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PRIMER NOTE

Isolation of polymorphic microsatellite loci from the flea beetle *Phyllotreta nemorum* L. (Coleoptera: Chrysomelidae)

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Abstract

Ten microsatellite markers for the flea beetle *Phyllotreta nemorum* were developed using di- and trinucleotide repeat-enriched libraries. Each of these primer pairs were characterized on 96 individuals. Expected heterozygosities ranged between 0.11 and 0.84 and the number of alleles ranged between two and 14 per locus. These microsatellite markers are the first published for any *Phyllotreta* species.

Keywords: *Barbarea vulgaris*, Denmark, flea beetle, microsatellites

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The interaction between an oligophagous flea beetle, *Phyllotreta nemorum*, and its host plants has become a model for the study of the influence of geographically variable selection and gene flow on the geographical- and host plant-related distribution of adaptive traits (Thompson 2005). The flea beetle is polymorphic in its ability to use *Barbarea vulgaris* ssp. *arcuata* (G-type) as a host plant (De Jong & Nielsen 1999). The genetic basis of this ability has been studied to some extent, and involves major genes (Nielsen 1997; De Jong *et al*. 2000). The geographical distribution of the different beetle phenotypes in Denmark shows a striking pattern, with extensive variation in the proportion of *Barbarea*-resistant, and susceptible beetles among samples collected on spatially disjunct plant patches of the different host plants (De Jong & Nielsen 1999). Moreover, the frequencies of occurrence of the different phenotypes appear to be changing through time (Nielsen & de Jong 2005). These observations have lead to the question to what extent dispersion or migration, and selection influence the limited spread of the *Barbarea*-resistant trait in the flea beetles. Initial allozyme-work has indicated weak population structure in the flea beetles (De Jong *et al*. 2001). Drawbacks of allozyme-markers, however, include the relatively low number of loci that can be sampled due to the limited size of the beetles, and hence finite amount of enzyme that can be extracted, and the relatively low allelic variability per locus. Microsatellite markers show none of these drawbacks and are codominant making them the ideal candidate markers to further elucidate the roles of migration and selection in the distribution of the *Barbarea* resistance in *P. nemorum*. Here we report the development of microsatellite primers for this species.

Di- and trinucleotide repeat-enriched libraries of *P. nemorum* genomic DNA were constructed by a selective hybridization procedure (Karagyozov *et al*. 1993), using the method described by Van der Schoot *et al*. (2000). Briefly, genomic DNA was digested with *Alu*I, and ligated to a blunt adaptor (21-mer: 5′-GTTTCAGATCGGCTCATCGC and a 25-mer: 5′-ACACCAAAGTCTAGACCGA-GTAGCG). The fragments were separated on a 1% agarose gel and those ranging between 300 and 1000 bp were isolated from the agarose gel using an extraction kit (QIAquick Gel Extraction Kit, QIAGEN). After enrichment for microsatellites by hybridization, the polymerase chain reaction (PCR) fragments were cloned into a pGEM-T vector (Promega) according to the instructions of the supplier and subsequently transformed to *Escherichia coli* XL2-Blue competent cells (Stratagene). Insert-containing colonies were transferred onto Hybond N+ membranes (Amersham) and screened for microsatellites by hybridization to a mixture of microsatellite oligonucleotides at 65 °C. Three hundred and thirty-seven

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Table 1 Polymorphic microsatellite markers for *Phyllotreta nemorum*

Locus (GenBank ID)	Repeated motif †	Primer sequences (5′–3′)	Size range	Amplification conditions (T_a/no. of cycles)‡	No. of alleles (Effective no. of alleles)	H_O H_E
PnA03	$(CA)_{10}$	F: HEX-CAACGAGCAATCGATACAATTCG	205–225	55/35	11	0.93
(DQ507809)		R: ACATTCTGCGCCGAGATTGG			(6.07)	0.84
PnA04	$(GCA)_6$	F: 6-FAM-AATTACGAGAGCAACATGTCGG	174–180	57.5/35	3	0.31
(DQ507810)		R: ACTGTTGCTGTTGGGTTTGC			(1.63)	0.39
PnBB08	$(CAA)_4$	F: HEX-CCTGATCCATTACCAGAACCTCC	215–281	62/35	13	0.64
(DQ507811)	$N_{12}(CAA)_5N_{22}(CAA)_5$	R: ATGGTGACTGTGACTGTGAGATGG			(5.22)	0.81
PnAB12	$(GCT)_{10}(GTT)_2$	F: NED-GAGATTGAGACGATTGCTGGG	176–218	62/35	14	0.75
(DQ507812)	$(GCT)_2GTT(GCT)_4$	R: CTCAACAGTTGCATTACCAGC			(5.67)	0.83
PnD04	$(GCT)_7$	F: HEX-CTCGAGCTTGACTCACTACTGC	136–160	55/35	7	0.68
(DQ507813)		R: CCAGTTCCAGTGATTCGAGC			(3.13)	0.68
PnD06	$(CAG)_7$	F: 6-FAM-CTTCTTCAGCAGCCTGATGG	157–184	50/32	9	0.79
(DQ507814)		R: ATCACGTTCGGCACCACCTG			(4.00)	0.75
PnD09	$(GCA)_4N_{26}$	F: 6-FAM-GCTCCAACTACACCAAACTCG	167–193	55/30	10	0.77
(DQ507815)	$(GCA)_6$	R: TCGCGTACCCGTAATAGTGG			(4.47)	0.78
PnE11	$(GCA)_5N_3$	F: HEX-GTACAGTCATGTCTTGGAACGC	211–247	57.5/35	12	0.89
(DQ507816)	$(GCA)_8$	R: CTCGATTGCGTAGTAGCCGG			(5.48)	0.82
PnH09	$(GCG)_6$	F: NED-CGTGAGGCTTGTAGTATTTGG	162–165	55/30	2	0.10
(DQ507817)		R: CTACCATCCGATGATGAACG			(1.13)	0.11
PnH12	$(CCG)_5$	F: NED-AACCCTGGACGCCTATTCTGC	140–167	55/30	9	0.32
(DQ507818)		R: CGCGAATTACGGTTATCAGG			(1.47)	0.32

†The number after 'N' denotes the number of nucleotides in between repeat sequences.
‡PCR cycle program: 1 cycle 3 min at 94 °C, no. of cycles (30 s at 94 °C, 45 s at T_a °C, 1 min at 72 °C) (for details see Table 1), 10 min at 72 °C.

of 1920 clones were positive and 96 of those positive clones were sequenced using the DYEnamic ET Terminator sequencing mix and analysed on an ABI PRISM 3700 sequencer (Applied Biosystems). Primer pairs for 22 microsatellite repeats were designed using the software package VECTOR NTI ADVANCE 10 for Windows (Invitrogen). The primers were tested using genomic DNA extracted by the PUREGENE DNA Purification Kit (Gentra Systems) from 96 individuals of *P. nemorum* collected in Lynæs, Kværkeby, Vigersted, Ejby and Taastrup (Denmark). Amplification was performed in a 25-µL reaction volume containing 5 µL of Go*Taq* flexi buffer, 500 nM of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 0.625 U of Go*Taq* DNA polymerase and at least 4 ng of *P. nemorum* genomic DNA. The forward primer of each pair was 5′-fluorescently labelled with either 6-FAM, HEX or NED (Applied Biosystems) (Table 1). The markers were tested using different PCR protocols (Table 1) on a Mastercycler gradient (Eppendorf). All PCR products were run on an ABI PRISM 3700 sequencer and analysed using GENESCAN and GENOTYPER software (Applied Biosystems).

Out of the 22 tested markers, 10 primer pairs were polymorphic in *P. nemorum*; they showed two to 14 alleles per locus (effective number of alleles was 1.13–5.67 per locus) and the expected heterozygosities ranged between 0.11 and 0.84 (Table 1). The presence of null alleles, scoring errors due to stuttering and large allele dropout was checked with MICRO-CHECKER version 2.2.3 (University of Hull. Available at www.microchecker.hull.ac.uk/). Locus PnBB08 showed the possible presence of null alleles only when tested with the samples of Vigersted, due to a heterozygote deficit. There was no evidence for the presence of null alleles for all other loci. Scoring errors due to stutter bands and large allele dropout were not detected. GENEPOP software version 3.4 (available at http://wbiomed.curtin.edu.au/genepop/) was used to test deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium. All loci were in linkage equilibrium and only PnBB08 (consistent with MICRO-CHECKER, P value: 0.000732) and PnAB12 (P value: 0.006563) were not in HWE, with an observed heterozygosity lower than the expected heterozygosity (Table 1). The lower observed heterozygosity value for PnAB04 was not significant. These 10 characterized microsatellite markers should prove useful to study the distribution of the *Barbarea* resistance in *P. nemorum*.

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