Chapter 3

Biochemical Separation of Plum Curculio Populations by RAPD-PCR

Introduction

Plum curculio is an endemic pest of pome and stone crops east of the Rocky Mountains. It is one of the major pests of these crops in the eastern and central United States (Racette et al. 1992) and the most destructive pest of peaches and plums in the southeastern United States (Yonce et al. 1995).

Two morphologically identical strains have been reported: a univoltine northern strain and a multivoltine southern strain (Chapman 1938, Bobb 1952, Racette et al. 1992). The line dividing these populations, estimated by Chapman (1938), runs through the fruit-growing region of western Virginia. Assuming multivoltine larvae may be present in Virginia fruit at harvest (based on the presence of the multivoltine strain in the state), trade barriers have been imposed against the importation of Virginia fruit by California and foreign countries such as Brazil. Thus, identification of the two strains is important in order to document the distribution of these strains in Virginia.

The adults enter an orchard in the springtime from hedgerows and woodlots (Butkewich and Prokopy 1993, Prokopy et al. 1999) where they have overwintered. The adults feed on leaves and twigs until fruits are formed. Then both sexes feed upon, and the females oviposit in, the immature fruits. Feeding and oviposition damage worsen early abscission (June drop) (Bobb 1952) and cause corky scars on the fruits remaining in the tree (Chapman 1938, Bobb 1952, Racette et al. 1992), making it unmarketable. In areas where the multivoltine strain occurs the fruits remaining on the tree may contain live larvae at harvest, resulting in exportation of this pest to other states or countries where plum curculio is not endemic. This prospect has caused trade barriers to be raised against states, like Virginia, that are suspected or known to have a multivoltine strain of plum curculio in the fruit growing regions.

Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) has been successfully used to differentiate populations of several extant insects: alfalfa weevil, *Hypera postica* (Gyllenhal) (Erney et al. 1996), a weevil pest of sugar beet, *Aubeonymus mariaefranciscae* (Taberner et al. 1997), whiteflies (Gawell and Bartlett 1993), and the alfalfa leafcutting bee, *Megachile rotundata* (Lu and Rank 1996).

Polymerase chain reaction (PCR) techniques have also been successfully used on ancient insects (DeSalle et al. 1992, Cano et al. 1993, Farrell 1998).

RAPD-PCR is based on analysis of the separation patterns in agarose or polyacrylamide gels of amplified DNA fragments (amplimers) produced by small primers (10 nucleotides in most studies) of generally arbitrary sequence. These small primers bind to homologous sites within the genome. If the primers bind at opposing sites at least 3000 base pairs apart with the 3' ends oriented toward each other, amplification occurs (Loxdale and Lushai 1998). If there are mutations in the binding sites between individuals or between strains of a species, the primer is less likely to bind and no amplification will occur, resulting in the lack of a band on an electrophoretic gel. This allows dominant genes to be profiled (Loxdale and Lushai 1998) and sampled individuals from populations to be differentiated.

The objective of this study was to differentiate between a univoltine strain (from Massachussetts) and a multivoltine strain (from Georgia) of plum curculio using a RAPD-PCR assay.

Methods & Materials

Insects

Univoltine plum curculio adults were obtained from Massachusetts (Dr. Ron Prokopy, U. MA, Amherst), and multivoltine plum curculio adults from Georgia (Dr. Dan Horton, U. GA, Athens).

DNA extraction

Weevils were placed in 300 ?l of TE (Tris-HCl, EDTA) buffer and frozen at –80°C for storage. The insects were thawed and the TE buffer was removed before starting extraction. The DNA extraction protocol was modified from a protocol developed for *Drosophila melanogaster* by Ashburner (1989). Weevils were placed in individual 1.5 ml colorless microcentrifuge tubes and ground with Kontés pestles in 300 ?l of homogenization buffer (10 mM Tris-HCl (pH 7.5), 60 mM NaCl, 10 mM EDTA with 5% sucrose) and homogenized. A volume of 300 ?l of lysis buffer (300 mM Tris-HCl -pH 7.5), 100 mM EDTA, 0.625% Sucrose, 1% DEPC) was added and the samples

were incubated for 15 min at 70° C. When cooled to room temperature, 90 ?l of 8 M potassium acetate was added and the samples were incubated for 30 min on ice. The samples were then centrifuged at 20800 *g* at 4°C 10 min. The supernatant was removed to a fresh microcentrifuge tube without disturbing the surface lipid or pellet. The supernatant was extracted twice with an equal volume of 1:1 phenol/chloroform and spun at 20800 *g* at 4°C for 5 min. One extraction was done with chloroform only to remove residual phenol in the supernatant. DNA was precipitated by adding 2 volumes of absolute ethanol and incubated at room temperature for 5 min. The samples were centrifuged at 20800 *g* at room temperature for 5 min. The supernatant was discarded. The pellet was washed with 400 ?l of 70% ethanol, allowed to dry, and then was resuspended in 50 ?l of TE (Tris- HCl EDTA) buffer.

RNA was removed by adding 1.5 ?l of RNAse Cocktail A (Ambion, Austin) to each sample. Samples were then column-purified (Microcon® Centrifugal Filter Devices, Millipore Corporation, Bedford) to remove RNA fragments. The samples were electrophoresed on an 0.8% agarose gel containing ethidium bromide to visually determine the quality of the DNA and tested using a GeneQuant DNA Calculator (Pharmacia Biotech, Piscataway) spectrophotometer to estimate the concentration of DNA from its absorbance at 260 nm. Quality of DNA was inferred from the appearance of a single, discrete, high molecular weight band following electrophoresis and from the ratio of absorbance at 260 nm (DNA and RNA) to the absorbance at 280 nm (protein). The ideal range of this ratio is 1.4-1.8 and the lowest ratio of quality accepted in this study was 1.4. Samples with a lower ratio were not used in this study.

RAPD-PCR

PCR was carried out in a total volume of 25 ?l. Each reaction consisted of 2.5ul 10x PCR Gold buffer (PE Applied Biosystems, Foster City), 200 ?M dNTP mix (PE Applied Biosystems), 1.5 mM $MgCl_2$, 0.5 units of AmpliTaq^{TM} Gold (PE Applied Biosystems), 6.25 ng plum curculio DNA, 1 ?M primer (Table 1), and ddH_2O to a volume of 25 ?l. For the univoltine weevils only, 0.1% gelatin and 1% Triton X-100 were added to the PCR reaction. Amplification was completed with the following thermal 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, final extension 72°C for 7 min and held at 4°C.

The primers were taken from Haymer 1994, Hsiao 1996, and Taberner et al. (1997; Table 1).

Table 1 RAPD-PCR primers

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[1]
IT1	5'-AGA ACG CAG C-3'	Hsiao 1996[1]

1. Modified from the ITS3 primer

RAPD-PCR Analysis

RAPD-PCR amplimers are visible as discrete banding patterns following electrophoresis. These patterns can be used to differentiate individuals from different populations and, hence, permit inferences to be drawn regarding the populations. Using Using RAPDistance (Armstrong et al. 1994) and the occurrence of the amplimers produced by each primer for the two populations, genetic distance of the individuals in the populations can be estimated. These distances were calculated by RAPDistance using the equation:

$$2*n11/((2*n11)+n01+n10),$$

where n11= number of positions where both individuals have the band, n01= number of positions where only individual "y" has the band, and n10= number of positions where only individual "x" has the band. Genetic distances can range from 0.00 to 1.00; values closer to 0.00 indicate high level of genetic similarity. Values closer to 1.00 indicate greater genetic distance.

Results

Of 15 primers tested, only four primers (OPE-01, OPE-03, OPE-04, OPE-07) yielded consistent banding patterns from gel to gel and individual to individual. The approximate molecular size (base-pairs) of each amplimer unique to all tested individuals in a population is listed in Table 2.

Primer	Massachusetts Weevils	Georgia Weevils	Figures
OPE-01	385	934	5 & 6
OPE-03	1070, 455, 415	940, 870, 385,	7 & 8
OPE-04	726, 406, 350, 330, 295	580, 440	9 & 10
OPE-07	720, 415	1700, 990, 860, 620	11 &12

Table 2. Approximate molecular-weights (base pairs) of unique amplimers obtained from each population of plum curculio.

Figures 5-12 show examples of the patterns of amplified DNA fragments obtained with each primer for samples from each population. Of the 59 bands that were scored, 21 (35.6%) were informative for distinguishing between the populations. Genetic distances between individuals were calculated from the number of amplimer differences detected between each pairwise comparison (Table 3).

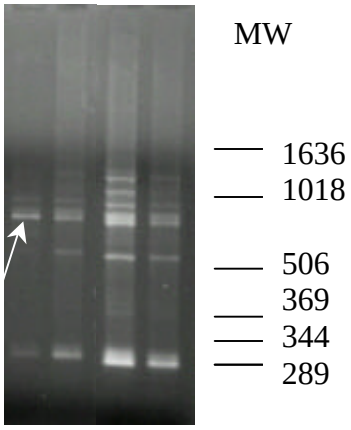

Figure 5. Primer OPE-01 with DNA from Georgia samples 23, 24, 28, and 30. Arrow marks the unique band- 934 bp.

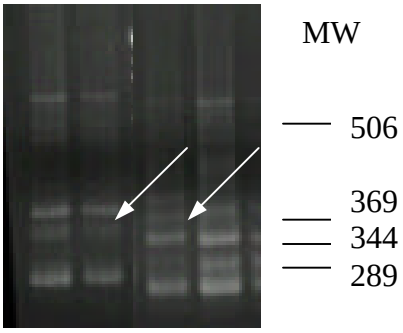

Figure 6. OPE-01 with DNA from Massachusetts samples 12, 11, 7, and 8. Arrows mark the unique band- 385 bp.

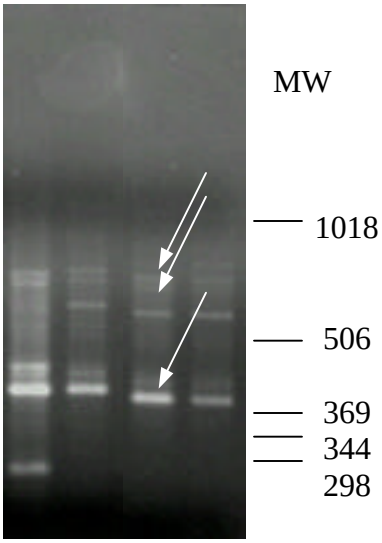

Figure 7. OPE-03 with DNA from Georgia samples 23, 24, 28, and 30. Arrows mark the unique bands- 940bp, 870 bp, 385 bp.

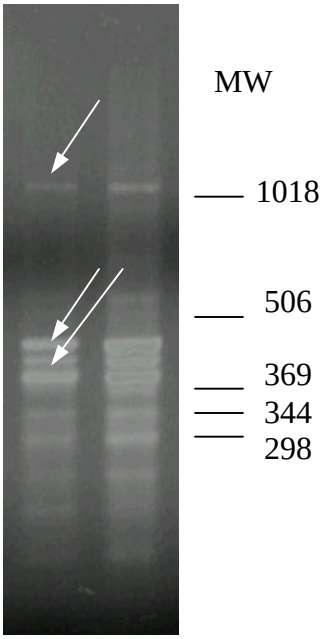

Figure 8. The lanes are OPE-03 with DNA from Massachusetts samples 7 and 8. Arrows mark the unique bands for primer OPE-03- 1670 bp, 455 bp, 415 bp.

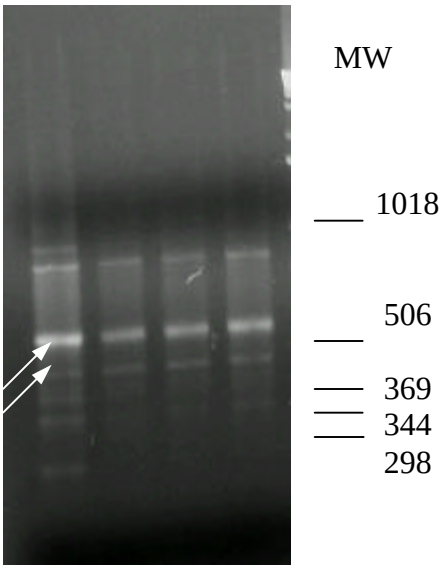

Figure 9. Primer OPE-04 with DNA from Georgia samples 23, 24, 28, and 30. Arrows mark the unique bands- 580 bp, 440 bp.

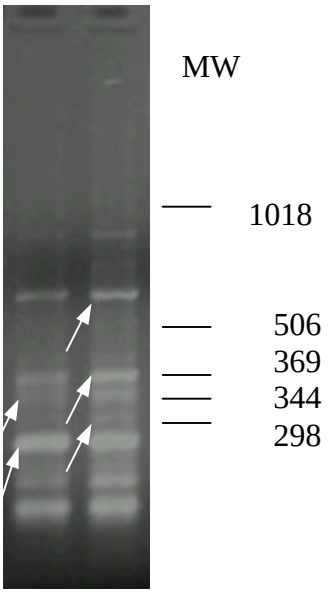

Figure 10. Primer OPE-04 with DNA from Massachusetts samples 7 and 8. Arrows mark the unique bands- 726 bp, 406 bp, 350 bp, 330 bp, 295 bp.

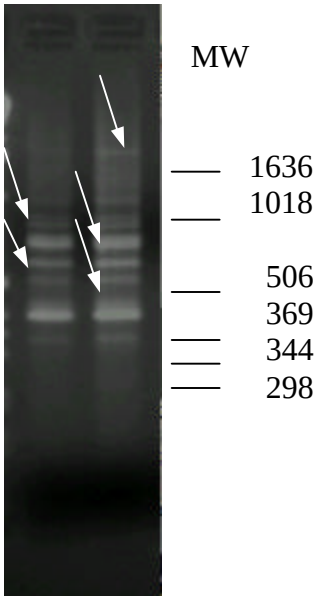

Figure 11. Primer OPE-07 with DNA from Georgia samples 28 and 30. Arrows mark the unique bands- 1700 bp, 990 bp, 860 bp, 620 bp.

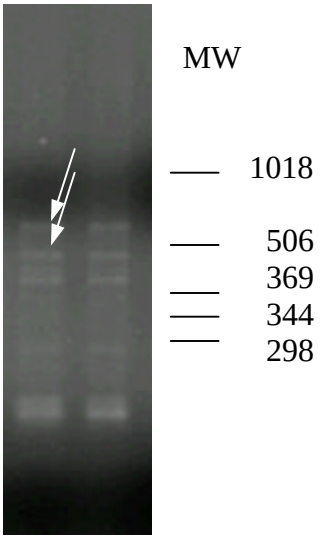

Figure 12. Primer OPE-07 with DNA from Massachusetts samples 7 and 8. Arrows mark the unique bands- 720 bp, 415 bp.

Table 3. Pairwise genetic distances between individuals from the univoltine and mutilvoltine populations of plum curculio.

	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	0.754386	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

Distances range from 0.00 to 1.00. Individuals most closely related have numbers closer to 0.00.

Discussion

The RAPD method has become popular for separating populations based on genetic differences because it provides information on molecular polymorphisms without a previous knowledge of DNA sequences from the organism. It is also a technically simple method and is faster than other methods employed in other studies, e.g. restriction length polymorphisms (RFLPs), amplified restriction length polymorphisms (AFLPs), single strand conformation polymorphisms (SSCPs; Loxdale and Lushai 1998).

The RAPDistance analysis verifies that the unique banding patterns from OPE-01, OPE-03, OPE-04, and OPE-07 can be used to distinguish individuals from Georgia and individuals from Massachusetts. The genetic distance matrix (Table 3) shows the individuals from Massachusetts, when compared to other individuals from Massachusetts (in the red box), have a value close to zero, indicating the individuals are genetically similar. Individuals from Georgia, when compared to other individuals from Georgia (in the blue box), also have a value close to zero. However, individuals from Georgia when compared to individuals from Massachusetts have a value closer to 1.0, indicating a

greater genetic distance. This supports the assumption that individuals from the same populations are more closely related to each other than to individuals from the other population (Table 3).

The primers used in this study provide a way to differentiate the two populations of plum curculio tested. Further testing of this assay on univoltine individuals collected from Michigan, Utah, New Jersey, and Ontario and on multivoltine plum curculios from North Carolina, South Carolina, and New Jersey needs to be done. If the results suggest that individuals from univoltine and multivoltine populations can be distinguished by this RAPD-PCR assay it will be applied to Virginia plum curculio populations.

Although Stevenson and Smith (1961) and Padula and Smith (1971) noted reduced fecundity in crosses between the strains of plum curculio, some offspring were produced, although they did not describe the phenotipy of the offspring. Theoretically some hybrid offspring will show phenotypical traits of the univoltine parent and some offspring will show traits of the multivoltine parent.

Because of the possibility of hybrid individuals in areas where both strains occur and because it is not known which part of the genome is being amplified, the RAPD-PCR assay may not be able to accurately quantify numbers of individuals of the multivoltine strain in the hybridization zone. DNA samples from hybrid individuals may produce a multivoltine banding pattern after electrophoresis, but exhibit univoltine phenotypes. Because of this, the amplimers that are diagnostic need to be characterized. Building a body of genetic information will allow researchers to look for more differences between the two strains of plum curculio on a molecular level. This could eventually payoff in greater understanding of the differences of behaviour between the two strains. A better understanding of the behaviours will allow for better management of this pest.

References

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