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PRIMER NOTE

The isolation of microsatellite loci in the Mediterranean fruitfly *Ceratitis capitata* (Diptera: Tephritidae) using

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Abstract

The Medfly (*Ceratitis capitata*) is a polyphagous dipteran pest which has spread from North Africa to the countries of the Mediterranean Basin and has also invaded tropical and subtropical regions throughout the world. Colonizing populations typically possess low levels of genetic variability. Microsatellites provide an effective means of investigating the population structure of such genetically depauperate populations, however, microsatellite markers traditionally require a long phase of development in new taxa. We used a biotin/streptavidin capture technique to isolate microsatellites directly from *C. capitata* genomic DNA and we describe here the identification of seven polymorphic microsatellite markers in *C. capitata*.

Keywords: *Ceratitis capitata*, enrichment protocol, Medfly, microsatellite

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The Mediterranean fruit fly (Medfly) *Ceratitis capitata*, a polyphagous multivoltine pest of great economic importance, invaded Spain from North Africa over 150 years ago (Hagen *et al*. 1981). The Medfly has since spread to most of the countries of the Mediterranean Basin and has also colonized tropical and subtropical regions throughout the world. To manage this pest, it is important to find genetic markers suitable for determining the geographical origin of *C. capitata* populations invading new areas. Because of founder effects and genetic bottlenecks, colonizing populations typically possess low levels of genetic variability. Microsatellites provide an effective means of investigating the population structure of such genetically depauperate populations. We have used a biotin/streptavidin capture technique (Refseth *et al*. 1997; Gardner *et al*. 1999) to isolate microsatellites directly from *C. capitata* genomic DNA and we have identified seven polymorphic microsatellite markers that are suitable for the analysis of the genetic structure and gene flow studies in *C. capitata* populations.

C. capitata genomic DNA was isolated using standard phenol/chloroform extraction with RNase (20 µg / mL) digestion (Maniatis *et al*. 1989). Five µg of Medfly genomic DNA were digested in a volume of 50 µL with 10 units of *Mbo*I (Promega) for 5 h at 37 °C, followed by heat inactivation of *Mbo*I at 65 °C for 30 min. The oligonucleotides, linker A, 5′-GGGTAGGATGGGGGATGGG-3′ (1.6 nmol) and linker B, 5′-GATCCCCATCCCCCATCCTACCC-3′ (1.6 nmol) were mixed and heat denatured for 5 min at 95 °C in a total volume of 60 µL containing 50 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, and allowed to cool slowly overnight to room temperature to generate the double-stranded *Mbo*I adapter.

This adapter (0.53 nmole) was ligated to 5 µg of *Mbo*I digested genomic DNA in a volume of 100 µL containing 30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 1 mM ATP, 10 µg bovine serum albumin (Promega) and 40 units T4 DNA Ligase (Promega). Excess adapter molecules and low molecular weight genomic DNA were removed by centrifuging the ligation reaction through a Micron 50 filter (Amicon®) at 16 100 *g* for 30 s. Cleaned elutant was recovered by inverting the sample reservoir and spinning at 16 100 *g* for a further 30 s into a 1.5-mL tube. Adapter ligated DNA was hybridized to 1 µg (15 nmol) of biotinylated probe in a total volume of 100 µL containing 50 µL of 2 × binding and washing (B & W) buffer (10 mM

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Table 1 The results of the PCR three-primer tests and DNA sequencing analyses on recombinant clones of *Ceratitis capitata* genomic DNA generated using a biotin/streptavidin magnetic enrichment protocol

	Probe			
	$(AC)_{10}$	$(AG)_{10}$	$(TGC)_{10}$	Total
No. of clones tested in three-primer PCR test	19	9	5	33
No. of clones yielding two or more PCR bands	13	3	2	18 (54.5%)
No of sequenced clones which contained microsatellites*	13	3	2	18 (100%)

*Recombinant clones which yielded two or more bands in the three-primer test were sequenced.

Tris-HCl, pH 7.5; 1 mM EDTA, 2.0 M NaCl: Dynal). The biotynlated probes used were 5′-$(AC)_{10}$GAGC[Biotin]A-3′, 5′-$(AG)_{10}$GCAC[Biotin]A-3′ and 5′-$(TGC)_{10}$ AGCG-[Biotin]A-3′. Following heat denaturation for 5 min at 95 °C, the hybridization mixture was rapidly cooled to the appropriate hybridization temperature (AC_{10} and AG_{10}, 50 °C; TGC_{10}, 55 °C). In a separate tube 100 µL of Dynabeads® M-280 Streptavidin (Dynal®) were washed three times in 100 µL of B & W buffer. The hybridization reaction was added to the prepared bead mix and incubated with gentle agitation at the hybridization temperature for 30 min. The captured fragments were washed three times in 100 µL of 1 × SSC at room temperature followed by three washes in 100 µL of 1 × SSC at 30 °C. Captured fragments were eluted from the beads by heating for 5 min at 95 °C and were purified using a Micron 50 filter (Amicon®).

The microsatellite enriched retentate was polymerase chain reaction (PCR) amplified in a 50-µL PCR reaction containing 1 × PCR buffer (Promega), 4 mM $MgCl_2$, 10 mM dNTPs, 10 pmol of linker A and 1 unit of *Taq* polymerase (Promega). PCR was carried out in a Perkin-Elmer 2400 Thermal cycler with one cycle of denaturing at 94 °C for 5 min followed by 35 cycles of 95 °C for 45 s, 63 °C for 45 s and 72 °C for 90 s, ending with one cycle of 72 °C for 10 min. PCR products were cloned using the TOPO TA cloning system® (Invitrogen). Recombinant clones were tested for microsatellite repeat sequences by a three-primer PCR amplification test (Gardner *et al*. 1999). Products from clones that yielded two or more bands in the three-primer test were purified using the StrataPrep™ kit (Stratagene) and were sequenced on a ABI Prism® 310 Genetic analyser. The results of the three-primer tests and DNA sequencing are presented in Table 1. PCR primers were designed for eight *C. capitata* microsatellite loci and seven of these loci were polymorphic (Table 2). Approximately 20 individuals from five *C. capitata* populations were

Table 2 Characteristics of seven microsatellite loci of *Ceratitis capitata*

Locus	Motif	Primer sequence (5′–3′). F:forward, R:reverse	Size* (bp)	Allele size range (bp)	T_a (°C)	D_A	H_O	H_E	GenBank Accession no.
dccap1	$(CA)_2CTGC(CA)_4$	F: ACATACACACTGACATCCGCTAAGT R: CCAATAACGACGACAATCACC	152	277–281	56	3	0.63	0.43	AF267491
dccap2	$(TGCCGC)_2(TGC)_{11}CAC(TGC)_2$	F: GCAACAACAAAGCAAAGCAA R: ATCGGGTAACGGCTGAGTA	214	288–312	58	4	0.41	0.33	AF267489
dccap4	$(AT)_4(CA)_8$	F: CTAGGGAACCCTGGGGGAGG R: CTTCCCTTTATGCCCGTATGTAT	184	284–344	58	4	0.51	0.45	AF267494
dccap5	$(AC)_2TA(TG)_3AT(TG)_5C(TG)_6TC(TG)_3$	F: GCAATGAAAGCAAGCAACAA R: GCCGTGAAAGGTGAATGAC	223	336–344	56	3	0.16	0.35	AF267287
dccap6	$(AT)_2AG(AT)_4(AC)_2(AT)_2(AC)_3$	F: AGCCTGTTTTGACCAACGTC R: CGTCACTTAGCGGATGTTCAG	164	287–229	58	4	0.58	0.4	AF267493
dccap1.1	$(TA)_2TG(TA)_2CATG(TA)_2CAT(AC)_2GTC(TG)_4$	F: TGCCAATAACGACGAAATC R: AGCCGAAGAATTGGCATTTA	152	277–279	56	2	0.6	0.44	AF267492
dccap9	$(TGCCGC)_2(TGC)_4TAC(TGC)_2CGC(TGC)_2$	F: AGTGTCTGAAAACACAACAGCAAC R: GTTGTATTGTTGCACGAGGATATG	239	306–324	58	4	0.5	0.46	AF267490

The locus name, repeat motif, primer sequence, annealing temperature, sequenced allele size and GenBank accession no. for microsatellite loci isolated are given. The number of distinct alleles (D_A) and levels of heterozygosity (H_O = observed proportion of heterozygotes, H_E = expected proportion of heterozygotes) are based on data from 20 individuals from five populations.

assessed. Genomic DNA from single flies was isolated using the DNeasy™ Tissue Kit (Qiagen). Microsatellite loci were amplified in 25 μL PCR reactions containing 50 ng of *C. capitata* genomic DNA, 1 × PCR buffer (Promega) (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 200 μm of each dNTP, 10 pmol of each primer and 1 unit of *Taq* polymerase (Promega). Amplifications were carried out in a Perkin-Elmer 2400 Thermal cycler with one cycle of denaturing at 94 °C for 5 min followed by 35 cycles for 45 s at 95 °C, 45 s at the primer-specific annealing temperature (Table 2), 72 °C for 90 s, ending with one cycle of 72 °C for 10 min. Products were electrophoresed on standard sequencing gels (6% acrylamide, 8 M urea, in 1 × TBE) and visualized using the Silver Sequence™ DNA Staining System (Promega).

Analyses of genetic diversity were carried out using GENEPOP software (Raymond & Rousset 1995). Numbers of distinct alleles ranged from one to four per locus with observed and expected heterozygosities ranging from 0.32 to 0.63 (Table 2). Null alleles were identified in the locus dccap5. The overall genetic diversity (Nei 1987) found in this study (G_D = 0.51) is comparable with values reported in Mediterranean *C. capitata* populations by Bonizzoni *et al*. (2000). These seven microsatellite loci are currently being used to analyse gene flow and mutation processes in *C. capitata* populations from the Mediterranean Basin.

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