Vitis 35 (2), 87–89 (1996)

Extraction of DNA from eggs of grape phylloxera (*Daktulosphaira vitifoliae* FITCH) for use in RAPD testing

by

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Summary: Three different extraction methods for preparing DNA from single grape phylloxera (*Daktulosphaira vitifoliae*) eggs for use in PCR-RAPD assay were compared. Pulverization in a glass micro-grinder produced much more clear and consistent RAPD results than when the egg DNA was extracted with a Chelex lysis or a proteinase-K$^+$ lysis. Multiple single egg extractions from the same parthenogenetic colony were done with all three methods; no RAPD assay differences were detected. This technique will allow evaluation of the roles population diversity, mutation rates, and reproductive mechanisms play in the *Vitis*/phylloxera interaction.

Key words: Vitis, DNA extraction, RAPD, phylloxera, Daktulosphaira vitifoliae, parthenogenesis.

Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* FITCH) are North American aphid-like insects that feed on roots and leaves of *Vitis* L. species. *Vitis vinifera* L., the commonly cultivated wine and table grape, is extremely susceptible to root feeding by phylloxera and successful cultivation depends upon phylloxera resistant rootstocks. These rootstocks were developed in the late 1800s after phylloxera were inadvertently imported into Europe, and consequently destroyed much of European viticulture. Phylloxera were also imported into California about this time, and grape growers there were soon using European rootstocks to combat the pest. The rootstock AXR#1 excelled in many rootstock trials and seemed to have adequate phylloxera resistance (LIDER 1958). As a result of these trials AXR#1 became widely used in California in the large scale replantings of the 1960s and 1970s.

Currently, Californian AXR#1 is under attack from more aggressive strains of phylloxera named biotype B (GRANETT *et al.* 1983) to distinguish them from less aggressive biotype A strains. Recent work using RAPD (randomly amplified polymorphic DNA) analysis revealed that there was as much polymorphism within biotypes as among biotypes (FONG *et al.* 1995). Given that phylloxera were only recently imported into California and that they are thought to be entirely parthenogenetic in this state, the level of observed genetic diversity needs explanation. To begin studying how and why phylloxera vary in California and how this variability might affect the resistance of grape rootstocks, it is necessary to be able to extract and analyze DNA from single individuals. Such assessments would allow evaluation of mutation rates, the stability of parthenogenesis, and the genetic diversity of field populations.

The polymerase chain reaction (PCR) technique provides a powerful tool in molecular genetic studies. One of its advantages is its ability to amplify nanogram amounts of DNA. As a result, DNA extraction methods such as Chelex lysis and proteinase-K$^+$ lysis have been developed for studying extremely small organisms such as single bacteria, fungal spores (LEE and TAYLOR 1990) and nematode eggs (BARSTEAD *et al.* 1991; WILLIAMS *et al.* 1992). However, effective DNA extraction and reliable amplification are largely dependent on the nature of materials being studied. The major problems encountered have been the effective release of DNA from nuclei and removing interfering substances from extracts. The study reported herein proposes a simple and reliable method of DNA extraction from a single, very small (0.3 x 0.15 mm) grape phylloxera egg. It also compares the efficiency and reliability of three DNA-template extraction methods using RAPD analysis.

Materials and methods

All phylloxera eggs for this study were from a single parthenogenetic colony, A STD (FONG *et al.* 1995), provided by J. GRANETT, Department of Entomology, University of California, Davis. The 0.3 x 0.15 mm eggs were derived from one or more females feeding on root pieces of *V. vinifera* Cabernet Sauvignon. Three DNA extraction methods using either single or about 75 (hereafter called multiple) egg samples were conducted to compare the efficiency and reliability of their use in RAPD amplification.

Grinding method: A single egg of phylloxera was transferred to a micro-grinder (Radnoti Glass, Arcadia, CA) containing 3 µl of DNA extraction buffer (100 mM Tris-HCl pH 7.5, 10 mM EDTA, 350 mM Sorbitol), and was homogenized. The micro-grinder was then given a 2 s pulse in a mini-centrifuge to spin down the extract, after

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which the supernatant was transferred to a 500 μl sterile Eppendorf tube. The micro-grinder mortar was washed twice with an additional 5 μl of the extraction buffer. All extracts were combined with the supernatant in the Eppendorf tube. 20 μl of DNA lysis buffer (2 M NaCl, 2 % w/v CTAB, 50 mM EDTA) and 20 μl of 5 % sarcosyl were mixed with the extracts. The homogenate was then incubated at 65 °C for 30 min. Following this incubation 50 μl of chloroform:isoamyl alcohol (24:1) was added to each tube and mixed well. Tubes were centrifuged at 8000 rpm for 5 min, the upper aqueous phase was carefully collected. This chloroform phase was then re-extracted by adding 20 μl of DNA extraction buffer. The secondary aqueous phase was pooled with that from the first extraction, and 10.5 μl of 5 M NaCl and 140 μl of ethanol were added. Tubes were chilled at -20 °C for 1 h and centrifuged at 14,000 rpm for 5 min. The supernatant was discarded, precipitated DNA was air-dried and redissolved in 20 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNAs were then ready for assay.

DNA was also extracted from the same parthenogenetic colony using a multiple egg sample so that comparisons with DNA from the single egg extractions could be made. Multiple eggs were also homogenized in a micro-grinder. The procedure was essentially the same as the single egg DNA extraction except that final precipitated DNA was redissolved in 50 μl of TE buffer.

Chelex lysis method (WALSH *et al.* 1991): A single egg was transferred into a 500 μl sterile Eppendorf tube containing 15 μl of Chelex™ 100 (BioRad Richmond, CA). The mixture was then incubated at 95 °C for 20 min followed by centrifugation at 14,000 rpm for 5 min. The supernatant was carefully transferred to a new tube and the extraction was adjusted to a final volume of 20 μl with sterile water in preparation for RAPD assay. DNA was also extracted from multiple egg samples in the same way as the single egg was except that 60 μl of Chelex™ 100 resin was used. After incubation at 95 °C for 20 min, the sample was extracted once with 60 μl of chloroform:octanol (24:1) and DNA was precipitated as described in the grinding method. The DNA was resuspended in 50 μl of TE buffer.

Proteinase-K^+ lysis method (modified from BARSTEAD *et al.* 1991): A single egg was transferred into a 500 μl sterile Eppendorf tube containing 20 μl of lysis buffer (60 μl/ml proteinase-K^+, 50 mM KCl 0.45 % Tween-20, 0.05 % gelatin in 10 mM Tris-HCl pH 8.0). The tube was first frozen at -70 °C for 15 min, then incubated at 60 °C for 60 min, followed by incubation at 95 °C for 20 min, and finally by centrifugation at 14,000 rpm for 5 min. The supernatant was carefully transferred into a new tube and the final volume was adjusted to 20 μl with sterile water in preparation for RAPD assay. DNA was extracted from the multiple egg sample using the same protocol as described above except that 60 μl of lysis buffer was used. After incubation at 95 °C for 20 min, the sample was mixed with 60 μl of chloroform:octanol (24:1). The upper phase containing DNA was collected and precipitated as described in the grinding method. The extracted DNA was resuspended in 50 μl of TE buffer.

DNA samples extracted from multiple egg samples using the above three methods were analyzed with a spectrophotometer at 260 nm and 280 nm. Samples were diluted to 20 ng/μl based on the A_{260} absorbance. DNA purity was estimated by their A_{260}/A_{280} absorbance ratio.

RAPD analysis: Two tests were carried out to evaluate the single egg DNA extractions. The first test compared the three different extraction methods of single and multiple egg DNA. The second test evaluated the reproducibility of the single egg extractions using the grinding method. Three primers were used in this study: OPA-11$^{5'}$-CAATCGCCGT-$^{3'}$, OPA-12$^{5'}$-TCGGCGATAG-$^{3'}$, and OPA-15^{5}-TTCCGAACCC-$^{3'}$ (Operon Tech. Alameda, CA). Amplification reactions were carried out as described by WILLIAMS *et al.* (1990) in 25 μl containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.1 % Triton X-100, 3.0 mM $MgCl_2$, 0.2 mM each of dATP, dGTP, dCTP and dTTP (Boehringer Mannheim, Germany), 0.5 mM primer, and 1.0 unit of Taq DNA polymerase (Promega Corp., Madison, WI). About 40 ng of phylloxera DNA from the multiple egg extractions or 2 μl of DNA from the single egg preparations were added into each reaction mixture to make 25 μl of final reaction volume, respectively. To minimize variations in background reactions, a master mixture containing all reaction components except DNA being tested was prepared. Therefore differences between reactions were due to the DNA added. To ensure reproducibility of RAPD amplification, multiple replicated reactions were performed.

Amplification was performed in a PTA-100 Thermal Controller (MJ Research Inc., USA) for 1 min at 94 °C and 4 cycles of 1 min at 94 °C, 1.5 min at 36 °C and 2 min at 72 °C, then followed by 25 cycles of 0.5 min at 94 °C, 0.5 min at 37 °C and 1 min at 72 °C. Amplified DNA fragments were incubated at 72 °C for 5 min. Amplification products were analyzed by electrophoresis in 1.7 % agarose gel and detected under UV light after staining with ethidium bromide.

Results and discussion

The results of RAPD analysis of the single egg DNA extraction methods showed that only the grinding method produced strong and consistent banding patterns (Figure). The Chelex lysis and the proteinase-K^+ lysis methods only occasionally produced clear and consistent bands, in most cases their RAPD products were very faint bands or non-detectable bands. The multiple egg DNA extractions, however, resulted in visually uniform amplified banding patterns among the three tested extraction methods. Spectrophotometer measurement indicated that the three methods had similar A_{260}/A_{280} ratios (between 1.7 to 1.85) following a single chloroform extraction.

To further check the efficiency and the reliability of the grinding method, independent DNA extractions from single parthenogenetic eggs were assayed. Identical band-

ing patterns were obtained from these separately extracted DNA samples. The inability of the Chelex and proteinase-K^+ methods to produce consistent and adequate DNA for RAPD testing may have been largely due to their lack of efficiency at extracting DNA from small single eggs. The protective cuticle layers of phylloxera eggs, enriched in waxes and chitin (CHAPMAN 1969), may be responsible for this. In contrast, we have successfully extracted and amplified plant leaf tissues using Chelex and proteinase-K^+ methods. The grinding method in combination with cell lysis buffers seemed to be effective at rupturing the cells of single phylloxera and releasing enough DNA for amplification.

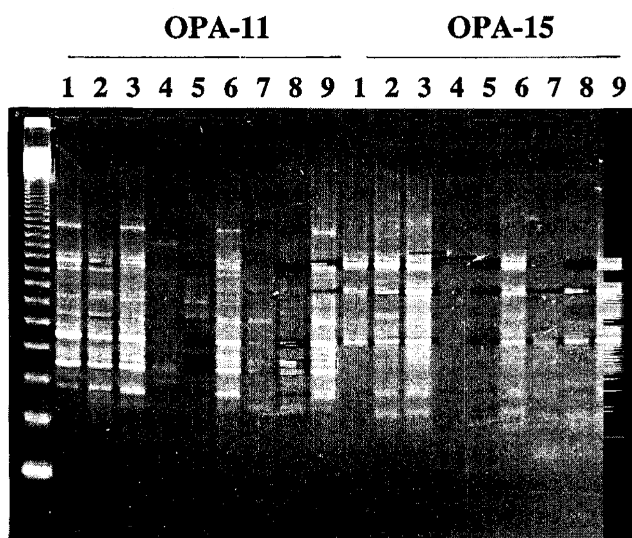

Figure: Agarose gel (1.7 %) electrophoresis of RAPD products from three different DNA extraction methods of grape phylloxera eggs. The single and multiple (about 75) egg samples were taken from a single parthenogenetic colony, A STD (FONG *et al.* 1995), and primers OPA-11 and OPA-15 were used. The lane on the far left = 123 bp DNA molecular ladder; lanes 1 and 2 = DNA from a single phylloxera egg extracted with the grinding method; lane 3 = DNA from multiple phylloxera eggs extracted with the grinding method; lanes 4 and 5 = DNA from a single phylloxera egg extracted with the Chelex method; lane 6 = DNA from multiple phylloxera eggs extracted with the Chelex method; lanes 7 and 8 = DNA from a single phylloxera egg extracted with the proteinase K^+ method; lane 9 = DNA from multiplephylloxera eggs extracted with the proteinase K^+ method.

Optimization of PCR reactions was carried out by varying concentrations of dNTPs and Mg^{2+}, or by altering the concentration of the DNA. In all cases, DNA from the grinding method provided the best templates for PCR amplifications. DNA from each single egg extraction was diluted to a final volume of 20 µl , which provides six to seven runs of PCR-based assay from a single egg. In addition, single egg DNAs dissolved in TE buffer after chloroform partitioning have proved to be stable when stored at -20 °C for at least three months in contrast to Chelex and proteinase-K^+ methods. DNA from these last two methods typically has a shorter life span.

The extraction method described here was developed for the genetic study of grape phylloxera, but appropriate modifications may allow it to be used with similar small organisms. Study of single phylloxera DNA will clarify our understanding of the observed diversity in Californian isolates (FONG *et al.* 1995) and provide the means to study how phylloxera adapt to grape rootstocks and *Vitis* species. Studies of single egg DNA will also allow evaluation of the roles mutation rates, population diversity within a site or on a root, variability in asexual reproduction, and the possibility of an unobserved sexual cycle play in the *Vitis*/phylloxera interaction.

Acknowledgements

Financial support from a specific cooperative agreement with the USDA/ARS-Fresno, the American Vineyard Foundation, the California Table Grape Commission, the Wine Group Inc., and the California Grape Rootstock Improvement Commission are gratefully acknowledged. We also wish to thank Dr. JEFFREY GRANETT and his laboratory for supplying phylloxera eggs.

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Received February 22, 1996