Molecular Ecology Notes (2006) **6**, 191–193 doi: 10.1111/j.1471-8286.2005.01189.x

PRIMER NOTE

Isolation of microsatellite markers for *Contarinia nasturtii*, a European pest invading the New World

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Abstract

Microsatellite markers were developed for epidemiological studies on *Contarinia nasturtii* (Diptera, Cecidomyiidae), a native European pest that was introduced to the New World in 1996. Nine loci were found to be polymorphic and suitable for the analysis of 56 male individuals that were collected using pheromone traps. These markers all conform to Hardy–Weinberg expectations in at least one of the two tested populations, and carry an average number of 11 alleles among populations and observed levels of heterozygosity ranging from 0.32 to 0.86. Primers for all markers also successfully amplified fragments from *Contarinia pisi* and *Contarinia tritici*.

Keywords: agriculture, Brassicaceae, *Contarinia pisi*, *Contarinia tritici*, epidemiology

Received 11 August 2005; revision accepted 7 September 2005

The Swede midge *Contarinia nasturtii* (Kieffer) is a serious insect pest of vegetable crops. Females of this species infest various Brassicaceae, such as cauliflower, cabbage and Brussels sprouts, by laying eggs onto the terminal bud of the plant. The developing larvae feed near the growing point, causing deformations of leaves and flower buds and paving the way for secondary disease infections. First evidence for an introduction of this native European midge into the New World was reported in 1996, when farmers in Ontario, Canada experienced heavy yield losses in broccoli. Initially misdiagnosed as symptoms of a nutritional deficiency, damage was later identified as being caused by the Swede midge after the insect was found and identified in 2000. In late summer 2004, *C. nasturtii* appeared for the first time south of the Canadian border, in New York state, USA, on pheromone traps newly developed to detect this species in low densities (Frey *et al*. 2005; Hillbur *et al*. 2005; J. Kikkert, personal communication). In this study, we report nine microsatellite markers that can be used to study the epidemiological dynamics of this spreading insect.

Loci were isolated from genomic DNA of approximately 50 reared *C. nasturtii* individuals obtained using the GenElute DNA extraction kit (Sigma). The first method we used was a protocol based on Glenn *et al*. (2000) and Hamilton *et al*. (1999), modified as described in Brunner & Frey (2004). Microsatellite enrichment was conducted in two mixes of 5′-biotinylated primers $(GCT)_8$ with $(CA)_{10}$ and $(CCT)_8$ with $(CT)_{10}$. Later, we changed to the FIASCO (fast isolation by AFLP of sequences containing repeats) microsatellite-enrichment technique (Zane *et al*. 2002), using the $(AC)_{17}$ primers as well as a mixture of $(GCT)_8$ and $(CT)_{10}$ (Gallini *et al*. 2005). The relative amount of the four amplified fragment length polymorphism (AFLP) adaptor-specific primers was unvaried as no bands were observed using an equal mix.

From each of the two protocols 160 clones were sequenced. The yield of plasmids including microsatellites was highly comparable between the two methods (85 vs. 84%). However, a considerable amount of plasmids of the first method shared the same fragment, possibly due to an over-amplification of the digested DNA (Zane *et al*. 2002). Also, the amount of successful recombinant clones was many-fold greater using the FIASCO approach, where we included an A-addition (QIAGEN) prior to ligation.

Specific primer pairs for 25 loci were manually constructed or designed using the web-based PRIMER 3 program (Rozen & Skaletsky 2000), and then tested in uniplex amplifications with nonlabelled primers (Microsynth GmbH). A total of 22 of the pairs (88%) successfully amplified polymerase chain reaction (PCR) products from

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Table 1 Genetic characterization of nine polymorphic microsatellite loci in *Contarinia nasturtii*, tested on 56 male individuals from two populations in Switzerland, Kerzers (K) and Treiten (T). Primer pairs that have been combined in multiplex reactions show equal superscript numbers following the locus name. The annealing temperature of all primers is 55 °C

Locus	Primer sequences 5′–3′, fluorescence labelling	Repeat motif	Allele range (bp)	No. of alleles K/T/total	H_O K/T	H_E K/T	GenBank Accession no.
cn-bf8[1]	F: PET-AACTCATCATCTGGTGCCGA R: GGCCTAATTTACAGTGACAAATAC	$(TG)_{17}$	174–198	9/7/10	0.71/0.61	0.73/0.74	DQ152909
cn-bf45[1]	F: 6FAM-AATCCATCACTTTTGTGTTGAGGT R: TGAACATTGATCCAGTGATAAAATTG	$(AG)_{13}$	204–212	4/5/5	0.57/0.79	0.72/0.78	DQ152910
cn- bf82[2]	F: NED-CTTGGCGATCTTGTCGATCT R: TGCTTTGACTGGTGGACGT	$(GYT)_7(GCT)_{43}$ $CTT(GYT)_6$	182–185	2/2/2	0.46/0.32	0.43/0.44	DQ152911
cn-C1-13[1]	F: VIC-ACTTGAACGAGCAAACAACAAC R: GATGAAGACGACGATGAGCA	$(CT)_5TT(CT)_{18}$	124–168	12/12/14	0.86/0.86	0.88/0.86	DQ152912
cn-C2-1[3]	F: NED-CCAAATCGCTAGTTTGCTCAC R: TTTTGGTGTGGTGCTGTTTC	$(CA)_8(TA)_2(CA)_6$	145–175	7/6/7	0.68/0.82	0.79/0.80	DQ152913
cn-D1-5[2]	F: VIC-CAAAGGCGAACTAGACAAGACA R: CGTTGCACCCGTTGTTATC	$(AG)_3AC(AG)_4$ … $(AG)_{15}$	183–227	14/12/15	0.79/0.82	0.80/0.84	DQ152914
cn-D1-13[2]	F: 6FAM-CCTTTCCAGTTGCGAATATGA R: TCTACCGCATCCGCTATTGT	$(AG)_{18}$	243–283	14/12/17	0.82/0.79	0.85/0.78	DQ152915
cn-D1-18[4]	F: PET-CGGAATATAGCGAAAAGTGTACC R: TGTTCGAGTGGGACAAATACA	$(TC)_5CC(TC)_{17}(TG)_{11}$	159–197	12/15/17	0.71/0.86	0.88/0.90	DQ152916
cn-D1-22[1]	F: NED-CACGATTTGGGAGTGAACCT R: TTCGCCGCATTATTATCCAC	$(CA)_4CC(GA)_9TA(GA)_6$	147–183	10/10/12	0.54/0.57	0.77/0.88	DQ152917

H_O, observed heterozygosity; H_E, expected heterozygosity.

FIASCO, compared to only 10 pairs (40%) from the first approach. The lower success rate of the first approach may be due to an excessive concatamer formation caused by the polymerase used as well as the manual primer design.

A subselection from the 32 successful primer pairs was made by testing their ability to amplify in one individual each of *Contarinia pisi* and *Contarinia tritici*. Seventeen primer pairs were able to produce fragments in both species, and eight primer pairs worked at least for *C. pisi*. We selected 16 primer pairs of the first group to be tested in multiplex reactions using fluorescent-labelled forward primers (Applied Biosystems).

Sixty male individuals from two Swiss populations (5 km apart) caught with pheromone traps (Hillbur *et al*. 2005) were used to evaluate the genetic characteristics of the 16 loci. For each individual and each of four multiplex reactions with four loci each, PCRs were run in a total volume of 10 μL using the QIAGEN Multiplex PCR Kit following the manufacturer protocol, amplifying in 35 cycles with an annealing temperature of 55 °C. One microlitre of a 1/100 dilution of the PCR products was electrophoresed in 15 μL formamide on POP7 gel matrix using a 3130xl Genetic Analyser (Applied Biosystems) along with GeneScan 500 LIZ size standard. All loci amplified well; however, only nine loci produced polymorphic and heterozygous alleles in the tested males. Allele size data for all loci could be analysed for 56 of the 60 individuals (28 individuals of each of the two populations) using GENEMAPPER version 3.7 (Applied Biosystems).

Heterozygosity estimates as well as exact *P* values for deviations from the expected Hardy–Weinberg equilibrium (HWE) were evaluated using the Markov chain algorithm (100 000 steps) implemented in ARLEQUIN 2.001 (Schneider *et al*. 2000) for each locus and for both populations. Genotypic linkage disequilibrium (LD) was tested between all pairs of loci across populations based on a permutation procedure in the same program (10 000 permutations). There was a significant LD detected between one pair of loci ($P = 0.020$, cn-bf82/cn-D1-05) in the population of Kerzers, and two others ($P = 0.047$, cn-bf45/cn-C1-13; $P = 0.031$, cn-D1-18/cn-C1-13) in the population of Treiten. Significant deviation from HWE was observed for cn-C2-1 ($P = 0.017$) and cn-D1-18 ($P = 0.024$) in Kerzers and for cn-D1-22 ($P < 0.001$) in Treiten. The microsatellites showed moderate to high polymorphism with four to 17 alleles per locus and population, with the exception of the trinucleotide repeat locus cn-bf82, which showed only two alleles (Table 1).

Acknowledgements

We thank Beatrice Frey, Nathalie Inauen and Franziska Lampart for their valuable technical assistance, Patrick Brunner for his technical advice, Brion Duffy and Bettina Harr for their comments on the manuscript, and Robert Baur, Reinhard Eder and Stefan Rauscher for providing trapped insect specimens.

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