

## PRIMER NOTE

# Isolation and characterization of trinucleotide repeat microsatellite markers for *Plutella xylostella* L.

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*Plant Research International, Wageningen UR, PO Box 16, NL-6700 AA Wageningen, the Netherlands***Abstract**

Thirteen microsatellite markers generating high quality patterns have been developed and characterized for diamondback moth (*Plutella xylostella* L.), of which 11 are based on trinucleotide repeats. These markers are polymorphic, generating up to 15 alleles in a test set of 12 caterpillars. The markers will be useful to assess the differentiation of *P. xylostella* populations and the exchange of pest populations between sites with crops, green manure crops and weeds.

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Diamondback moth, *Plutella xylostella* L. (Yponomeutidae = Plutellidae) is the most serious insect pest of crucifers worldwide (Talekar & Shelton 1993). It uses a wide range of plant species within the family Brassicaceae including vegetable brassicas, ornamental brassicas and weeds. Although this cosmopolitan pest is able to migrate and disperse across long distances, very little is known about its local dispersal patterns. Landscape structure may affect the abundance of Lepidopteran pests in field crops (den Belder *et al.* 2006). Microsatellite markers are useful tools to assess genetic variation within and among populations and to study movement between populations or crops. Recently, six microsatellite markers from *P. xylostella* were published (Endersby *et al.* 2005). All but one are based on dinucleotide repeats. Unfortunately, dinucleotide repeat microsatellite markers may typically produce 'stutter' bands for single alleles due to polymerase slippage, which may hamper unambiguous scoring (Esselink *et al.* 2003).

We have isolated trinucleotide repeat microsatellite loci from a genomic DNA library using an enrichment procedure after Karagoyozov *et al.* (1993) as modified by van de Wiel *et al.* (1999) and Zavodna *et al.* (2002). Genomic DNA was isolated from four caterpillar heads of a breeding line using the DNeasy Tissue Kit (QIAGEN). Five hundred ng genomic DNA was restricted/ligated with *AluI*, *RsaI* or *HaeIII*, pooled and amplified as described before (Esselink *et al.* 2003). Microsatellite-containing fragments were selected

by hybridization to Hybond N<sup>+</sup> membranes with separately spotted synthetic oligonucleotides: (TCT)<sub>10</sub>, (TGT)<sub>10</sub>, (GAG)<sub>8</sub>, (GTG)<sub>8</sub>, (TGA)<sub>9</sub>, (AGT)<sub>9</sub>, (CGT)<sub>8</sub>, (GCT)<sub>8</sub>, (GCC)<sub>7</sub>. Fragments were eluted from the membranes with increasing washing stringencies, yielding three different fractions enriched for microsatellite repeats: (i) the fraction eluted at low stringency (0.5 × SSC, 1% SDS for 30 min at 62 °C); (ii) the fraction eluted at increased stringency (0.2 × SSC, 1% SDS for 30 min at 62 °C); and (iii) the fraction eluted at high stringency (0.1% SDS for 10 min at 100 °C). Eluted fragments were precipitated and reamplified. To obtain microsatellites with a high number of repeats but also containing sufficient flanking sequence, elution fraction 1 was used as source for cloning DNA fragments containing repeat motifs with 30% CG content, fraction 2 for motifs with 60% GC, and fraction 3 for motif CGG (100% CG content). Amplified fragments were cloned and for each motif 192 colonies were screened as described before (van de Wiel *et al.* 1999; Van der Schoot *et al.* 2000). The most abundant trinucleotide repeat was GCC (85% positive clones) followed by GCT (71%), CGT (58%), TGA (34%) and GTG (10%). For TCT, TGT, GAG, AGT no repeats were isolated. From 541 microsatellite-containing clones, the insert was amplified and 138 size-selected inserts were sequenced from both sides. After making contigs, 77 sequences with a unique repeat were obtained. We designed 33 primer pairs using DNASTar (Lasergene). For comparison, we also included six loci developed by Endersby *et al.* (2005). Reverse primers were PIG-tailed (Brownstein *et al.* 1996). Pattern quality and degree of polymorphism was assessed on DNA of six samples from breeding lines from Italy,

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**Table 1** Characteristics of 14 selected microsatellite loci from *Plutella xylostella* (L.) tested on 12 individuals

Locus	GenBank Accession No.	Repeat motif*	Primer sequences 5'–3'	Dye	$T_a$	Allele size range (bp)	No. of alleles	$H_O$	$H_E$	$F_{IS}$	Gene diversity	HWS
DBMPRI02	AM237401	(TGA) <sub>42</sub>	F: ATCGCGATATCTGTTGTTTCGAGT R: GTTTCATTGCGAGGAAACCTACAAATCT	NED	54	180–326	8	0.33	0.77	0.60	0.83	$P < 0.05$
DBMPRI03	AM237402	(CA) <sub>31</sub>	F: AGATGGCGGTGGAGGGTTCTCGT R: GTTTGTGGCCGAGGCCGAGGACAGGTA	6-FAM	61	176–282	10	0.36	0.88	0.62	0.95	$P < 0.05$
DBMPRI12	AM237403	(GTC) <sub>22–6</sub>	F: TTGCAGCTTAAAGCTCATTGACTC R: GTTTCTGCGCCCGTAATAGTTGAAACTT	HEX	57	193–274	15	0.50	0.91	0.48	0.97	$P < 0.05$
DBMPRI13	AM237404	(CGT) <sub>20</sub>	F: TGTTTGATAACGAGTATTTCGTACA R: GTTTTATGAAAGCGCTGAAGATGTATTCT	6-FAM	52	111–193	6	0.33	0.48	0.34	0.51	$P < 0.05$
DBMPRI14	AM237405	(CGC) <sub>9</sub>	F: GCGCTGTAGTGGCGGCATACGT R: GTTTAAGTTCGCCGCGGACAAAGTGG	HEX	62	189–211	8	0.58	0.75	0.27	0.80	$P < 0.05$
DBMPRI15	AM237406	(CCG) <sub>11</sub>	F: AAAGAGCGGGATTGTCTGTGATCG R: GTTTGGCCGAAGGTACCCTGGGGTTGT	NED	59	160–179	10	0.67	0.84	0.25	0.89	NS
DBMPRI16	AM237407	(CCG) <sub>11</sub>	F: TGCCTACATTAACGGGTGACCTT R: GTTTCATCGCGGAGGCAATCTAATCT	NED	58	167–175	4	0.50	0.68	0.28	0.69	$P < 0.05$
DBMPRI17	AM237408	(CCG) <sub>10</sub>	F: ACTGCGAGCATGCTACGGCATAAG R: GTTTCGCGGTGGAGATTAGTTGAGTCCG	HEX	61	93–120	8	0.67	0.79	0.20	0.83	NS
DBMPRI21	AM237409	(GT) <sub>21</sub>	F: ACCGGATTACATAAGTGGTGGTAA R: GTTTCAGCGAAGACTGAAGAAATAGGT	6-FAM	54	234–252	7	0.42	0.81	0.52	0.86	$P < 0.05$
DBMPRI28	AM237410	(TGA) <sub>75</sub>	F: GGAGACCCGTGCTCCAGCCAGTCCG R: GTTTGCGTCAGTGCAGCGGCAGTAGG	6-FAM	60	336–352	5	0.33	0.57	0.45	0.61	NS
DBMPRI29	AM237411	(TGA) <sub>33</sub>	F: AGAGACCTACGTCCAGCAGTGGAA R: GTTTAGAAATGGCGCAAAACCTTAGACC	HEX	53	95–111	7	0.75	0.75	0.04	0.78	NS
DBMPRI30	AM237412	(GCA) <sub>19–5</sub>	F: ACTCCGAATCCGTTGTTGAGTTAC R: GTTTGTTCAGCTGACTGATTTGATTG	6-FAM	56	173–203	8	0.33	0.82	0.62	0.88	$P < 0.05$
DBMPRI31	AM237413	(TGA) <sub>15–1</sub>	F: AGGCCGAGGACCGAGTGTTTTAAAG R: GTTTCTGCTGGGGAGGAAAGAGATGTT	HEX	55	129–188	10	0.33	0.84	0.63	0.90	$P < 0.05$

\*, minus denotes mismatch; †, forward (F) primers were dye-labelled; reverse (R) primers were pig-tailed (GTTT added); §, NS, not significantly different from the expectation of HWE in a permutation test. The 12 individuals tested are not from one population, and four of them are from inbred laboratory lines.

$T_a$ , annealing temperature; GENEPop version 3.4 (Raymond & Rousset 1995) was used to calculate  $H_O$  (observed heterozygosity) and  $H_E$  (expected heterozygosity),  $F_{IS}$  and gene diversity.

Germany and the Netherlands. Amplification was performed in 20- $\mu$ L reaction volume containing 10 ng of genomic DNA, 4 pmol of each primer, 100  $\mu$ M of each dNTP, 10 mM Tris-HCl pH 9.0, 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 0.01% Tween 20, 1.5 mM  $\text{MgCl}_2$  and 0.4 U Goldstar *Taq* DNA polymerase (Eurogentec). Polymerase chain reaction (PCR) conditions were 94 °C for 3 min followed by 30 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 3 min. Amplification products were separated on a 6% denaturing polyacrylamide gel and visualized by silver staining (Promega Silver Sequence DNA Sequencing System). Three loci failed to amplify, eight showed multiple banding patterns and three were monomorphic. Twelve loci showed no amplification for three or more samples, even after 40 cycles at 2.5 mM  $\text{MgCl}_2$ . The remaining 13 loci generated high quality patterns (no stutter bands). Two of these loci, originating from the TCT enrichment, contained dinucleotide repeats; all other loci are trinucleotide repeats. Only three loci of Endersby *et al.* (2005) (DBM01, DBM06 and DBM09) amplified in all samples.

The 13 newly developed markers were analysed using a panel of 12 samples (the six previous samples and six samples from four different agricultural fields in the Netherlands), using fluorescently labelled (6-FAM, HEX or NED) forward primers on an ABI PRISM 3700 DNA analyser, and genotyped with GENOTYPER 3.6NT. All markers generated unambiguously scorable profiles on this system as well, with four to 15 (average 8.15) alleles per locus (Table 1). No product was observed for one of the samples with marker DBMPRI03, which could indicate the presence of null alleles. For all loci except DBMPRI29, the observed heterozygosities were much lower than expected. This may partly be the result of the inclusion of four individuals from inbred laboratory lines. Alternatively, null alleles could be present in these markers as well. Endersby *et al.* (2005) found no evidence for absence of Hardy–Weinberg equilibrium in 48 Australian individuals tested with their six microsatellite markers. Larger samples from natural populations will be required for accurate assessment of allele frequencies and Hardy–Weinberg equilibrium. No linkage disequilibrium was detected between loci. In conclusion, the results show that *P. xylostella* contains high numbers of microsatellites, particularly with CG content of 60% or higher. Surprisingly, the CCG motif turned out to be very promising in generating polymorphic markers, as nearly all (19 out of 20) sequences

obtained were unique, and four out of five designed primer pairs for CGG are included in the final set. The high quality of the patterns and the high degree of polymorphism (gene diversity 0.61–0.97) show considerable promise for the study of differentiation of *P. xylostella* populations and of exchange of pest populations between sites with crops, green manure crops and weeds.

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