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Application of RAPD-PCR Markers for Identification and Genetic Analysis of American Elm (*Ulmus americana* L.) Selections[1]

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Abstract

We used randomly amplified polymorphic DNA (RAPD) markers from DNA polymerase chain reactions to differentiate selected American elms. DNA profiles made from leaf DNAs of parental trees were identical to profiles produced by DNA from leaves, roots, vascular tissues, callus or suspension-cultured cells from the same trees or their ramets. In addition, several variations in the technique did not appear to alter the genotype-specific DNA profiles from a given elm selection. RAPD polymorphisms were inherited as dominant Mendelian factors in the progeny of a cross between two American elm selections. We conclude that the construction of RAPD DNA profiles is a reliable and easy method for selection identification, genetic analysis, and the assembly of linkage maps in American elms.

Index words: polymerase chain reaction, arbitrary primers, DNA polymorphism, plant identification, genetic markers, breeding.

Species used in this study: American elm (*Ulmus americana* L.).

Significance to the Nursery Industry

We determined how DNA markers can be used to identify American elm trees as well as their vegetative and sexual progeny. DNA technology can be used as a management tool for cultivar identification, certification of germplasm sources, and for establishment of markers in plant breeding. Our efforts in biotechnology and plant breeding with American elms required a simple, reliable technique to identify ramets from selected trees, unselected wild trees, cultured cells, and small tissue samples. We found that the application of RAPD DNA profiles, which is gaining wide acceptance in agriculture, medicine and forensic sciences, can be easily used for the identification of American elms selected for disease tolerance. In addition to accurate identification of individual selections, we are using DNA profiles of American elms to measure relatedness, in taxonomic studies, for construction of linkage maps, and in genetic analysis to identify markers associated with desirable traits.

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Introduction

American elms (*Ulmus americana* L.) were the most widely planted landscape tree in North America because of their adaptability to urban and rural sites, rapid growth rates,

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and desirable forms. A Dutch elm disease (DED) epidemic caused by the accidental introduction of the Eurasian fungus *Ophiostoma ulmi* (Buisman) Nannf. eliminated the highly susceptible American elm as a significant landscape tree. Various authors have concluded that the best hope for long-term control of DED in American elm depends on selection of disease-tolerant trees.

Attempts to screen seedling populations for DED resistance in the 1940s met with very limited success, both in the numbers of resistant trees identified and their levels of resistance (18). Subsequently, American elms surviving in areas of high disease incidence were used as starting material in selection efforts for DED resistance (22) and in breeding programs (19, 21). Trees from these selection efforts, considered to be tolerant hosts for DED (20), have been vegetatively propagated as root or shoot cuttings on numerous occasions (17), and cells of some selections have been cultured for micropropagation (6). Until recently a reliable protocol for identification of different American elm selections was not needed because the bulk of the propagation efforts were done by a small number of investigators.

There are characteristic responses of selected American elms to *O. ulmi*, both *in vivo* and *in vitro*. Artificial inoculation of ramets from selected elms resulted in relatively low levels of disease symptoms in the crown and limited dieback compared to unselected seedlings (20, 25). Cultured cells from DED-tolerant trees inhibited the growth of the pathogen when compared to fungal growth on cells from unselected trees (3). Pijut et al. (16) also showed that the extent of elm cell growth on fungus culture filtrate was highly correlated with field levels of resistance in the ramets.

In some of our recent attempts to reproduce these observations, the reactions of selected elm ramets and cultured cells with DED fungi were different than expected. We needed to determine if the periodic variations in these reactions were due to physiological or genetic differences caused by propagation (7), or to technical differences in the application of the test methods. Because American elm trees, their ramets, or cultured cells were morphologically indistinguishable, we required a simple, reliable method to identify the different genotypes. Aside from being screened for crown resistance to DED, there have been only minor attempts to identify distinctions in selected elms. Scanning electron microscopy (12) or flavonoid chemistry (8) are of limited value in elm selection identification.

RAPD technology has been successfully applied to the identification of closely related cultivars in many important horticultural plants including such diverse species as roses (23) or coffee (15) and hardwood species such as poplars (2, 13), willows (13) and red maple (11). The versatility and ease of the technique indicated that it might be a useful method to distinguish American elms.

In this report, we define a set of DNA profiles which provide reliable genetic markers for individual American elms. We demonstrate the use of this technique to identify ramets or callus cells of questionable authenticity. We also analyzed the segregation and independent assortment of RAPD markers in an American elm cross. Based on differences detected in RAPD DNA profiles, we concluded that the unexpected variations of American elm selections in reactions to DED fungus were probably caused by improper labeling during propagation. The differences in DNA profiles did not likely reflect genetic modifications produced in propagation or culture, since the over-all pattern of dissimilar bands would require changes at a very large number of chromosomal sites.

Materials and Methods

Plant materials. Elm selections included five trees which have shown significant DED-tolerance in previous studies: No. 680, No. 8630, R18–2, 'Delaware 3' and 'Delaware' (formerly 'Delaware 2') (20, 21, 22). The original trees or their ramets growing at the USDA Forest Service Laboratory in Delaware, OH, (formerly the Ohio Research Site of the US National Arboretum) were used as the sources of plant material. A DED-susceptible tree, 'A,' was selected at random from seedlings growing near Delaware, OH; propagated cuttings of 'A' were used as the source of plant material. Elm selections were propagated by vegetative cuttings (4) and grown in a soil, peat, perlite mix (1:4:4 by vol) under 16-hr artificial lighting in the greenhouse. Leaves were used as starting sources for cell culture (9). Sixteen F_1 seedlings were obtained from a controlled cross between 'Delaware' (maternal parent) and No. 680 (pollen parent).

To examine DNA profiles from different tissue sources, we used young leaf tissue, leaf midribs, roots, and leaf cells propagated as callus as described in the text or figure legends. To examine the capacity of *O. ulmi* to grow on elm cells, callus was inoculated with fungus spores, as described by Domir et al. (3). Callus sectors were defined by the presence (sample A) or absence (sample B) of visible hyphae, while uninoculated callus was defined as sample C. Intact tissues were washed in a 1% sodium dodecyl sulfate solution for 15 min., rinsed in deionized water, frozen in liquid nitrogen, and stored at –70C (–158F). Sterile cultured cells were frozen immediately.

Nucleic acid extraction. Total nucleic acids were isolated from 250–500 mg of fresh or frozen elm tissue using the hot CTAB (cetyltrimethyl ammonium bromide)/selective precipitation method (5) or by using cesium chloride isodensity gradients as described by Baker et al. (1). Restriction fragment length polymorhisms (RFLP) were performed using standard procedures described previously (10).

DNA amplification protocol development. RAPDs were performed and analyzed according to the methods of Williams et al. (26) as modified by Nelson et al. (14). The reaction buffer used was that defined by Promega, Inc. (Madison, WI) using 1.85 mM magnesium chloride. Changes in magnesium concentration of the reaction buffer altered individual DNA profiles but maintained detectable distinctions. We observed no detectable variations in DNA profiles when we used the 35-position, 0.5-ml tube, Tempcycler II (Coy Corp., Grass Lake, MI) or the 48-position, 0.5-ml tube, DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT). DNA profiles did not differ appreciably based on the commercial source of *Taq* DNA polymerase: Boehringer-Mannheim (Indianapolis, IN), Gibco-BRL (Bethesda, MD) or Promega. The use of an extraction method which yielded pure DNA (1) or one which yielded total nucleic acids including both RNA and DNA (5) did not detectably affect the PCR-RAPD profiles of the elm selections tested (unpublished observations). In our hands an accurate assessment of DNA concentration, determined by fluorimetry after staining with Heochst 33258 or by spectrophotometric absorbance (10), was key to the reliable identification.

Primers for the PCR-RAPDs were taken from a pre-selected set of 96 UBC-series decanucleotides, a subset of the 700 primers defined previously (24). Some of the primers did not produce detectable amplification of elm sequences while others did not produce readily distinguishable polymorphisms. Primer selection for the DNA profiles shown here was based on both the number and frequency of the polymorphisms produced as well as the display of clearly distinguishable DNA banding profiles among distinct elm genotypes. The sequences of primers used in this study were UBC-114 (5'TGACCGAGAC3'), UBC-116 (5'TACGATGACG3'), UBC-119 (5'ATTGGGCGAT3'), UBC-146 (5'ATGTGTTGCG3'), UBC-376 (5'CAGGACATCG3') and UBC-578 (5'GGTGTCCACT3'). Each decanucleotide was synthesized commercially by Biosynthesis, Inc. (Lewisville, TX).

Results and Discussion

DNA profiles produced from parental seedlings or archival ramets. Pilot reactions with American elms indicated that there was a high degree of genetic polymorphism detectable with the RAPD procedure within this species. The RAPD DNA profiles from parental trees No. 680 and No. 8630 and from archival ramets 'Delaware' and 'A' are presented in Fig. 1. The polymorphisms produced with this small set of decanucleotide primers has allowed us to distinguish every American elm genotype analyzed to date. Consistent, reproducible results were obtained using standardized reaction conditions. However, occasional gel-to-gel fluctuations in DNA profiles made it necessary to include archival DNA reactions alongside each batch of tested samples.

Application of RAPD-PCR to identify ramets. Initial DED-susceptibility trials with the ramets of elm selection No. 8630 had indicated a relatively high level of DED tolerance, but trials subsequent to 1989 revealed that ramets of No. 8630 were highly susceptible to DED (Schreiber and Townsend, personal communication). DNA, isolated from 0.2 g of leaf

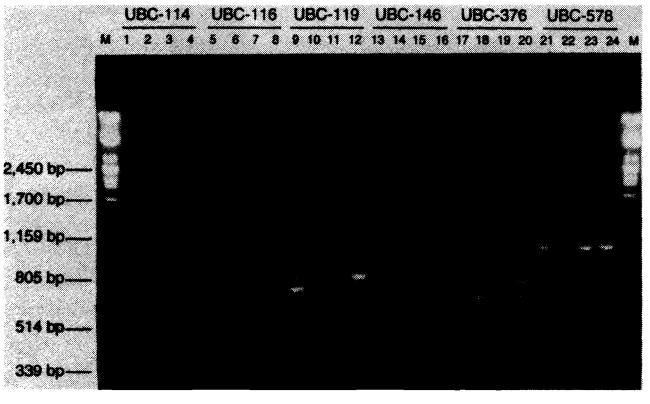

Fig. 1. Comparison of RAPD DNA profiles between selected American elms. The DNA fragments produced by RAPD reactions from four American elm selections (No. 680, No. 8630, 'Delaware,' and 'A') are presented in order using the indicated primers (UBCs 114, 116, 119, 146, 376, and 578) in lanes 1–24. PCR products were separated by agarose gel electrophoresis, gels were stained with ethidium bromide and then photographed over ultraviolet light; lane M contained the molecular weight size marker PstI digested Lambda phage DNA; sizes in base pairs (bp) indicate migration of known size markers.

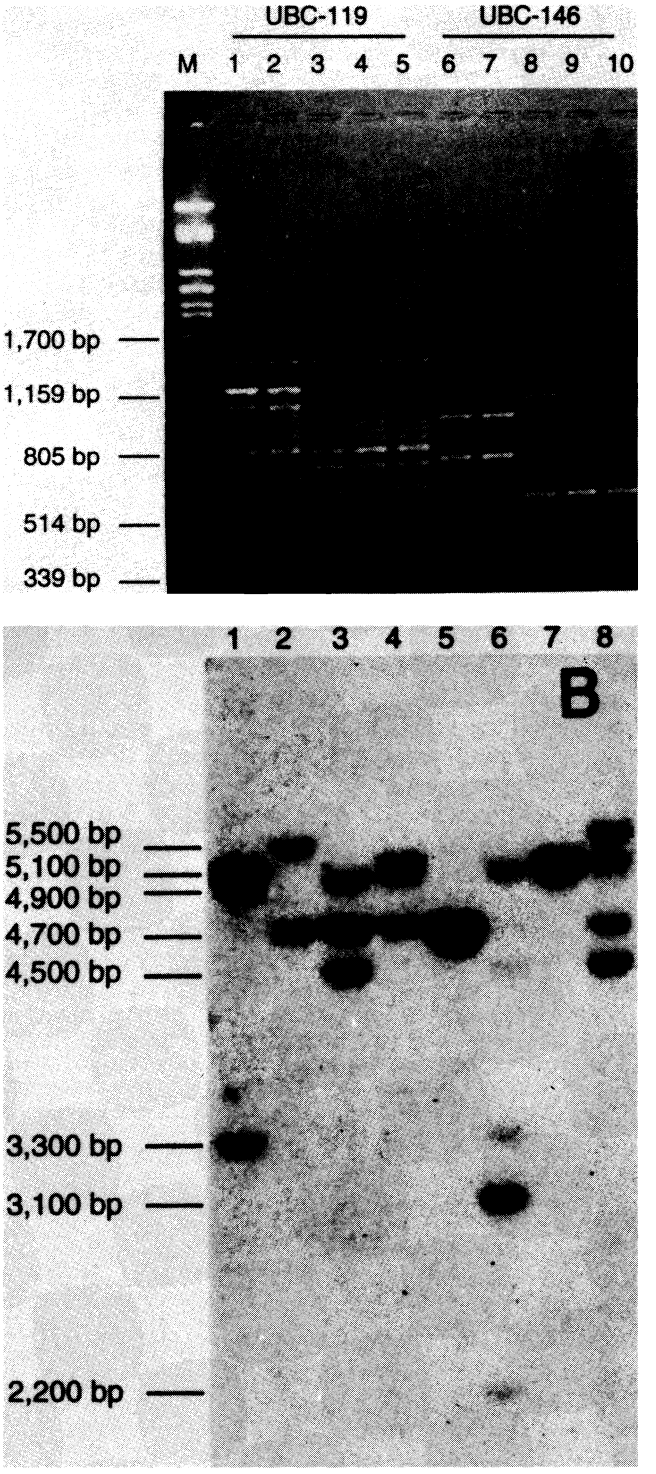

Fig. 2. Comparison of DNA profiles between putative No. 8630 ramets and the parental American elm tree. DNA profiles, as described in Fig. 1, from parental No. 8630 tree and putative ramets, obtained by (A) PCR amplification with decanucleotides UBC-119 (lanes 1–5) and UBC-146 (lanes 6–10), or by (B) RFLP after molecular hybridization. In (A) Lanes 1, 2, 6 and 7 are amplification products from two parental No. 8630 DNA preparations; lanes 3–5 and 8–10 are from three No. 8630 ramet DNA templates. Fragments in (B) are displayed by autoradiography after filter hybridization. Sizes in bp indicate the migration of DNA size markers. Genomic DNAs were digested with HindIII and gel blots were hybridized to elm clone #1–5. Lane designations: putative No. 8630 ramet (lane 1); parental selections No. 8630 (lane 2), No. 680 (lane 3), 'Delaware 3' (lane 4), 'R18-2' (lane 5), and unselected wild elms (lanes 6–8).

tissue from each tested tree in a 1993 field trial (Schreiber and Eshita, unpublished), showed that the ramets were genetically identical clones, as represented with two primer reactions shown in Fig. 2A lanes 3–5 and 8–10. However, the DNA profiles of duplicate parental tree DNA template preparations (Fig. 2A lanes 1–2 and 6–7) differed from the tested ramets.

To obtain an independent confirmation of the results with RAPD profiles, we analyzed DNAs from the putative No. 8630 ramets and from the parental tree using restriction fragment length polymorphisms (RFLP). Hybridization with an anonymous Pst I/HindIII restriction fragment (Fig. 2B) also showed differences between the putative No. 8630 ramets, represented here by tree #7 (lane 1), and the parental No. 8630 tree (lane 2). This RFLP assay also demonstrated polymorphisms with American elm selections No. 680, 'Delaware 3,' and R18–2 (lanes 3–5, respectively) and with unselected wild American elms (lanes 6–8). We concluded that the putative No. 8630 ramets used in the 1993 field trial were not propagated from the original parent tree.

Application of RAPD-PCR to identify elm callus cells. Domir et al. (3) showed that the relatively high rate of fungal growth on callus from 'A' ramets was highly correlated with the field susceptibility of ramets of the 'A' elm. However, in a recent experiment a set of putative elm 'A' callus (designated JK1) appeared unable to sustain fungus growth. DNA profiles from five elm 'A' ramets (Fig. 3, lanes 1–5) and from older callus which had supported fungus growth (lane 9) were identical. However, DNA profiles from the JK1 callus samples (lanes 10–11) were different. Similarly, suspension culture cells derived from another putative 'A' callus (designated VG1) were checked for authenticity (lanes 12–13). The variations in DNA profiles suggested by the polymorphisms shown in Fig. 3 were an indication that both the questionable callus and the suspect suspension cultures were derived from elms other than 'A'.

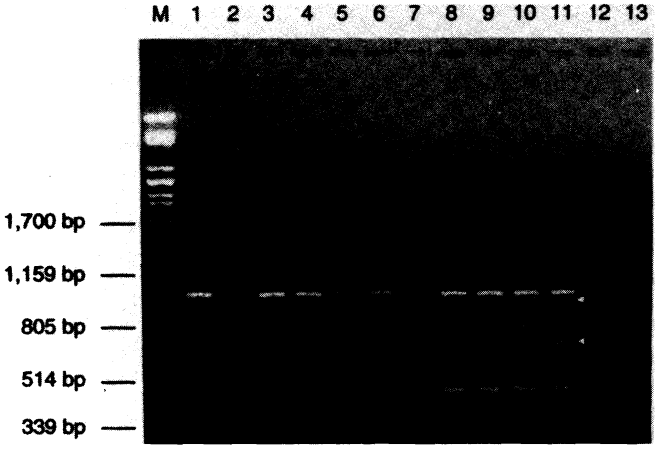

Fig. 3. Contrasting RAPD DNA profiles of intact tissues from an archival American elm 'A' ramet and from long-term callus cultures. DNA profiles obtained with UBC-116 and template DNAs taken from the leaves of five elm 'A' ramets (lanes 1–5); leaf mesophyll cells (lane 6); leaf midribs (lane 7); roots (lane 8); putative 'A' callus which supported *O. ulmi* fungus growth (lane 9); putative 'A' callus which would not support fungus growth, study JK1 (lanes 10–11), and putative 'A' suspension culture cells from study VG1 (lanes 12–13). White arrows indicate DNA polymorphisms consistently detected between archival 'A' tissues and putative 'A' callus from the JK1 study.

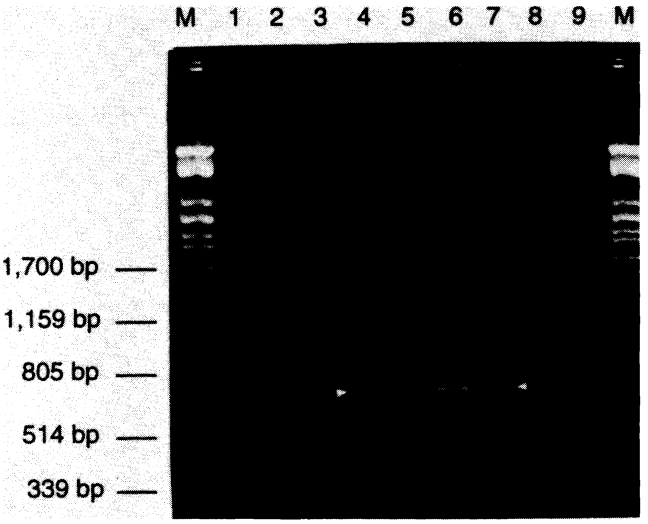

Fig. 4. DNA profiles from mixed callus and fungus cells. DNA profiles obtained with UBC-119 and template DNAs taken from American elm leaves or from callus with (sample A) and without (samples B or C) detectable *O. ulmi* fungus growth: leaves of archival American elms 'A' (lane 1) and 'Delaware' (lane 9); uninoculated callus (sample C) of each elm genotype ('A' in lane 2, 'Delaware' in lane 8); from inoculated callus with no detectable fungal cells ('A,' sample B in lane 3) or with detectable fungus ('A,' sample A in lane 4, 'Delaware,' sample A in lanes 6 and 7); DNA from shake cultured fungus (lane 5). White arrows indicate the migration position of the potential *O. ulmi* DNA marker band.

Because leaf DNA contains much more chloroplast DNA than do non-green tissues, it was possible that differences in the concentrations of chloroplast DNAs altered the DNA profiles obtained from callus cells, which were cultured in darkness. To test this possibility, DNA profiles from leaf mesophyll cells (lane 6), leaf midribs (lane 7), and elm 'A' roots (lane 8) were compared to leaf DNA profiles. Profiles from the non-green and the green tissues were identical, indicating that distinctions detected in the newer 'A' callus profiles were not related to differences in chloroplast DNA content. We concluded that the newer 'A' callus was derived from a different American elm.

DNA profiles from mixed callus and fungus cells. DNA profiles were constructed from inoculated callus with visible fungus (sample A), inoculated callus with no detectable fungus (sample B), and uninoculated callus (sample C) as described in Materials and Methods. We consistently observed a polymorphism in the DNA profiles with primer UBC-119 which was correlated with the presence of detectable fungal growth on callus (sample A) from both elm 'A' and 'Delaware' (Fig. 4, lanes 4, 6, and 7). This DNA band of about 650 bp was also detected in the UBC-119 DNA profile of *O. ulmi* (Fig. 4 lane 5) but was absent in the profiles of callus cells with no detectable fungus (Fig. 4 lanes 1–3, 8 and 9). If the 650 bp DNA band contains fungus sequence, it may be useful as an assay for the presence of *O. ulmi* in elm tissue.

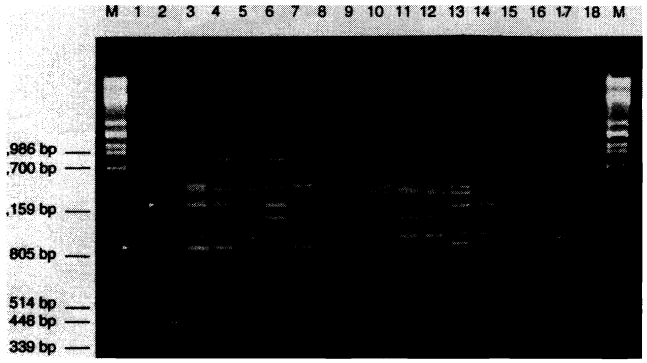

Fig. 5. Genetic inheritance and segregation of RAPD markers in American elm F_1 progeny. DNA profiles obtained with UBC-116 and template DNAs taken from the leaves of parental trees No. 680 (pollen parent, lane 1), 'Delaware' (maternal parent, lane 2) and 16 F_1 seedlings (lanes 3–18). White arrows indicate segregating RAPD markers from No. 680 (875 bp) and from 'Delaware' (1200 bp).

American elm PCR-RAPD polymorphisms were inherited as dominant Mendelian alleles. To determine the pattern of inheritance of the RAPD polymorphisms in American elm, DNA profiles were obtained from 16 progeny of a controlled cross between 'Delaware' and No. 680. The DNA profiles produced using primer UBC-116 are presented in Fig. 5. We followed the inheritance of an 875 bp DNA polymorphism unique to No. 680 and a 1200 bp polymorphism unique to 'Delaware'. If each DNA polymorphism was a heterozygous dominant marker in a cross with a homozygous recessive, we expected 8 of the 16 progeny to inherit each polymorphism. We observed that 7 of the 16 progeny inherited the No. 680 polymorphic band while 9 of 16 inherited the 'Delaware' polymorphism, therefore we concluded that a set of the RAPD polymorphisms were inherited as dominant Mendelian allelles. In addition, we concluded that the loci for these polymorphisms were not linked because they assorted independently: 4 of the 16 seedlings inherited both bands while 4 of 16 seedlings did not inherit either band. Elm DNA profiles based on RAPDs will be valuable in genetic analysis, particularly in plant breeding efforts aimed at germplasm improvement for DED, elm yellows, and elm leaf beetle resistance.

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