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Identification of *Acer rubrum* Using Amplified Fragment Length Polymorphism*

ABSTRACT: Amplified fragment length polymorphism (AFLP) analysis of botanical forensic evidence provides a means of obtaining a reproducible DNA profile in a relatively short period of time in species for which no sequence information is available. AFLP profiles were obtained for 40 *Acer rubrum* trees. Leaf material from five additional species was also typed. Genomic DNA was isolated using the DNeasy Plant Miniprep Kit (Qiagen, Valencia, CA), double-digested by two restriction endonucleases (*Eco*RI and *Mse*I) and ligated to oligonucleotide adapters. Two consecutive PCR reactions (pre-amplification and selective amplification) were performed using a modification of the AFLP protocol described by Gibco (Invitrogen, Rockville, MD). The DNA fragments were separated by capillary electrophoresis using the CEQ 8000 DNA Fragment Analyzer. A number of *Acer rubrum* species-specific peaks were identified. In addition, within this closed set of samples, 15 of 16 (93.8%) blind samples were correctly identified. AFLP data can be used to determine the species of botanical evidence or to associate a sample to a source. This information can be used in forensic investigations to link a piece of evidence with a particular location or suspect.

KEYWORDS: forensic science, forensic botany, amplified fragment length polymorphism, AFLP, *Acer rubrum*

The goal of forensic science is to extract as much information as possible from any and all types of evidence so that one may reconstruct a series of events as accurately as possible. Botanical evidence has the potential to provide valuable information to the forensic scientist by linking a person to a location (1,2).

Plant systematics and plant anatomy attempt to classify or identify species based on morphological characteristics. Species identification by either method is not always possible, since it may be limited by the amount and/or condition of the evidence received, but if distinctive cells are present, determination of a genus or species can be achieved (2). The determination of a plant species can be used to link suspect(s) with a specific location or to a broader geographic region. The specificity of the link is based on the distribution of the species in question, i.e., the less dispersed the species, the more useful the information. In contrast, plant fragments present at a crime scene that are inconsistent with local vegetation may indicate a prior location of either the suspect or victim, which would provide information critical for accurate crime reconstruction (2).

Morphological characteristics, while helpful in classifying or identifying evidence, do not usually provide enough detail to individualize a source of evidence. Individualization of evidence involves determining a unique source for a piece of evidence and the exclusion of all other possible origins. The evolution of forensic science has moved in the direction of individualization techniques wherever possible.

Molecular techniques that utilize DNA provide information that can be used to individualize many forms of biological evidence. The genetic markers used for human identification have been well characterized. The STR loci used in forensics are widely distributed throughout the genome, moderately polymorphic, exhibit low mutation rates, and are in Hardy–Weinberg and linkage equilibrium. While PCR-STR is currently the most widely used method for DNA typing in humans, its use with other species is limited since some knowledge of the DNA sequence is necessary in order to develop the primers for PCR amplification. STRs can be isolated, and their primer sequences determined, but these processes can be lengthy, labor intensive, and inefficient, especially in species with low STR frequencies in the genome (3).

Random amplified polymorphic DNA (RAPD) (4) and amplified fragment length polymorphism (AFLP) (5) are techniques that permit one to genotype an organism when very little information is known about its genome. RAPD was used to link plant material from a source tree to a suspect in a homicide investigation (6). However, the low stringency PCR conditions allows nonspecific binding of the primers and produces nonreproducible bands in the final product (7). Thus, results obtained with RAPD are not as reproducible and the resolution of genetic difference is not as high as the forensic community typically demands (7).

AFLP is a powerful method that combines techniques from classical hybridization-based (8) and PCR-based (9) genotyping strategies. AFLP can be used to genotype DNAs of any origin and complexity. The process involves three steps: restriction digestion of genomic DNA and ligation of oligonucleotide adapters, selective amplification (via two consecutive PCR reactions) of sets of restriction fragments, and electrophoretic analysis of the PCR fragments. Typically 50–100 restriction fragments are amplified and detected, making the AFLP technique a powerful genotyping method. The AFLP technique has several advantages: (1) reproducibility over a wide range of template concentrations due to the high stringency annealing conditions (5); (2) production of a large number of observable molecular markers due to the fact that the entire genome is subject to restriction digestion (10,11); and (3) a high discriminative capacity since multiple combinations of selective nucleotides can be used to observe additional markers (11).

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AFLP is currently being used in breeding studies, germplasm management, variety identification, backcross breeding in plants, animal genetics, as well as fungal and bacterial taxonomic identification and epidemiology (11). Genetic mapping and linkage experiments have been performed using AFLP for a variety of plants (12–16). In the fight against drugs, a national AFLP database is currently being developed and evaluated for *Cannabis sativa* to determine the amount of genetic variation within and between plants seized by authorities (1). In addition forensic scientists are beginning to recognize the potential of AFLP. Coyle et al. (17) are in the process of developing an AFLP method for the individualization of marijuana samples.

The Red Maple, *Acer rubrum*, is a native species in the United States and is distributed throughout much of the US and Canada. Most studies involving *Acer rubrum* have focused on ecological and physiological issues (18–22). *Acer rubrum* has not been studied genetically to any extent. Thus, the wide distribution and lack of sequence data made *Acer rubrum* a good choice for the study described herein. Plant material from the ubiquitous Red Maple adhering to a victim and/or suspect may expedite forensic investigations in both rural and urban areas. In our study AFLP analysis was used to identify the species and to associate a sample with a source within a closed set of trees.

Materials and Methods

Samples

Five leaves from each of 40 different Red Maple trees located in Hamilton Township, NJ, were collected, placed in coded envelopes and transported on ice to the laboratory. Young leaves were collected since they contain more cells per weight, have fewer polysaccharides and polyphenolics, and are easier to handle.

Leaves were collected from five additional species to serve as non-*Acer* control samples. The control samples included Norway Maple (*Acer platanoides*), Japanese Maple (*Acer japonicum*), American Elm (*Ulmus americana*), Sweetgum (*Liquidambar* spp.), and Oak (*Quercus* spp.).

The leaves were washed (5 min, 3×) in sterile 1× phosphate-1 × buffered saline (0.15 *M* NaCl, 0.02 *M* KH_2PO_4, pH 7.2) solution to remove any contaminants from the outer surface of the leaves. Washed leaves were placed in coded bags and stored at −70°C.

At a later date using sterilized mortars, pestles, and spatulas the samples were ground in liquid nitrogen and placed into 1.5 mL sterile Eppendorf tubes. Approximately 100 mg of sample was placed into each tube; three to six tubes were made for each sample tree. The samples were stored at −70°C.

Duplicate samples from each of the Red Maple and non-*Acer* control trees were run through the protocol from DNA extraction to capillary electrophoresis. Results from the *Acer rubrum* samples were used to establish a searchable DNA database. In addition, 16 Red Maple samples were selected at random, recoded and run through the entire protocol as blind samples. *Arabodipsis thaliana* was to be used as the positive control. However, after running multiple samples it was determined that the *Arabodipsis thaliana* DNA provided in the AFLP Core Reagent Kit (Invitrogen, Carlsbad, CO) was degraded. Thus, one of the sample trees (B6), which gave reproducible results, served as the positive control. Sterile distilled water was used as a negative control.

DNA Extraction

DNA was extracted using the DNeasy Plant Miniprep Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with the following modifications: (1) the optional centrifugation in step four was performed for five minutes at 13.2 × 1000 rpm to remove leaf debris which may clog the column; (2) centrifugation in steps 8, 9, 12, and 13 was performed at 8.2 × 1000 rpm; (3) DNA was eluted twice in separate steps using 100 and 50 μL of elution buffer AE (provided with the kit); and (4) eluates were not pooled—the second elution was retained only as a precaution.

Five μL of the first elution was run on a 0.8% agarose gel (MBG, Fisher Scientific, Hampton, NH) at 75 V for 90 min to check the quality of the DNA. The DNA was quantified using a SmartSpec 3000 spectrophotometer (BIO-RAD, Hercules, CA).

Restriction Digestion

Complete digestion of template DNA is crucial for the reproducibility of AFLP profiles. A pilot study was conducted to optimize the conditions for digestion. Single and double digests were performed on both sample DNA and the pUC19 vector (1,000 μg/mL, New England Biolabs, Beverly, MA) with *Mse*I (10,000 U/mL, New England Biolabs) and *Eco*RI (20,000 U/mL, New England Biolabs) to ensure that the enzymes cut and to determine the time required for complete restriction.

AFLP

The AFLP protocol described by Gibco (Invitrogen, Rockville, MD) provided the starting point for the AFLP procedure used herein. The AFLP Core Reagent Kit (Invitrogen) containing 5× Reaction Buffer [50 m*M* Tris-HCl (pH 7.5), 50 m*M* Mg-acetate, 250 m*M* K-acetate], Adapter/Ligation Solution [*Eco*RI/*Mse*I adapters, 0.4 m*M* ATP, 10 m*M* Tris-HCl (pH 7.5), 10 m*M* Mg-acetate, 50 m*M* K-acetate], T4 DNA Ligase [1 U/μL in 10 m*M* Tris-HCl (pH 7.5), 1 m*M* DTT, 50 m*M* KCl, 50% (v/v) glycerol], and TE Buffer [10 m*M* Tris-HCl (pH 8.0), 0.1 m*M* EDTA] used in the restriction and ligation steps. The *Eco*RI and *Mse*I provided in the kit were not used since the results of the pilot study indicated that the concentrations of the enzymes provided were insufficient for complete restriction in a reasonable amount of time.

Restriction digestion was performed in sterile 1.5 mL Eppendorf tubes. The restriction reaction contained 5 μL of the 5× reaction buffer, 18 μL of sample DNA, 1 μL of *Eco*RI (20,000 U/mL, New England Biolabs), and 1 μL of *Mse*I (10,000 μg/mL, New England Biolabs). The concentration of DNA used ranged from 4–56 ng/μL. A full 18 μL of sample DNA was always used in the restriction reaction, regardless of the DNA concentration, because a minimum quantity of DNA was required to obtain a reproducible profile, while excess DNA did not affect the results. The digests were incubated at 37°C for 3 h as determined by the pilot study (not for 2 h as specified in the Gibco protocol). Following restriction, the enzymes were heat inactivated (70°C, 15 min).

Ligation of Adapters

After inactivation of the restriction enzymes, ligation was performed in the same tube. Twenty-four μL of Adapter/Ligation solution and 1 μL of T4 DNA ligase were added. The samples were vortexed, pulse centrifuged, and incubated at 20°C for 2 h. Following ligation, a 5 μL aliquot was diluted 1:5 (not 1:10 as described in the Gibco protocol) with TE buffer. The ligation reactions and dilutions were stored at −20°C.

Preamplification

The preamplification reactions were set up as described by Gibco and contained 5 μL of diluted DNA from the ligation reaction, 40 μL of AFLP pre-amp primer mix (E-A/M-C; *Eco*RI 5′-GACTGCGTACCAATTC**A**-3′/*Mse*I 5′-GATGAGTCCTGAGTAA**C**-3′, Invitrogen), 5 μL of 10× PCR buffer [200 m*M* Tris-HCl, (pH 8.4), 500 m*M* KCl, 15 m*M* $MgCl_2$], and 1 μL of *Taq* DNA polymerase (5 U/μL, Invitrogen). DNA was amplified using the GeneAmp PCR System 2400 (Perkin Elmer, Wellesley, MA) as follows; 20 cycles of 94°C 30 sec, 56°C 60 sec, 72°C 60 sec, final extension 72°C 7 min, 4°C hold. The PCR products were not diluted 1:50 as described in the Gibco protocol.

The primers E-A/M-C consist of sequences that are complementary to the adapters and adjacent restriction sites plus one additional selective nucleotide (**A**, **C**) at the 3′ ends. Thus, only a subset of the *Eco*RI/*Mse*I fragments with sequences complementary to the two selective nucleotides were amplified.

Selective Amplification

Selective amplification reactions were set up according to the manufacturer's instructions with the following modifications: (1) the pre-amplification reaction was diluted 2:5 with TE; (2) 5 μL of the 2:5 dilution was added to 5 μL of mix 1 and 10 μL of mix 2. Mix 1 contained 5 μL of labeled *Eco*RI primer (E-ACT, *Eco*RI 5′-(dyeD4)GACTGCGTACCAATTC**ACT**-3′, Proligo, Boulder, CO) diluted to 27.8 ng/μL and 45 μL (6.7 ng/μL) of *Mse*I primer M-CAT (*Mse*I 5′-GATGAGTCCTGAGTAA**CAT**-3′, Invitrogen). Mix 2 contained 79 μL of distilled water, 20 μL of 10× PCR buffer with Mg, and 1 μL of *Taq* DNA polymerase (5 U/μL, Invitrogen). PCR was run using the following protocol: one cycle of 94°C 30 sec, 65°C 30 sec, 72°C for 60 sec, followed by touchdown PCR for 13 cycles. The annealing temperature was decreased by 0.7°C in each cycle. These cycles were followed by 23 cycles of 94°C 30 sec, 56°C 30 sec, 72°C 60 sec, final extension 72°C 7 min, 4°C hold.

The E-**ACT**/M-**CAT** primer pair had three selective nucleotides at the 3′ ends—two in addition to the one used for the pre-amp primers. The labeled *Eco*RI primer contained WellRED dye D4 (Beckman Coulter, Fullerton, CA), since this fluorophore produces the highest fluorescent yield in the CEQ 8000 system. The choice of the selective nucleotides (nt) is arbitrary. However, since eukaryotic genomes are typically AT rich (5), we chose primers with A/T at four of the six positions. The primer design and amplification strategy ensure that only a subset of the *Eco*RI/*Mse*I fragments amplified in the pre-amp step are preferentially amplified.

Capillary Electrophoresis

Amplified fragments were separated by CE using the CEQ 8000 from Beckman Coulter. The loading buffer was prepared using 350 μL of ultrapure formamide (J. T. Baker, Phillipsburg, NJ) and 4.38 μL of CEQ DNA Size Standard—400 base pair ladder (Beckman Coulter). Forty μL of loading buffer was combined with 1 μL of sample from the selective amplification and overlayed with one drop of mineral oil. The Frag 3 method (capillary temperature 50°C; denaturation 90°C 90 sec; injection 20 kV 30 sec; separation 60 kV 35 min) analysis set was chosen since these parameters are optimal for fragment analysis using the 400 base pair ladder.

Fragment Analysis

To develop AFLP profiles with the CEQ software (version 5.0.360), a study must be created to run the primary analysis. An AFLP study was created using the default conditions listed in Table 1 plus the requirement that fully populated bins and samples without qualifying peaks were not excluded. Fully populated bins were not excluded because species-specific peaks could be found in those bins. Samples without qualifying peaks were not excluded because data needed to be collected for analysis of the negative controls.

The CEQ 8000 software calculates a threshold based on 10% of the second highest peak (the highest peak is due to dye artifacts from unincorporated primers). An AFLP study creates a binary representation of the electropherogram data. Peaks are analyzed using a Dominant Scoring software tool and scored as either present ''1'' (above threshold) or absent ''0'' (below threshold). One large AFLP study was created for both replicates of each of the 40 Red Maple samples and the five non-*Acer* controls, the positive and negative controls, and the 16 blind samples. This study was used for comparison of the samples, identification of species-specific peaks, and identification of the blind samples.

A software program called Twin Peaks (proprietary) was developed to establish a searchable database of AFLP profiles. The program uses a series of pairwise comparisons between a query AFLP profile and the profiles in the database to generate a list of matches (hits) and give a measure of the strength of the match (percentage of identical fragments). Using this list the electropherogram from the query was visually compared to the most closely matching database electropherograms in order to determine a match.

Results and Discussion

AFLP Profiles

The DNeasy Plant Miniprep procedure yielded purified, high molecular weight DNA suitable for AFLP (data not shown). In our hands, AFLP profiles were obtained with a minimum DNA input of 72 ng (28 ng less than that recommended by Invitrogen). This corresponds to approximately 1/2–3/4 of a leaf. Further studies are

TABLE 1—*AFLP study analysis parameters.*

Default Fragment Analysis Parameters
General
Peak criteria
Slope threshold—10
Relative peak height threshold—10%
Size estimation and allele ID confidence
Confidence level—95%
Analysis methods
Size standard—400
Model—cubic
Migration variable—migration time
Quantitation
ID standard—time
Calculate using—area
Advanced
Mobility
Dye mobility calibration—no correction
Standard mobility reference—none
Dye spectra—use calculated dye spectra

ID, identity; AFLP, amplified fragment length polymorphism.

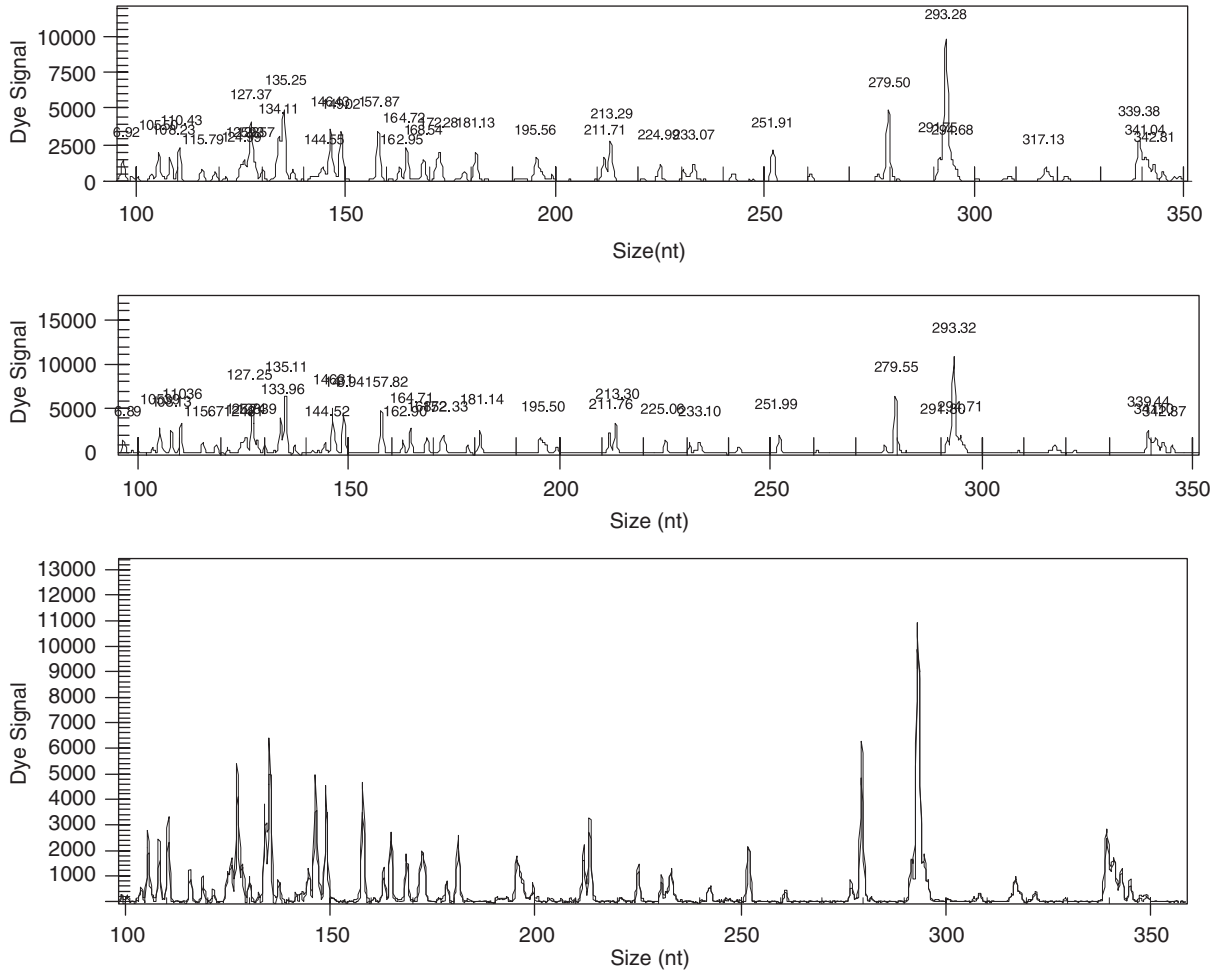

FIG. 1—*Red Maple profiles obtained from duplicate analysis of leaves from the same tree (D2, top, and middle) and overlay of top and middle electropherograms (bottom).*

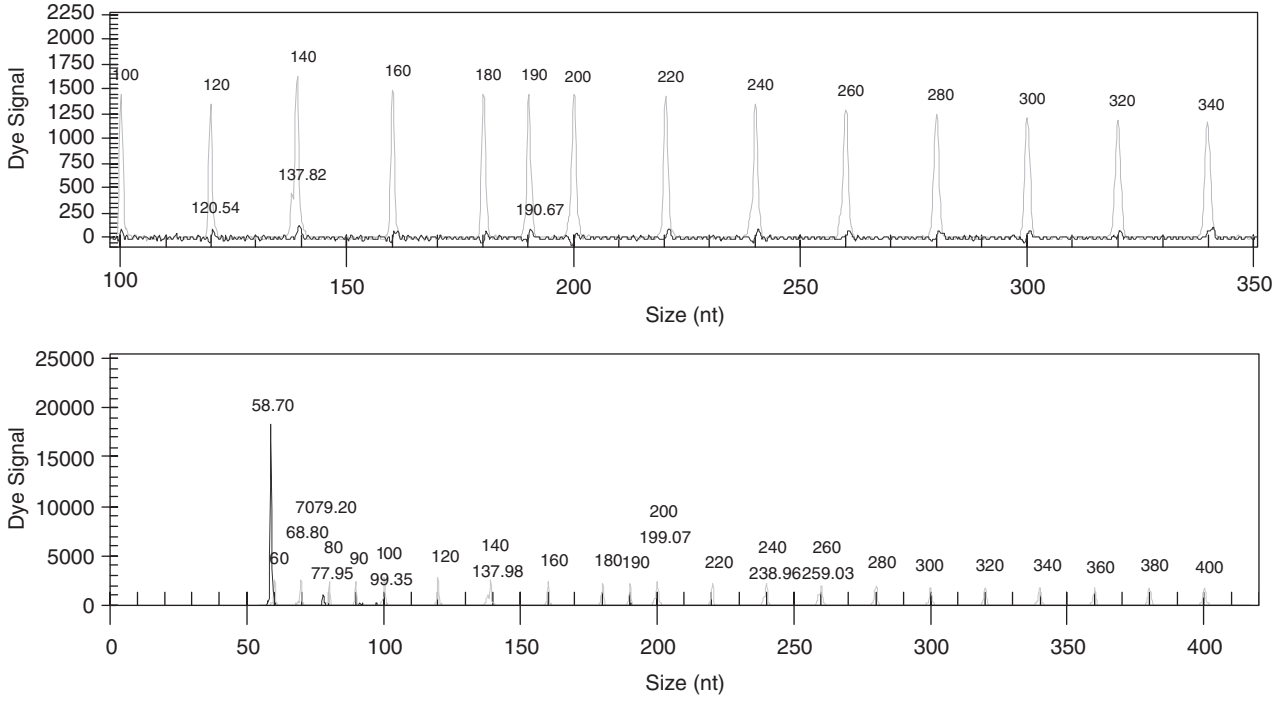

FIG. 2—*Negative control electropherograms. DNA ladder in light gray, control fragment peaks in black.*

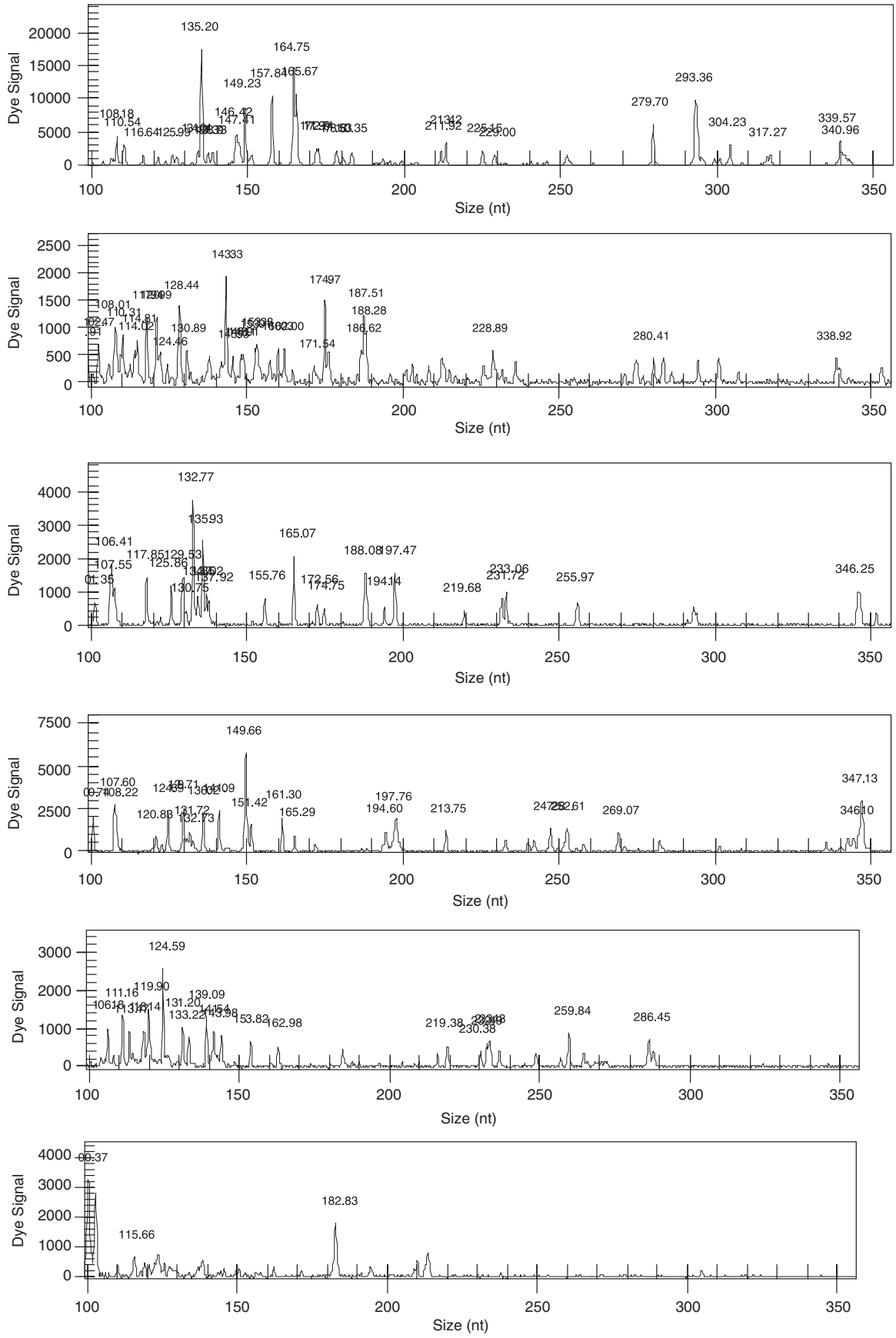

FIG. 3—*Red Maple and controls. From top: Red Maple (B6), Oak, Japanese Maple, Norway Maple, Sweetgum, and American Elm.*

needed to optimize the extraction and AFLP protocols. Reducing the extraction elution volume and increasing the concentration of the primers, *Taq*, and the number of amplification cycles may reduce the amount of starting material required for successful typing.

AFLP fragments ranging in size from 100–350 nt were used in data analysis since artifacts were frequently present in the samples at the high and low ends of the standard range (50–400 nt). Data, in the form of electropherograms, were obtained from duplicate runs of each of the 40 Red Maple sample trees and the five non-*Acer* control species, as well from the single runs of the 16 blind samples.

Duplicate runs produced concordant AFLP profiles for the Red Maple, non-*Acer* species controls, and the positive control. The differences encountered in the duplicate samples were mainly due to slight shifts in mobility, which sometimes manifests in the fragments being placed in different bins in the AFLP data. These shifts are variable, and may be due to differences in intercapillary conditions within or between runs. This lowers the percent matching bins for duplicate samples. These fragment shifts are not eliminated by inclusion of an internal size standard. We have discussed this issue with Beckman Coulter. The vendor is modifying the software (analysis parameters) to eliminate the problem. Differences in AFLP data were also due to slight differences in relative peak heights. Changes in the relative intensities of the second highest peak or peaks near the 10% threshold may result in inconsistent peak determination by the software. Altering the threshold to 5% and 20% did not eliminate this situation. Direct observation of the electropherogram data will, however, resolve these issues.

Figure 1 shows the electropherograms from duplicate samples of Red Maple tree D2. Note in the overlay electropherogram that the overall pattern is the same, as are peak groupings and peak height ratios. Thus, the 40 profiles from the Red Maple samples were entered into the database used by Twin Peaks to analyze the non-*Acer* species control and blind samples.

The negative controls (Fig. 2) sometimes contained fragments, usually less than 100 nt, that appeared in the negative control (Fig. 2, bottom electropherogram), but not in the positive control or in the samples. This situation is not unique. Van der Wurff et al. (23) previously reported the presence of artifactual bands in the AFLP negative control reactions that did not appear in the positive control reactions. We hypothesize that the primers may be forming less favorable secondary structures when no other DNA is present. Analysis of the primer structure using Oligo Analyzer (24) indicated that primer dimer formation and self-annealing of the *Eco*RI primers is possible. This may account for the peaks below 100 nt in the negative controls.

Species-Specific Peaks

A series of species-specific peaks were identified for the Red Maple. Peaks at 108, 111, 135, 165/166, 212/213, 280, 293, and 340 nt are present in all Red Maple samples (Fig. 3). In this instance tree B6 (the positive control) was the source tree for the Red Maple profile. Comparison of the profiles for Red Maple trees D2 (Fig. 1) and B6 (Fig. 3) show the presence of the species-specific peaks. The discrepancies in the two Red Maple profiles reflect individual differences. Peaks at 105, 127.25, 181, 195, 233, and 251 are present in D2 but absent in B6.

The fragment sizes that comprise the Red Maple species peaks may be present in the other species tested, but none contain all of the peaks. For example, the Norway Maple has peaks at 135, 165, and 340 nt, but not at 108, 111, 166, 212/213, 280, and 293 nt.

Identification

Of the 16 blind samples run, 15 (93.75%) were correctly matched with samples from the same tree. Figure 4 shows the electropherograms of blind sample X4 and the correctly identified source tree D7. In contrast, results for blind sample X3 were inconclusive. Based on a visual analysis of the electropherogram of X3 shown in Fig. 5, the source tree was narrowed down to two possibilities, trees C10 and G2. After checking the key, X3 was identified as having come from tree C10. This result was confirmed by a visual comparison of the X3 and C10 peak profile patterns. According to the Twin Peaks data, X3 had 95.17% matching fragment sizes with tree G2 and 94.48% with tree C10. Note that there are fewer peaks in the electropherogram of X3 than in the electropherogram of tree C10. This affects the Twin Peaks calculation of the percent matching bins and results in matches of less than 100% for samples from the same tree. Ad-

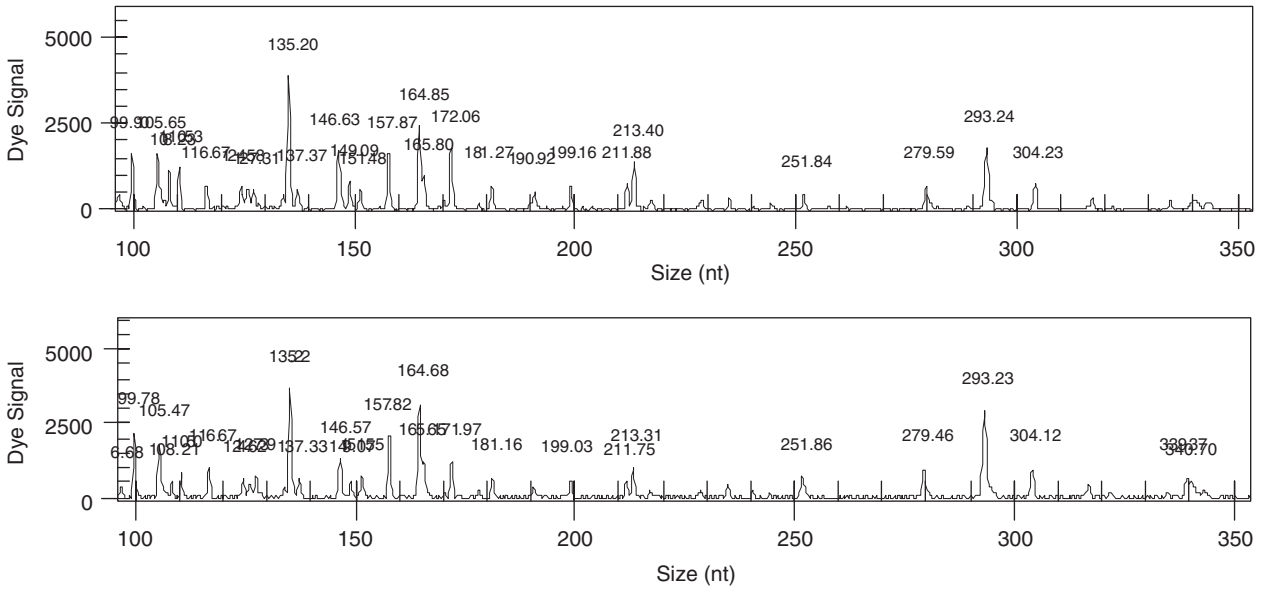

FIG. 4—*Blind sample profile top (X4), database profile from same tree below (D7).*

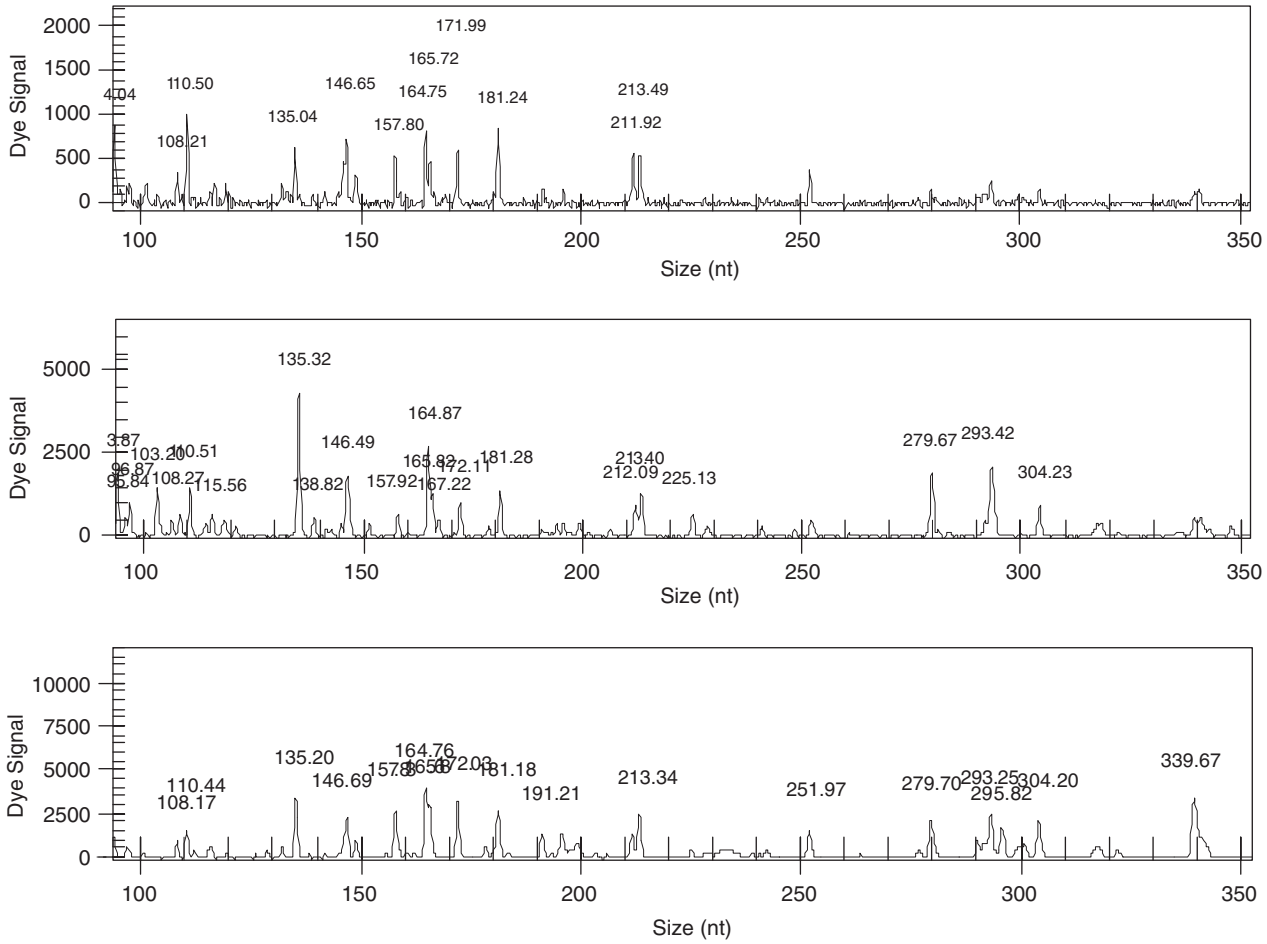

FIG. 5—*Inconclusive blind sample. Top: X3, Center: Tree G2, Bottom: Tree C10.*

justing the injection parameters (time and/or voltage) would mostly likely resolve issues of nonconcordance.

The inability to conclusively identify blind sample X3 may have been due to insufficient input DNA. For all 40 samples and their duplicates as well as the control samples and their duplicates, sufficient DNA was obtained such that at least an extremely faint band was visible on a yield agarose gel. This amounts to at least 4 ng/μL of DNA in the DNeasy eluate or 72 ng/μL in the AFLP reaction. However, upon visualizing the agarose gels run for blind samples X1–X16 it was found that no band was visible for samples X3, X7, X8, X9, and X10. Thus, for these samples no dilutions were performed after the ligation or after pre-amplification. Electropherograms of sufficient quality were produced for each of the samples. Of these samples, all but X3 could be identified despite the extremely small amount of DNA used in the AFLP reaction, which may have resulted in allelic dropout. Lowering the elution volume in the DNeasy DNA isolation protocol would most likely provide sufficient DNA concentration. In addition, as discussed above, the profiles for Red Maple trees D2 (Fig. 1) and B6 (Fig. 3) show the presence of the plant specific peaks. Thus, within this closed set, a particular AFLP profile could be associated with a specific tree.

Summary

The data indicate that identification is possible within a closed set of samples. The database for this study contained 40 trees, and as the number of trees increases, identification will become more difficult due to the increased probability that two trees will exhibit similar profiles for a particular primer pair combination. However, additional markers may be observed by using different primer pair combinations in tandem analyses. Changing the selective nucleotides of the unlabeled primer used in the selective amplification would provide additional information to discriminate among trees, based upon a different set of AFLP fragments, without the need for an additional sample, since only a small aliquot of ligation and pre-amplification mixture is needed for analysis.

For forensic samples that do not contain sufficient information to morphologically identify species, a small AFLP database of morphologically similar species from a particular location may provide the information necessary to determine species using species-specific peaks. With that information full AFLP analysis can be performed, limited to individuals of that species. For the closed set of plants from the limited geographic range studied herein, the species-specific peaks identified are present in all Red Maple samples and not in any of the other species studied. However, the variation of Red Maple species peaks over large geographic ranges and between additional species is not known since the samples in this study were from a localized region in central New Jersey and limited to five non-*Acer* species.

The results indicate that the AFLP technique is a viable and valuable technique for identification and association of plant samples to their source plants. Species may be determined and/or association with a particular source tree near a crime scene may be possible. The AFLP protocol designed by Vos et al. (5) provides a useful starting point for the procedure, although some optimiza-

tion may be necessary. The technique provides comparable results for multiple samples in a relatively short period of time. In addition, less than 100 ng of DNA is required for reproducible results. Kits are available for successful DNA isolation and for the AFLP procedure. PCR primers for AFLP are available commercially. The AFLP technique has several advantages for forensics—the method is rapid, robust, and many steps can be automated.

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