interrupted by nonrepeat motifs (full sequences available in GenBank). Several loci showed moderate to high levels of allelic diversity, to which rare alleles made a substantial contribution, with the result that gene diversities (Table 1) were not particularly high for microsatellites. Allele size distributions were generally consistent with a stepwise mutation model but many alleles also fell outside the expected sizes, possibly due to nontriplet slippage or single base indels. In general, observed heterozygosities (H_O) were similar to overall gene diversity [expected heterozygosity (H_E)], reflecting low genetic structuring in this sample. However, permutation tests of F_{IS} in FSTAT (Goudet 2001) indicated that at Biston 8 and Biston 13 H_O is significantly smaller than H_E, possibly reflecting the occurrence of null alleles at these two loci (although no nonamplifying null homozygotes were detected). Apparent differences between H_O and H_E at Biston 3 and 5 were not significant and were probably due to small sample sizes.

The development of microsatellite markers has been particularly difficult in some Lepidoptera (Nève & Meglécz 2000). The reasons are varied and not altogether clear but include low frequency in the genome, low polymorphism, high degree of complexity and gene duplication (A. van't Hof, unpublished data). In the present study, low polymorphism and nonspecific amplicons were the main limitations to isolating a larger number of informative microsatellite loci but these are common obstacles in other taxa. There are now several studies demonstrating moderate success in developing dinucleotide and trinucleotide microsatellite markers in butterflies and moths (Flanagan *et al*. 2002

and references therein), suggesting that, while problems may be encountered in some species, the microsatellite approach should not be discounted as an inefficient marker strategy in Lepidoptera.

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