NOTE

Use of Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) to Differentiate Populations of Plum Curculio, *Conotrachelus nenuphar* (Herbst)[1]

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Plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae), is an endemic pest of pome and stone crops east of the Rocky Mountains. Two morphologically identical strains have been reported: a univoltine northern strain and a multivoltine southern strain (Chapman, 1938, New York State Agric. Experimental Stn. Bull. 684; Bobb 1952, Va. Agric. Expt. Stn. Bull. 453; Racette et al., 1992, Phytoprotection 73: 85-100) The line dividing these populations, estimated by Chapman (1938), runs near the fruit-growing region of western Virginia. Because detection of multivoltine larvae in fruit at harvest can result in the establishment of trade barriers to fruit export from affected regions, definitive identification of the two strains is essential to limit the economic impact of this pest. First generation occurs early in the season; if oviposition occurs while fruit are very small, larvae are crushed by the expanding apple fruit. If fruit are sufficiently expanded at the time of oviposition (as may happen late in a first generation, or during a second generation), then larvae may successfully complete development in apple.

Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), which employs small primers (10 to 12 nucleotides in length) to amplify random fragments of genomic DNA, has been successfully used to differentiate populations of several extant insects: alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) (Erney et al., 1996, Ann. Entomol. Soc. Am. 89: 804-811); a weevil pest of sugar beet, *Aubeonymus mariaefranciscae* Roudier (Coleoptera: Curculionidae) (Taberner et al., 1997, J. Molec. Evol. 45: 24-31); whiteflies (Homoptera: Aleyrodidae) (Gawell and Bartlett, 1993, Insect Molec. Biol. 2: 33-38); and the alfalfa leafcutting bee, *Megachile rotundata* (F.) (Hymenoptera: Megachilidae) (Lu and Rank, 1996, Genome 39: 655-663). Based on these successes, we hypothesized that RAPD-PCR could be utilized to differentiate between univoltine and multivoltine plum curculios.

To test this hypothesis, univoltine plum curculio adults were obtained from Mas-

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sachusetts (R. Prokopy, U. MA, Amherst) and multivoltine plum curculio adults were obtained from Georgia (D. Horton, U. GA, Athens). Genomic DNA was extracted from individual weevils using a protocol modified from Ashburner (1989, *Drosophila*: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press). Briefly, weevils were placed in colorless microcentrifuge tubes and homogenized in 10 mM Tris-HCl (pH 7.5), 60 mM NaCl, 10 mM disodium ethylenediamene tetraacetate (EDTA) with 5% sucrose. An equivalent volume of lysis buffer (300 mM Tris-HCl, pH 7.5; 100 mM EDTA; 0.625% sucrose; 1% diethyl pyrocarbonate) was added and the samples were incubated for 15 min at 70°C. After cooling to room temperature, proteins were precipitated with 0.15 volume of 8 M potassium acetate. The resulting supernatant was extracted twice with an equal volume of 1:1 phenol/chloroform and once with chloroform alone prior to DNA precipitation with ethanol. The DNA pellet was washed with 70% ethanol, dried and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Contaminating RNA was removed by digestion with RNAse A and T1 (Ambion, Austin, TX) followed by microspin column filtration (Millipore Corporation, Bedford, MA).

RAPD-PCR was carried out in a volume of 25 µl. Each reaction consisted of 2.5 ul 10× PCR Gold buffer (PE Applied Biosystems, Foster City, CA), 200 µM dNTP mix (PE Applied Biosystems), 1.5 mM $MgCl_2$, 0.5 units of AmpliTaq™ Gold (PE Applied Biosystems), 6.25 ng plum curculio DNA and 1 µM primer (Table 1). For RAPD-PCRs with univoltine weevil DNA, 0.1% gelatin and 1% Triton X-100 were added to the

Table 1. Primers used in RAPD-PCR assays

Primer	Primer sequence	Reference
OPE-01	5′-CCC AAG GTC C-3′	Taberner et al. 1997
OPE-02	5′-GGT GCG GGA A-3′	Taberner et al. 1997
OPE-03	5′-CCA GAT GCA C-3′	Taberner et al. 1997
OPE-04	5′-GTG ACA TGC C-3′	Taberner et al. 1997
OPE-06	5′-AAG ACC CCT C-3′	Taberner et al. 1997
OPE-07	5′-AGA TGC AGC C-3′	Taberner et al. 1997
OPE-09	5′-CTT CAC CCG A-3′	Taberner et al. 1997
A04	5′-GAA ACG GGT G-3′	Haymer 1994
C01	5′-TTC GAG CCA G-3′	Haymer 1994
C06	5′-GAA CGG ACT C-3′	Haymer 1994
C15	5′-GAC GGA TCA G-3′	Haymer 1994
E7	5′-AGA TGC AGC C-3′	Haymer 1994
V1	5′-GTT GTC AAT GCA-3′	Taberner et al. 1997
1106	5′-CGA TGA CGC A 3′	Hsiao 1996*
IT1	5′-AGA ACG CAG C-3′	Hsiao 1996*

* Modified from the ITS3 primer. Haymer, 1994, Insect Molec. Biol. 3: 191-194. Hsiao, 1996, pp. 51-71 *in* WOC Symondonson, JE Liddell (Eds), The Ecology of Agricultural Pests. London: Chapman & Hall.

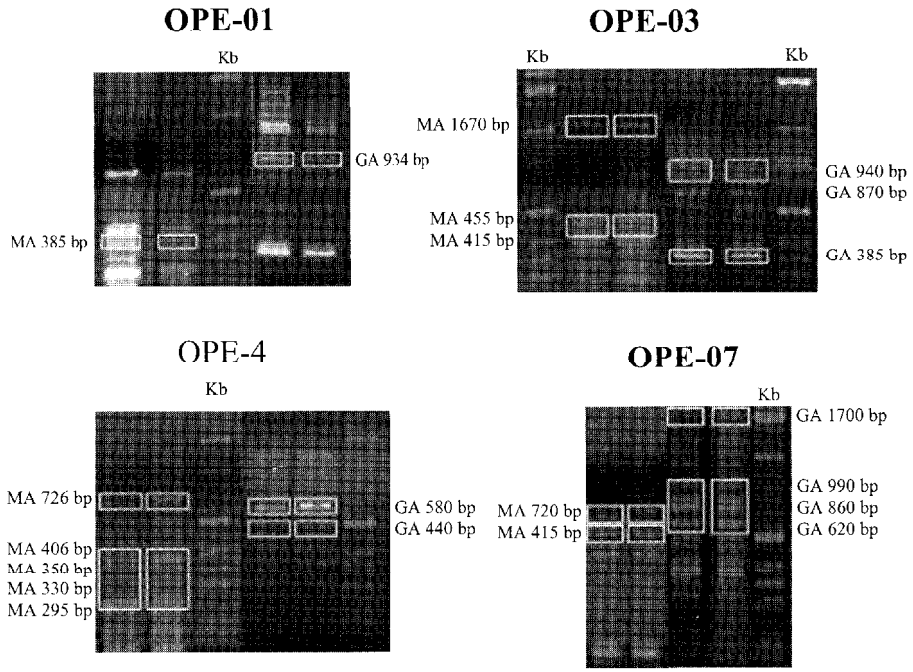

Fig. 1. Banding patterns from four primers (OPE-01, OPE-03, OPE-04 and OPE-07) showing differences in banding between northern and southern strains of plum curculio.

reaction mixture. Amplification was completed with the following cycling profile on a GeneAmp® PCR System 9700 (PE Applied Biosystems): 94°C for 10 min, then 45 cycles of 92°C for 1 min, 35°C for 1 min (12% ramping speed or 1° per 8 sec), 72°C for 2 min and a final extension 72°C for 7 min.

Of 15 primers tested (Table 1), OPE-01, OPE-03, OPE-04, and OPE-07 yielded consistent banding patterns from gel to gel and from curculio to curculio (Fig. 1). Of 59 bands that were scored, 21 (35.6%) were informative for distinguishing between

Table 2. Approximate sizes (base-pairs) of diagnostic amplimers obtained for the listed primers

Primer	Univoltine (MA) plum curculios	Multivoltine (GA) plum curculios
OPE-01	385	934
OPE-03	1070, 455, 415	940, 870, 385
OPE-04	726, 406, 350, 330, 295	580, 440
OPE-07	720, 415	1700, 990, 860, 620

Table 3. Genetic distances between univoltine (MA) and multivoltine (GA) plum curculios

	MA1	MA2	MA3	MA4	MA5	GA1	GA2	GA3	GA4
MA1	0								
MA2	0.125	0							
MA3	0.18755	0.1875	0						
MA4	0.151515	0.121212	0.151515	0					
MA5	0.15625	0.1875	0.1875	0.151515	0				
GA1	0.818182	0.745455	0.818182	0.754336	0.818182	0			
GA2	0.807692	0.769231	0.807692	0.740741	0.807692	0.209302	0		
GA3	0.781818	0.745455	0.781818	0.719298	0.781818	0.173913	0.069767	0	
GA4	0.777778	0.740741	0.814815	0.750000	0.777778	0.200000	0.142857	0.066667	0

Note: Boxes demarcate pairwise comparisons between individuals from the same population.

univoltine and multivoltine individuals (Table 2). Genetic distances between individuals were calculated using the RAPDistance equation [2*n11/((2*n11) + n01 + n10)], where n11 = number of positions where both individuals ("x" and "y") have the amplimer, n01 = number of positions where only individual "y" has the amplimer, and n10 = number of positions where only individual "x" has the amplimer (Armstrong et al., 1994, The RAPDistance Package 1.04. http://life.anu.edu.au/molecular/software/rapd.html). Hence, genetic distance is calculated as the number of amplimer differences detected between each curculio pair. Genetic distances closer to 0.00, such as those calculated between univoltine (MA) individuals and between multivoltine (GA) individuals, indicate high level of genetic similarity (values within boxes; Table 3), while values closer to 1.00 (unboxed values; Table 3), such as those calculated between univoltine and multivoltine individuals indicate greater genetic distance.

RAPDistance analysis verifies that the unique banding patterns from OPE-01, OPE-03, OPE-04, and OPE-07 can be used to distinguish between univoltine (MA) and multivoltine (GA) plum curculios. The genetic distance matrix (Table 3) also supports the assertion that GA and MA plum curculios have diverged genetically, consistent with the geographic separation of these populations. Analyses of additional plum curculios are currently underway to determine whether genetic distances are consistently smaller between individuals from separate populations of one strain than between individuals from separate populations of different strains.

Although Stevenson and Smith (1961, J. Econ. Entomol. 54: 283-284) and Padula and Smith (1971, Ann. Entomol. Soc. America 64: 665-668) noted reduced fecundity in crosses between univoltine and multivoltine strains of plum curculio, some offspring were produced. These observations suggest that hybridization zones exist between the strains, perhaps yielding progeny whose genomes contain both univoltine and multivoltine signatures. Because the regions of DNA that contribute to RAPD-PCR amplification are not known, RAPD-PCR assays may not accurately identify multivoltine individuals in the hybridization zone. For example, a hybrid individual may be characterized by a univoltine RAPD-PCR banding pattern, but exhibit a multivoltine phenotype. Hence, amplimers that appear to be diagnostic should be sequenced and carefully analyzed in standard PCR assays of hybrid offspring of known phenotype prior to use in large scale analyses of plum curculio populations.

Accumulated genetic data from univoltine and multivoltine strains of plum curculio will not only provide the foundation for development of convenient and rapid diagnostic strain assays, but will also provide insights into the reproductive phenology of this weevil at a molecular level. In addition to enhancing our understanding of plum curculio basic biology, a better understanding of reproductive behavior will likely allow for better management of this economically important fruit pest.