Genetic Fingerprinting of Grape Plant (*Vitis vinifera*) Using Random Amplified Polymorphic DNA (RAPD) Analysis and Dynamic Size-Sieving Capillary Electrophoresis

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Dynamic size-sieving capillary electrophoresis with laser-induced fluorescence detection (DSCE−LIF) was combined with random amplified polymorphic DNA (RAPD) analysis to demonstrate the feasibility of the genetic analysis of grape plant varieties and clones within a variety. Parameters of the genomic DNA extraction process, as well as those of the RAPD analysis, were optimized specifically for this application. Polymorphic DNA fragments were generated for four different grape plant varieties including Cabernet Franc, Cabernet Sauvignon, Merlot, and Chardonnay. Relative to slab gel electrophoresis (SGE) with ethidium bromide staining, DSCE−LIF provided superior separation efficiency and detection limits in the analysis of DNA polymorphic bands. Optimal DSCE−LIF analyses were achieved using a 10-fold RAPD sample dilution, hydrodynamic sample injection, and 100 ng/mL of YO-PRO-1 DNA intercalator in the dynamic size-sieving buffer solution. In addition, the reproducibility of both the DSCE−LIF and RAPD analyses were demonstrated.

Keywords: *Dynamic size-sieving capillary electrophoresis; polymorphic DNA fragments; random amplified polymorphic DNA analysis; grape plant; Vitis vinifera; genetic analysis*

INTRODUCTION

The specific clone of the grape plant employed in the wine-making process profoundly influences the aroma and flavor of the wine. Currently, there exist as many as 15 000 cultivars of grape plant worldwide, as a result of mutations and spontaneous crossings (Regner et al., 1998). Most likely, these cultivars originated from the domestication of wild vines. In the nineteenth century, the exchange of many varieties of grape plants occurred between the United States and Europe, changing names and blurring the genealogy lines of the grape plants (Lipkin, 1993). From an agricultural standpoint, the grape plant has an increasingly significant economic impact. As a result, viticulturists and vintners remain interested in the identification and characterization of specific grape plant varieties or clones within a variety. Not surprisingly, the latter represents a challenge within the wine industry.

Traditionally, the identification of grape plant varieties and clones for plant breeding, wine making, or the estimation of genetic relationships and diversity, has involved speculation, reliance on historical information, or the observation of morphological characteristics of the plant (ampelography). Such subjective means often lead to misidentification. More specifically, morphological features of the plant such as the leaf shape and size, berries, and the density of hair on shoot tips, can be both environmentally and developmentally dependent (Wolf et al., 1998). One solution to the characterization of grape plant has been the utilization of genetic analyses; significantly greater variability exists on the DNA level relative to that on the morphological level. Unfortunately, a relatively low number of genes has been identified for the grape plant due to the presence of endogenous chemicals that can interfere with the required experimental methodologies (Jobes et al., 1995).

Genetic analyses have progressed rapidly since the discovery of polymorphic regions, or loci with two or more alleles, within genomic DNA (Wyman and White, 1980). Variation in location, copy number, length, and base pair sequence of these highly repetitive DNA regions provide a rich source of markers for unique identification. Traditionally, restriction fragment length polymorphisms (RFLP) analysis has been employed (Bowers and Merdedith, 1996; Sivolap et al., 1993; Bowers et al., 1993). RFLP analysis is a robust and reliable genetic analysis technique. However, RFLP analysis is time-consuming, requires a large amount of clean DNA and considerable laboratory equipment, and often utilizes radioactivity for the detection of DNA fragments. In general, fewer alleles are detected using RFLP analysis than in using other methods. Amplified fragment length polymorphism (AFLP) analysis, a polymerase chain reaction (PCR)-based genetic typing technique, has been utilized as well (Vos and Hogers, 1995; Hill et al., 1996). Routinely, a relatively high number of polymorphic DNA markers is generated for each genome. However, similar to RFLP analysis, the technique is time-consuming and requires major laboratory facilities. Single-locus simple sequence repeat (SSR) markers have been developed for a number of species, including grapevine (Bowers et al., 1996; Thomas and Scott, 1993). The main advantage of the technique

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involves the ability to exchange and share quantitative genetic data, expressed as allele length. However, this process can be extremely time-consuming, beyond the scope of most laboratories, and impractical when applied to a large number of loci.

Alternatively, random amplified polymorphic DNA (RAPD) analysis has been applied to several aspects of the winemaking process (Chen et al., 1995; Couto et al., 1995). RAPD is a simple, yet powerful, PCR-based method that allows the experimenter to amplify randomly a previously unspecified segment of genomic DNA, creating unique fingerprints of polymorphic, double-stranded DNA markers (Williams et al., 1990; Welsh and McClelland, 1990). Variations in fingerprints produced by RAPD analysis stem from changes in sequences in the priming sites of different genomes; specific polymorphisms will amplify in one individual but not another (Benter et al., 1995**)**. A primer of an *arbitrary*, nonpalindromic sequence can be employed and shared universally. Fragment amplification occurs when the length spanned on opposite sides of the annealed primer is 5 kilobase pairs or less (Benter et al., 1995). The appearance of DNA polymorphic markers occurs with primers greater than 5 nucleotides in length (Caetano-Anolles et al., 1991). Primers range from 8 to12 nucleotides in length, with the most typical length being 10 nucleotides (Williams et al., 1990). Longer primers generate a greater number of polymorphic DNA fragments over a wider size range. Beyond 20 nucleotides in length, no additional polymorphisms are detected; presumably due to self-annealing (Ye et al., 1996). Furthermore, the pairwise combination of primers has proven beneficial (Welsh and McClelland, 1990; Micheli et al., 1993; Welsh and McClelland, 1991). RAPD analyses using two different primers in combination generate unique fragments relative to those created using each primer individually (Welsh and McClelland, 1990, 1991).

Unfortunately, RAPD analyses can be sensitive to reaction conditions such as the quality of the genomic DNA, the quality and quantity of the DNA polymerase, and the presence of RNA. Because of low-stringency annealing conditions (30−35 °C), some mismatches may be permitted. The latter accounts for the greater number of RAPD products than expected based on the size of the plant genome. The low-stringency PCR conditions have also been linked to reports of variable results between laboratories (Buscher et al., 1993). To improve reproducibility, the RAPD analysis has been modified according to higher sequence specificity and higher-stringency PCR conditions. Sequence-specific primers have been derived from cloned RAPD bands (Xu et al., 1995**)**, and single-tagged site (STS) primers have been developed (Botta et al., 1995).

The resolution and efficiency of the separation technique used to analyze the DNA molecular markers are expected to have a profound influence on the genetic results obtained. Traditionally, RAPD-generated fingerprints have been visualized using SGE with ethidium bromide staining. SGE is restrictive in this application, specifically, in that only the major polymorphic DNA fragments present in high concentrations are detected. A more sensitive detection technique would ensure the detection of low-concentration products and thereby ensure the integrity of the genomic fingerprint. Additionally, considerable genetic information can be lost if separation techniques of insufficient separation resolution and efficiency are employed in the analysis of complex RAPD analysis products. Large variations in band intensities in SGE have been reported, limiting reproducibility to a qualitative level in this mode (Chen et al., 1995). In addition, it is unclear as to whether ambiguous polymorphisms reported previously in RAPD analyses are a prohibitive feature of SGE, or of the RAPD analysis itself (Williams et al., 1990).

Owing to the potential complexity of genetic fingerprint patterns, as well as the genetic similarities of grape plant varieties and clones, we have investigated the use of DSCE−LIF in the analysis of RAPD-generated DNA fragments. To our knowledge, this specific application of DSCE has not been reported previously in the literature. DSCE was applied previously to the analysis of human RAPD samples in a brief report (Valentini et al., 1996). In capillary electrophoresis (CE), the efficient dissipation of joule heat allows the use of higher electrical field strengths (up to 900 V/cm) for more rapid and efficient separations (Ewing et al., 1989); one million theoretical plates per meter capillary are achieved routinely in this mode of electrophoresis. The superior separation efficiency of CE performed in microbore (50 to 100 μm i.d.) tubing is expected to accommodate the analysis of numerous polymorphic markers and demonstrate the potential utility of longer primers or multiple primers in combination. The displacement of low-viscosity, dynamic size-sieving polymer solutions from the separation capillary between analyses nearly eliminates run-to-run sample contamination (Grossman, 1994). Laser-induced fluorescence (LIF) provides a highly sensitive detection scheme (Nguyen et al., 1987). The more advanced RAPD analyses involving the use of sequence-specific and STS primers were beyond the scope of the current feasibility investigation.

EXPERIMENTAL PROCEDURES

Grape Plant Samples and DNA Standards. Grape plant leaves were obtained from the Williamsburg Winery (Williamsburg, VA), Sharp Rock Vineyards (Sperryville, VA), and Glebe Vineyards (Eastman, VA). Leaf collection occurred at bud break. Leaves were placed immediately in TRIS−EDTA buffer solution (89 mM Trizma base and 2 mM EDTA; Sigma Chemical Co., St. Louis, MO). Upon receipt, all samples were stored at −80 °C. The 1 kilobase pair PLUS DNA ladder standard was obtained from Life Technologies (Gaithersburg, MD) and diluted 1:10 with HPLC-grade water prior to injection. For the accurate size-calibration of polymorphic DNA markers, two linear double-stranded DNA fragments were added to the 1 kilobase pair PLUS DNA standard as internal standards. These DNA markers, obtained from Bioventures, Inc. (Murfreesboro, TN), were 150 and 1500 basepairs in length and were added to the RAPD samples just prior to sample injection. The final concentration of each internal standard marker in the 1 kilobase pair PLUS DNA standard and each RAPD sample was 3.3 and 0.89 ng/μL, respectively.

DNA Extraction Protocol. The genomic DNA extraction protocol employed was adapted from that described previously (Lodhi et al., 1994). The procedure was designed specifically for grape plant and is compatible with the RAPD analysis. Approximately 0.5 g of leaf tissue was ground with a mortar and pestle in the presence of liquid nitrogen. Stem parts were removed carefully from the leaf samples prior to grinding. The extraction buffer containing 100 mM TRIS−HCl, 20 mM EDTA, pH 8.0, 1.4 M NaCl (Fisher Scientific, Fair Lawn, NJ), 2% CTAB, and 0.2% 2-mercaptoethanol was added and mixed in the mortar. The 2-mercaptoethanol was added just prior to performing the extraction. The slurry was poured into microcentrifuge tubes, and poly(vinylpyrrolidone) (PVPP) (Aldrich Chemical Corporation, Milwaukee, WI) was added to a final

concentration of 100 mg/g of leaf tissue. The mixture was then incubated at 60 °C for 25 min, followed by cooling to room temperature. Chloroform/octanol (24:1, v/v) (Fisher Scientific) was added and mixed gently to form an emulsion, and the sample was centrifuged at 6000 rpm for 15 min. The aqueous phase was transferred to a new tube, 0.5 volume of 5 M NaCl was added, and the solution was mixed. Finally, two volumes of cold, 95% ethanol (Quantum Chemical Corporation, Tuscola, IL) were added and the sample was refrigerated at 4−6 °C for a minimum of 5 min. The precipitated DNA was centrifuged at 3000 rpm for 3 min and then at 5000 rpm for an additional 5 min. The resulting pellet was washed with cold, 76% ethanol. The DNA pellet was resuspended in TRIS−EDTA (TE) extraction buffer. The TE extraction buffer is composed of 10 mM Trizma base, 10 mM HCl, and 1 mM EDTA at pH 8.0. One microliter of a 10 mg/mL solution of RNAse A per 100 μL of solution was added, and the sample was incubated at 37 °C for 15 min. Both the concentration and purity of the extracted DNA were monitored using UV absorbance spectrophotometry. Extracted DNA samples with absorbance ratios ($A_{260\ nm}/A_{280\ nm}$) less than 1.7 were not used in the RAPD analyses. The resulting DNA was stored as recommended at −80 °C and −20 °C for long-term and short-term storage, respectively.

Polymerase Chain Reaction Methodology. All RAPD analyses methods were adapted from a previously reported protocol, employing an arbitrary primer sequence known to produce polymorphic bands for the grape plant genome (Ye et al., 1996). Each reaction tube contained 200 nmol of primer (OPA-03, 5′-AGTCAGCCAC-3′) (Operon Technologies, Alameda, CA), 250 ng of genomic DNA, 0.5 U of Native Taq polymerase (Perkin-Elmer, Norwalk, CT), 2.5 μL of the corresponding 10X enzyme buffer (10 mM Tris−HCl, 50 mM KCl, 2 mM $MgCl_2$) (Perkin-Elmer), 0.12 μL of a deoxynucleotide mixture (dATP, dCTP, dGTP, dTTP) (Promega, Madison, WI), (120 μM each dNTP (Promega, Norwalk, CT)), and HPLC-grade water to achieve a total volume of 25 μL. Amplifications were performed in a Gene Amp 2400 thermal cycler (Perkin-Elmer) with an initial dwell at 94 °C for 5 min. Then, the samples were cycled 35 times through the following reaction conditions: denaturation at 94 °C for 1 min, hybridization at 35 °C for 1 min, extension at 72 °C for 1 min 45 s, and a final extension at 72 °C for 7 min. Each sample was diluted 1:10 prior to injection.

Slab Gel Electrophoresis Methodology. RAPD samples (undiluted) were electrophoresed at 100 V using a submarine gel system Model EC370 (Fisher Scientific). The separation gel medium was 0.5% Trevigel-500 (Trevigen, Inc., Gaithersburg, MD) dissolved in TRIS−acetate−EDTA (TAE) buffer, pH 8.5. TAE buffer is composed of 89 mM Trizma base, 89 mM acetic acid, and 2 mM EDTA (Fisher Scientific). The gel and the run buffer each contained 10 μg/mL of ethidium bromide intercalator (Sigma). Gel loading dye (2 μL,; Sigma) was added to 10 μL of each of the RAPD reaction mixtures. A 1 kilobase pair standard ladder (Life Technologies) was also run on each gel; 1 μL of ladder sample was diluted in 9 μL of HPLC grade water, and 2 μL of loading dye was added prior to loading. Samples and standards were electrophoresed for approximately 45 min. Electrophoretic bands were visualized and photographed on a UV-transilluminator (Fisher Scientific).

DSCE Methodology. For all DSCE separations, an Advanced Technologies, Inc. (ATI) Unicam Crystal CE System (Boston, MA) was employed. The system temperature was maintained at 30 °C. For LIF detection, a Groton Technologies, Inc. (GTI) Spectrovision FD-300 Dual Monochromator fluorescence detector (Concord, MA) was mounted on an optical bench and retrofitted with a single line (488 nm), air-cooled argon ion laser (Uniphase, Ltd., San Jose, CA) as the light source. The laser light was directed by a mirror through a lens (Melles Griot, Irvine, CA), passed through a neutral density filter (o.d., 0.38), and focused on the detection window of the capillary. Emitted light passed through a 520 nm cutoff filter. The laser beam was aligned using standard solutions of fluorescein to give maximum detection sensitivity. The laser was operated at approximately 4 mW. The response time of the detector was 1.0 s. Data points were collected every 100 milliseconds and recorded using Axxiom Chromatography Model 737 data acquisition software (Moorpark, CA).

The fused-silica capillaries (Polymicro Technologies, Inc., Phoenix, AZ) were uncoated internally and had an internal diameter of 50 μm, a total length of 60 cm, and an effective length of 41 cm. The detection window was created by removing a 1-cm section of the polyimide coating using a butane lighter and carefully rinsing the capillary with methanol. All capillaries were conditioned before use with 0.1 M HCl for 4 h at 2000 mbar, followed by a 1-h rinse with HPLC-grade water at 2000 mbar. Capillary rinsing between electrophoretic runs was alternated between a 10-min and a 20 mi-rinse of 1.0 M HCl at 2000 mbar, each followed by a 5-min rinse with HPLC-grade water at 2000 mbar. This rinsing procedure was adapted from Yeung and co-workers and nearly eliminates electroosmotic flow in the fused-silica capillary (Fung and Yeung, 1995).

DSCE analyses were performed using 0.3% w/w solutions of hydroxyethyl cellulose (HEC) with a molecular weight range of 90 000−105 000 g/mol (PolySciences, Inc., Warrington, PA). This polymer was used as received. The polymer was dissolved in TRIS−HCl−EDTA (THE) buffer, pH 7.3. THE buffer is composed of 89 mM Trizma base, 89 mM HCl, and 2 mM EDTA (Fisher Scientific). The buffer solutions were filtered through 0.22μm cellulosic filters (Micron Separations, Inc., Westboro, MA). Polymer solutions were heated in a standard microwave oven and shaken mechanically until the polymer was fully dissolved; water loss due to heating was replenished gravimetrically with HPLC-grade water. The solutions were filtered through a 5-μm cellulosic filter (Micron Separations). The intercalator used for fluorescence detection was YO-PRO-1 (Molecular Probes; Eugene, OR). When not in use, solutions were stored in the refrigerator and covered in aluminum foil to prevent photobleaching.

Polymer solutions were loaded into the capillary for 4 min at 2000 mbar and electrophoresed at −367 V/cm (−22 kV) for 1 min prior to sample injection. Polymer solutions were sonicated before introduction into the capillary to remove air bubbles from the solution; air bubbles in contact with the electrode can eliminate the current and result in poor separations. Samples were injected as indicated. Prior to injection, the glass sample vial inserts were vortex mixed to ensure uniform sample concentration. The applied run voltage was −15 kV for a resulting field strength of −250 V/cm.

RESULTS AND DISCUSSION

Optimization of RAPD Parameters. The majority of RAPD analysis parameters were chosen on the basis of a previously reported protocol known to produce amplification products for the grape plant genome. (Ye et al., 1996). The optimal concentration of grape genomic DNA for the reaction mixtures was determined to be 250 ng. The use of 250 ng produced intense, visually discernible bands in SGE. Higher concentrations of genomic DNA appeared to overload the gel and promote band smearing. Contrastingly, the use of lower quantities of genomic DNA, such as 50 ng, generated faint bands that could be visualized by UV transillumination, but not by photography. Thus, all subsequent RAPD analyses were performed using 250 ng of grape plant genomic DNA.

The effect of the addition of gelatin in the form of 0.1% Bovine Serum Albumin (BSA) to the RAPD reaction mixture was investigated. Strommel et al. (1997) reported an improved reaction yield overall with the incorporation of BSA relative to gelatin-free RAPD reactions. These researchers suggest that gelatin-containing BSA promotes the stabilization of DNA/DNA hybrids. However, inconsistent effects of gelatin and BSA on RAPD amplification have been reported for several plant species. When BSA was used in our RAPD

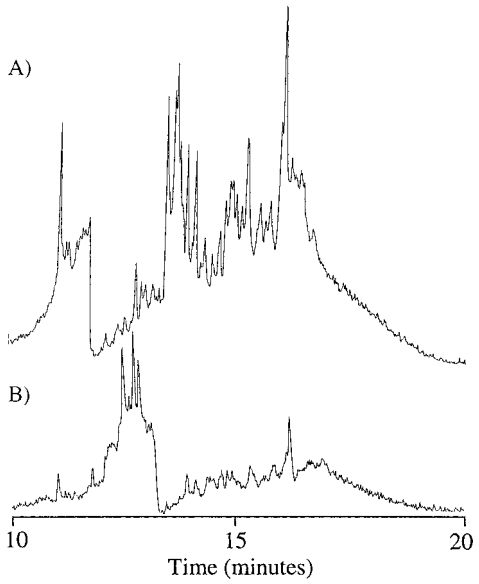

Figure 1. Reproducibility of DSCE−LIF analysis of diluted RAPD samples using electrokinetic injection. Duplicate DSCE separations of a 100:1 dilution of a Williamsburg Chardonnay RAPD sample. Sample was injected at −5 kV for 0.50 min.

reaction mixtures for grape plant amplification, tubes containing BSA displayed cloudiness after the RAPD analysis was performed. The presence of BSA did not improve significantly the banding intensity or fragment resolution. Therefore, BSA was eliminated from the reaction mixtures of subsequent RAPD analyses.

Optimization of DSCE−LIF Parameters. To achieve reproducible separations of RAPD-generated DNA fragments in DSCE, several experimental parameters were optimized. Initially, electrokinetic injection was investigated, because of the ability of this mode of injection to sample environments of low solute concentrations. However, the high ionic strength and extensive amounts of salt present in RAPD-generated samples adversely affected the reproducibility of DNA fragment injections. As noted by McCord et al. (1993), the salt ions (e.g. $MgCl_2$, KCl) prevalent in RAPD reactions harbor a higher mass-to-charge ratio than macroanionic DNA molecules. The high charge-to-mass ratio of small salt ions results in greater electrophoretic mobilities, and hence the preferential injection of such ions into the capillary. Over time, this phenomenon can cause sample depletion, and can decrease the reproducibility and sensitivity of the separation. To combat the high salt concentrations in the RAPD products, the simple procedure of sample dilution was employed. The sensitivity of the LIF detection system allowed for dilution of up to 100-fold while still showing peaks of adequate intensities. Unfortunately, the sampling bias owing to excess salts did not allow for the generation of reproducible fingerprints. As seen in Figure 1, duplicate DSCE runs of the same Chardonnay grape plant RAPD sample with the same experimental parameters produced two undeniably different electropherograms. It should be noted that the variability observed in injection quantities was random and did not appear to be a result of sample depletion in this case. Next, an attempt was made to remove excess salt using Microcon filters. However, as seen in Figure 2, the irreproducibility of different DSCE runs of the same RAPD sample was equally drastic. Additionally, the filtration of the RAPD samples was shown to reduce significantly the intensity

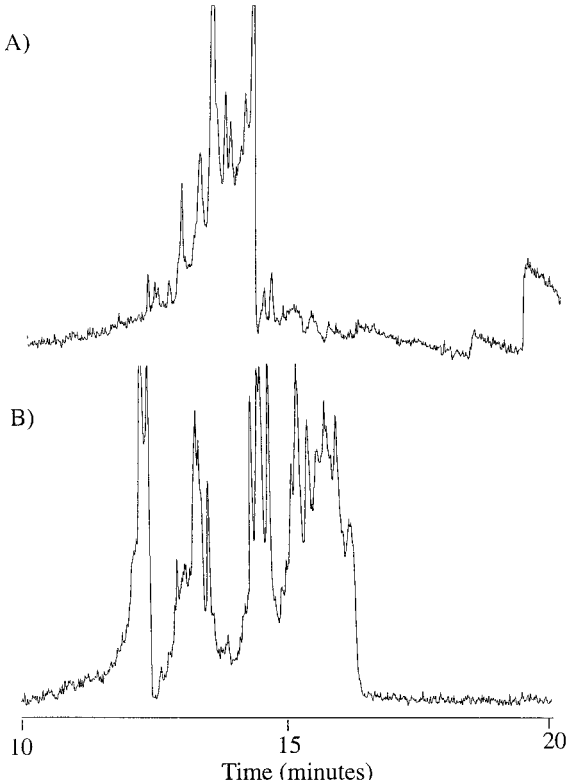

Figure 2. Reproducibility of DSCE−LIF analysis of Microcon-filtered RAPD samples using electrokinetic injection. Duplicate DSCE separations of a Williamsburg Chardonnay RAPD sample. Sample was injected at −5 kV for 0.50 min.

of various polymorphic bands. Therefore, the use of electrokinetic injection was abandoned and hydrodynamic injection parameters were investigated.

In conjunction with hydrodynamic injection, sample dilution was employed. Using this mode of injection, a 10-fold sample dilution was sufficient to yield reproducible fingerprints of the same sample (Figure 3). Additionally, a higher concentration of DNA intercalator in the separation buffer, 100 ng/mL of YO-PRO-1, was found to achieve more defined and intense peaks in the separation of RAPD generated fragments of the same sample (Figure 4). McCord et al. (1993) first demonstrated the advantages of using YO-PRO-1 intercalator for the dramatically enhanced detection of DNA in conjunction with LIF detection. The YO-PRO-1 intercalator is compatible with the 488 nm line of an argon ion laser, demonstrates high photostability, and has a high affinity for DNA. Negligible fluorescence is observed for YO-PRO-1-containing buffers unless the intercalator is structurally planarized upon incorporation into the double-stranded DNA helix. Thus, the use of hydrodynamic injection, sample dilution, and 100 ng/mL of YO-PRO-1 were determined to be requirements for achieving reproducible DSCE−LIF analyses of the RAPD generated DNA fragments.

Slab Gel Electrophoresis of RAPD Samples. Traditionally, the fingerprinting of genomes using RAPD markers is performed using SGE. This mode of electrophoresis is plagued by both limited resolution of DNA fragments and limited detection sensitivity. Additionally, this electrophoretic separation technique has been cited as one of the contributing factors in the relatively low reproducibility reported for RAPD analyses (Vidal et al., 1999). Figure 5 demonstrates the ability of SGE to separate RAPD generated fragments relative to the

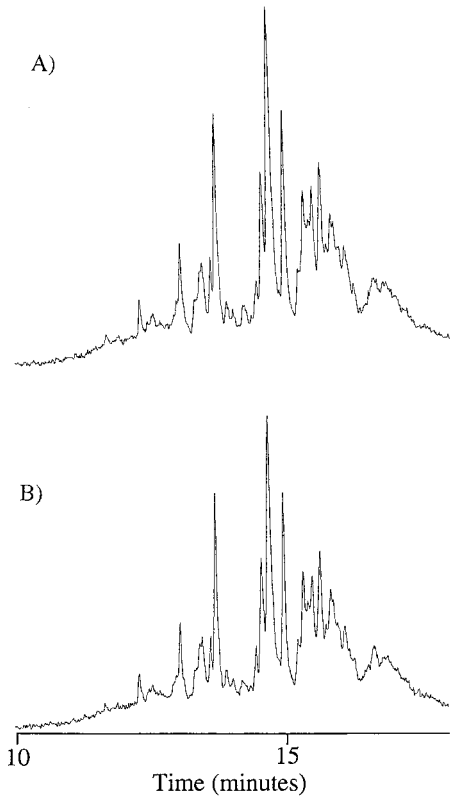

Figure 3. Reproducibility of DSCE−LIF analysis of diluted RAPD samples using hydrodynamic (pressure) injection. Duplicate DSCE separations of a 100:1 dilution of a Williamsburg Chardonnay RAPD sample. Sample was injected at 30 mbar for 0.50 min.

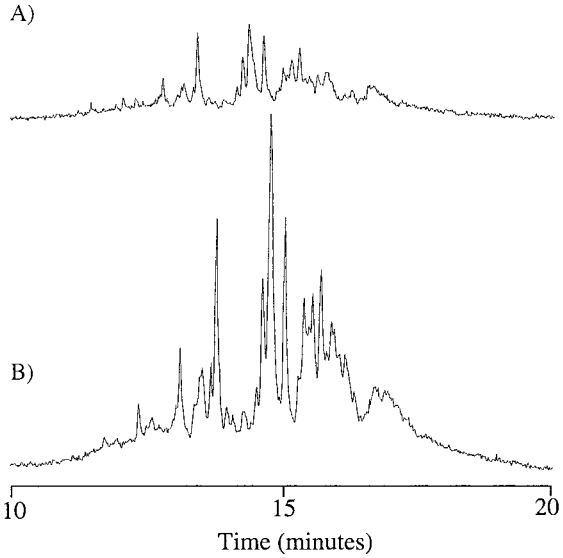

Figure 4. Optimization of concentration of DNA intercalator, YO-PRO-1. DSCE separations of a 10:1 dilution of a Williamsburg Chardonnay RAPD sample in 0.30% (w/w) HEC-90K in THE buffer, pH 7.5 containing (a) 10 ng/mL and (b) 100 ng/mL of YO-PRO-1. Sample was injected at 30 mbar for 0.5 min for both (a) and (b).

1 kilobase pair PLUS DNA sizing ladder. Lane 4 displays the negative control RAPD reactions which contained no grape plant genomic DNA. All other lanes are identified in the figure caption. In the slab gel format, the Chardonnay, Merlot, Cabernet Sauvignon, and Cabernet Franc varieties could be discriminated. There appears to be some similarity between varieties in terms of the dominant bands. Overall, many of the

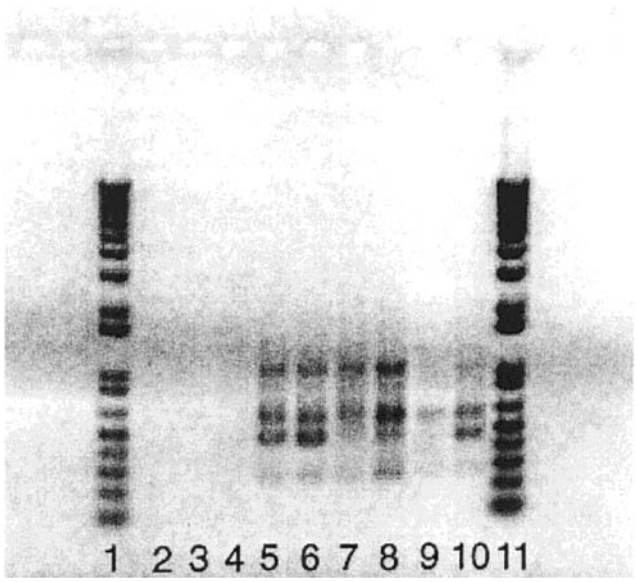

Figure 5. Slab gel electrophoretic separation of the 1 kilobase pair PLUS DNA standard relative to the separation of four grape plant varieties as well as three different Chardonnay samples in 0.5% Trevigel-500 TAE buffer, pH 8.5, containing 10 μg/mL of ethidium bromide intercalator, run at 100 V. Sample wells are indicated by an arrow. DNA fragment sizes are given in base pairs. Lane identification: (1) 1 kbp DNA ladder PLUS standard, (2) blank, (3) blank, (4) negative control (contains all RAPD reagents but not genomic DNA), (5) Glebe '91 Chardonnay, (6) Glebe '86 Chardonnay, (7) Williamsburg Merlot, (8) Williamsburg Cabernet Sauvignon, (9) Williamsburg Cabernet Franc, (10) Williamsburg Chardonnay, and (11) 1 kbp DNA ladder PLUS standard.

bands are light in intensity and are concentrated over a small base pair size range with poor resolution. To investigate the advantages of a more efficient, more sensitive electrophoretic technique, DSCE−LIF detection was performed on the same RAPD samples of the various grape plant varieties.

Slab Gel Electrophoresis versus Capillary Electrophoresis. Figure 6 depicts the DSCE−LIF analyses of the polymorphic DNA fragments of the various grape plant varieties using the previously described, optimized experimental conditions. The capillary electropherograms have been positioned on individual time scales, but aligned according to the migration times of the two internal standards. Similar to the results obtained using SGE (Figure 5), DNA fragments within the same size range (150−1500 base pairs) are observed for each sample. In addition, the major electrophoretic bands in Figure 5 correspond to those observed respectively for each sample using DSCE−LIF (Figure 6). However, DSCE−LIF revealed a greater number of resolved markers, as well as markers of relatively low concentration. Differences in peak intensities (and areas) are more discernible using DSCE−LIF. Significantly more complex and resolved fingerprints are revealed in DSCE−LIF (Figure 6D and 6E) versus SGE (Figure 5, Lanes 6 and 10) for the two different Chardonnay samples.

Sizing of Polymorphic DNA Markers Using DSCE−LIF. Because of the utilization of fused-silica separation capillaries in DSCE, the migration times of DNA fragments exhibit shifting which results from changes in the protonation state of the silica at the capillary surface. Calculated average standard deviations in migration times of a particular sample range from 0.06 to 0.15 min within the same day. Migration time shifting can be more pronounced from day to day;

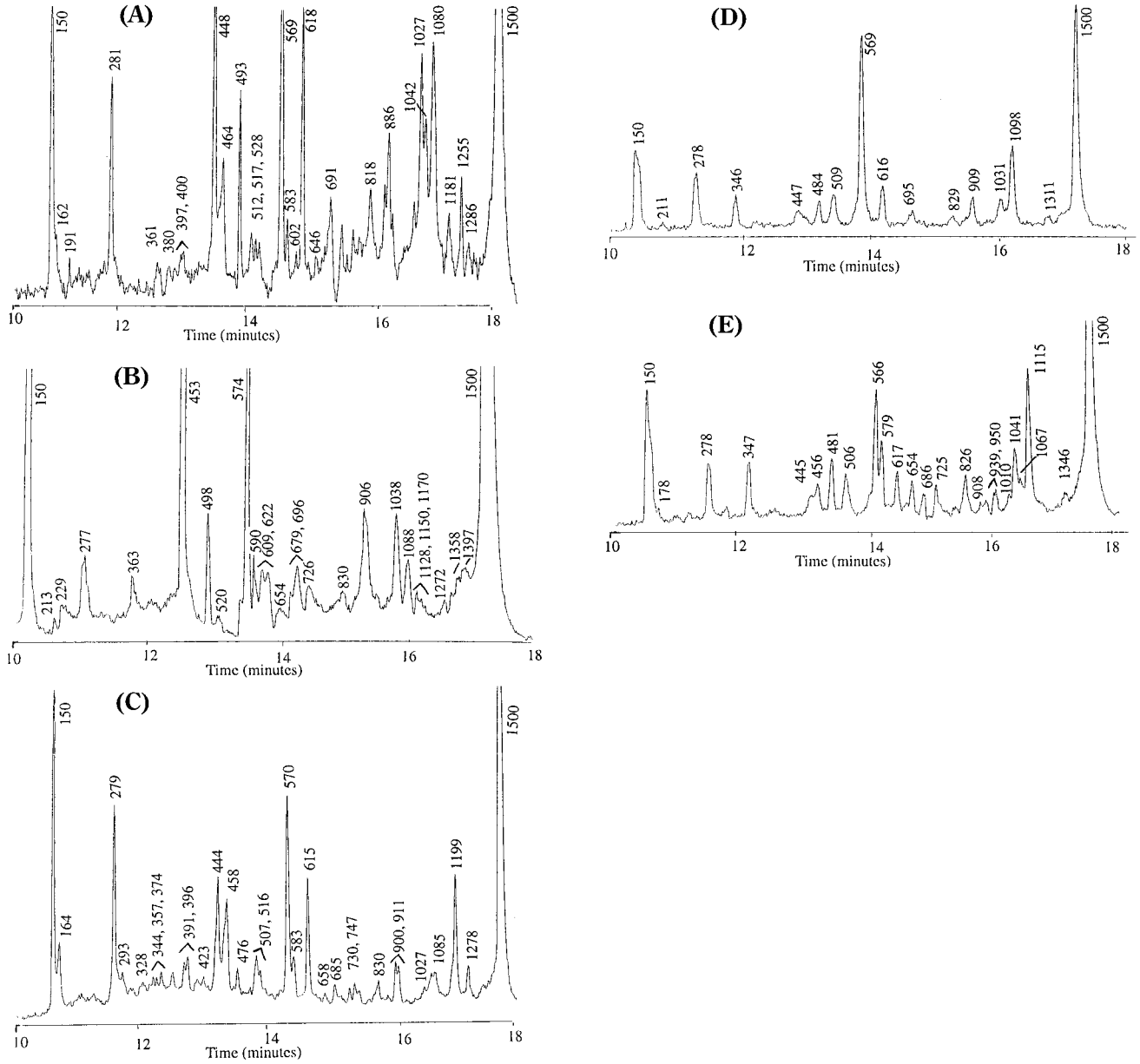

Figure 6. Sizing of DNA polymorphic DNA markers for (a) Williamsburg Cabernet Franc, (b) Williamsburg Cabernet Sauvignon, (c) Williamsburg Merlot, (d) Williamsburg Chardonnay, and (e) Glebe '86 Chardonnay (60 mbar for 2 min). All injection parameters are given in parentheses. All DNA fragment sizes are indicated in base pairs.

for example, the average standard deviation for the 1 kbp DNA standard ladder run on three different days is 0.35 min. These subtle shifts do not affect the patterns produced by the separation; however, they are significant enough to prevent accurate sizing of fragments generated in such a small base pair range.

Therefore, to accurately size the DNA polymorphic markers for the various grape plant samples, two internal standards of 150 and 1500 base pairs, were added (post RAPD reaction) to both the RAPD samples and to the 1 kilobase pair DNA PLUS ladder standard. The addition of such internal size standards was demonstrated by Butler et al. (1995) in the sizing of PCR amplified DNA fragments. Additionally, the 1 kilobase pair DNA PLUS ladder standard provides a more even distribution of peaks over the DNA size range bracketed by the polymorphic DNA markers, relative to the more commonly employed 1 kilobase pair DNA ladder standard. Figure 7 shows a representative DSCE separation of the 1 kilobase pair PLUS DNA ladder standard containing the two internal standards. A calibration curve was constructed by plotting DNA fragment size in base pairs versus migration time in minutes for the ladder standard. The calibration data were fitted to a third order polynomial equation (Figure 8). Then, migration times for peaks of the RAPD samples were used to calculate DNA fragment lengths in base pairs; the raw data were adjusted according to the migration times of the two internal standards. Each RAPD sample was electrophoresed in either duplicate or triplicate; these analyses were bracketed by electrophoretic analyses of the standard. The data for the standards were averaged, as were the data collected for each RAPD sample. Figure 6 shows the RAPD fingerprints for the Cabernet Franc, Cabernet Sauvignon, and Merlot varieties, as well as two Chardonnay samples; polymorphic markers have been labeled according to calculated sizes in base pairs. The electropherograms have been positioned on individual time scales and aligned according to the migration times of the two internal standards.

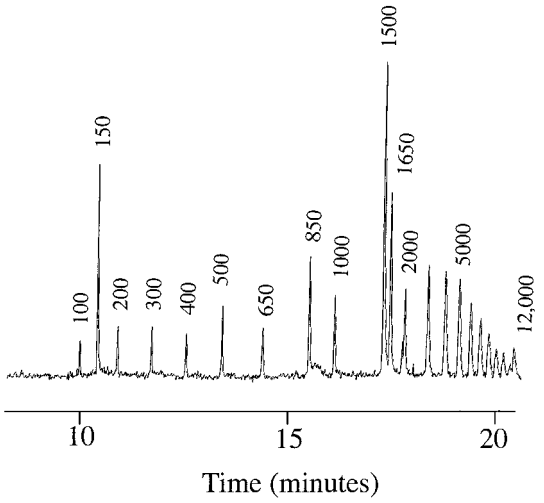

Figure 7. DSCE separation of the 1 kilobase pair PLUS DNA ladder standard containing the two internal standard DNA fragments. All DNA fragment sizes are indicated in base pairs. Sample was injected at 30 mbar for 0.24 min.

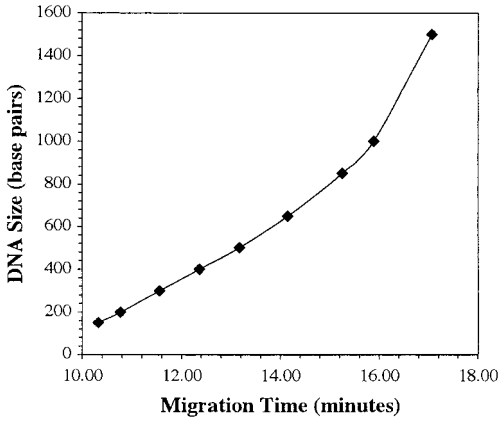

Figure 8. A representative calibration curve used for sizing DNA polymorphic markers of the Williamsburg Chardonnay RAPD sample. DNA size in base pairs was plotted versus electrophoretic migration time in minutes; data were fit to a third degree polynomial function. The standard deviation of each data point lies within each symbol. Each data point represents the average of duplicate analyses of each fragment of the 1 kilobase pair PLUS DNA ladder standard and internal standards. A separate calibration curve was constructed for each RAPD sample.

The criterion for the assignment of each peak was twofold. First, the peak had to be consistently reproducible. Second, the peak had to be three times greater than the average noise of the electropherogram. The error associated with the calculation of the DNA size in base pairs was less than 4% RSD.

The feasibility of combining DSCE−LIF and RAPD analyses has been established from an analytical standpoint. The conclusions that can be drawn from these preliminary data are limited from a genetics standpoint, owing to the use of a single RAPD primer. Nonetheless, some observations are noteworthy. Several DNA markers are common to the four grape plant varieties (280, 450, 570, 620, and 910 base pairs). Interestingly, the relative intensities (and peak areas) of these markers vary reproducibly from variety to variety. A great deal of similarity is noted between the Cabernet Sauvignon and the Cabernet Franc varieties (Figure 6A,B) in terms of the representative polymorphic DNA fragments. Bowers and Meredith (1997) demonstrated, with a very high degree of probability, using RFLP that the Cabernet Sauvignon variety is a progeny of the Cabernet Franc variety. Interestingly, an even greater genetic similarity has been revealed between the Cabernet Sauvignon and Merlot varieties (Figure 6B,C). This comparison involves both DNA fragment sizes in base pairs and relative peak intensities.

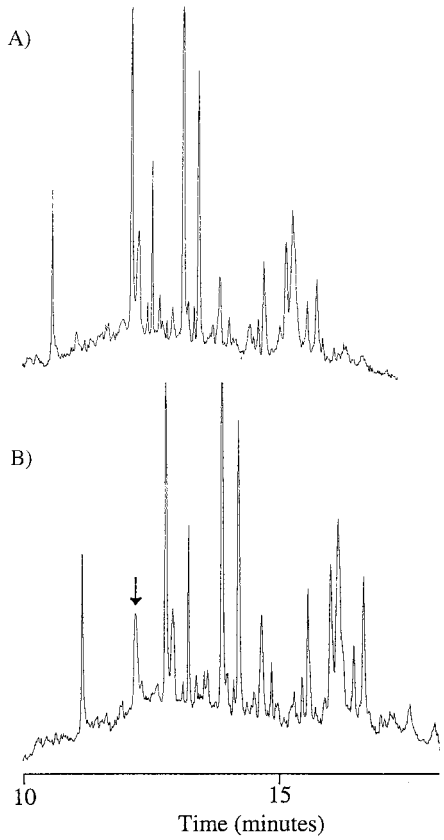

Figure 9. Reproducibility of RAPD analyses. DSCE separation of RAPD samples of the Williamsburg Chardonnay sample from (a) 2/2/98, and (b) 3/30/98. Samples were injected at 30 mbar for 1.5 min.

Remarkably, differences were noted within the Chardonnay variety. The Williamsburg Chardonnay RAPD sample (Figure 6D) exhibited the most complex pattern of polymorphic DNA fragments. The highly detailed baseline was characteristic of this sample and the majority of the smaller peaks were reproducible as well. The Glebe '86 Chardonnay RAPD sample (Figure 6E) displayed less baseline detail than the Williamsburg Chardonnay RAPD sample. More discussion of the analysis of the different Chardonnay samples is provided later in this paper.

Reproducibility of RAPD Fingerprints. Despite the success encountered in discriminating between grape plant varieties and clones within a single RAPD analysis, some uncertainty arises when the reproducibility of generated fingerprints of the same sample between different RAPD analyses is addressed. Pioneers of the RAPD technique affirm the reproducibility of DNA using a single arbitrary primer to create fingerprints and in mapping studies from many complex genomes (Williams et al., 1990; Welsh and McClelland, 1990). However, this affirmation assumes fidelity with respect to SGE separation; the possibility exists that the detection capability of DSCE−LIF surpasses the reproducibility limits of the RAPD technique. Figure 9 shows the DSCE fingerprints produced by two different RAPD analyses of the same sample. The arrow indicates

the only major peak that was not reproducible between RAPD analyses. The source of this peak is not known at this time. The key to high reproducibility appears to be related to the precision of adding the DNA polymerase to the RAPD reaction mixture. The concentration of the DNA polymerase is known to affect the discreteness of banding in SGE and is expected to result in similar effects in DSCE. Earlier investigations in our laboratory using less accurate pipettors resulted in markedly lower reproducibility of RAPD analyses (data not shown).

Polymorphic Markers of Chardonnay Clones. The subtlety of genomic differences between grape plant clones within the same variety presents a difficulty in their unequivocal identification. The frequency of unique polymorphic markers created during amplification of different clones will most likely be diminished with respect to those created during amplification of different grape plants varieties. Simply stated, fewer unique fragments will be created between clones than between varieties. The reduced intensity of a particular polymorphic marker in one clone versus another may suggest that the genomic sequence at their priming site is unique enough to cause a decline in primer binding affinity. Therefore, quantification of the relative peak areas could allow for the discrimination of grape plant clone fingerprints, using band or peak area as a polymorphic marker in addition to fragment length.

Because the RAPD analysis is a low-stringency PCR technique performed with a nonspecific primer, the probability of creating amplification products that are low in concentration owing to nonspecific annealing is increased. These amplification products that are low in concentration are just as likely to serve as polymorphic markers as products generated in higher concentrations. Thus, the detection of fragments present in low concentrations proves essential. Several investigators have noted the appearance of reproducible electrophoretic bands of relatively low concentration using RAPD analysis (Jean-Jacques et al., 1993; Loureriro et al., 1998; Moreno et al., 1995; Vidal et al., 1999); unfortunately, such low-intensity electrophoretic bands are often ignored during data analysis.

A number of investigators have attempted to discriminate between grape plant clones utilizing a variety of genetic typing techniques; many have been unsuccessful (Tessier et al., 1999; Collins and Symons, 1993; Jean-Jacques et al., 1993; Tschammer and Zyprian, 1994; Gogorcena et al., 1993; Botta et al., 1998; Loureriro et al., 1998). However, Regner et al. (1998) utilized SSR, RAPD, and AFLP markers, and were successful in detecting differences within clones of the Gruner Veltiner, Pinot Blanc, Morillion, and Chardonnay varieties. Using RAPD markers solely, Moreno et al. (1995) were able to discriminate between clones of *Vitis vinifera* to a limited extent.

Figures 10 and 11 represent the DSCE separations of several different Chardonnay samples gathered from local wineries in the state of Virginia. As stated previously, the conclusions that can be drawn from these preliminary data are limited from a genetics standpoint, owing to the use of a single RAPD primer. However, the DSCE analysis of these five samples reveals two distinctly different fingerprint patterns. The Group I samples ('86 Chardonnay, '91 Chardonnay, and 19-years-old Chardonnay) (Figure 10) yielded amplification products that resemble each other, and the Group II samples (Williamsburg Chardonnay and Sharp Rock Chardonnay, Clone 4) (Figure 11) produce amplification products that resemble each other.

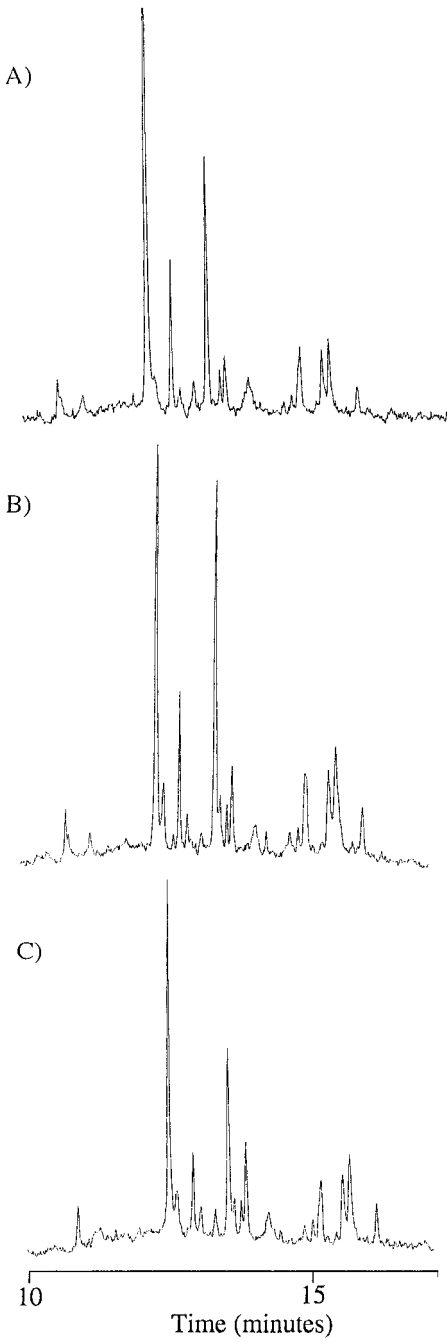

Figure 10. Group I Chardonnays. DSCE separation of RAPD fragments from Glebe Vineyards (a) '86 Chardonnay, (b) 19-year-old Chardonnay, and (c) '91 Chardonnay. Samples were injected at 30 mbar for 1.5 min.

CONCLUSIONS

The feasibility of combining DSCE−LIF with RAPD analysis for the genetic analysis of grape plant samples has been demonstrated. DSCE−LIF experimental conditions were optimized for the analysis of grape plant genomic fingerprints using 1:10 sample dilution, hydrodynamic injection, and 100 ng/mL YO-PRO-1 DNA intercalator. Run-to-run reproducibility has been achieved for both RAPD and DSCE−LIF analyses of grape plant fingerprints. The utilization of DSCE−LIF has proven

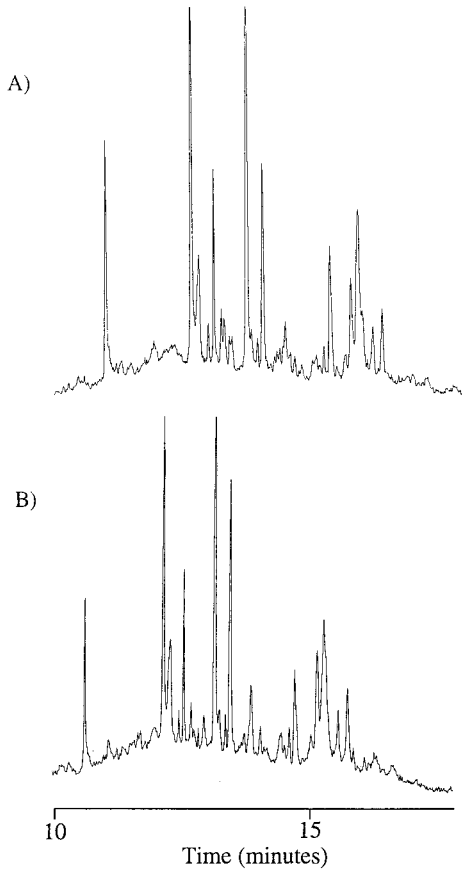

Figure 11. Group II Chardonnays. DSCE separation of RAPD fragments from (a) Williamsburg Chardonnay, and (b) Sharp Rock Clone 4 Chardonnay. Samples were injected at 30 mbar for 1.5 min.

highly advantageous versus SGE for the genetic differentiation of several grape plant varieties, as well as for the distinction of two types of Chardonnay clones. The full utility of RAPD analyses appears to be hampered by the insufficiencies of SGE as a separation technique.

ABBREVIATIONS USED

HEC, hydroxyethylcellulose; PCR, polymerase chain reaction; TRIS, tris[hydroxymethyl]aminomethane; THE, TRIS−hydrochloride−EDTA; RAPD, randomly amplified polymorphic DNA; DSCE, dynamic size-sieving capillary electrophoresis; LIF, laser-induced fluorescence; SGE, slab gel electrophoresis; CTAB, cetyltrimethylammonium bromide.

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