Teaching Methods

Laboratory Exercises on DNA Amplification Fingerprinting for Evaluating the Molecular Diversity of Horticultural Species

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Summary. **The primary objectives of these laboratory exercises are to familiarize advanced undergraduate and graduate students (and instructors) with the general concepts, techniques, and uses of DNA fingerprinting and to remove some of the perceived mystique underlying molecular genetics. The technique of DNA amplification fingerprinting (DAF) is partitioned into four independent laboratory exercises that include DNA isolation, DNA amplification, gel electrophoresis and silver staining, and data collection and analysis. Although the DNA amplification and gel electrophoresis exercises are emphasized, very detailed and easy-to-follow instructions and protocols are provided for all aspects of the DNA fingerprinting process. These exercises, or similar ones, have been successfully completed on the first attempt by several classes of novice graduate students and other researchers.**

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DNA fingerprinting can be defined functionally as a sampling procedure capable of reducing the extraordinarily complex genetic information contained in DNA. It is the aim of the method to produce a relatively simple and manageable series of bands or bar codes, which represent only selected, but defined, portions of a genome. Comparison of DNA profiles or fingerprints from different, but closely related (i.e., cultivars of plants or isolates of a fungus), organisms can reveal regions with unlike nucleotide sequences (polymorphisms) that uniquely identify individuals much like the patterns of a person's fingerprints. DNA fingerprinting has been used in genetic and physical mapping, map-based cloning, ownership rights, molecular systematics, phylogenetic analysis, marker assisted breeding, parentage testing, gene expression, and many other applications in the plant sciences.

Before the 1990s, DNA characterization required molecular hybridization (Southern, 1975) or selective DNA amplification (Erlich et al., 1991; Mullis et al., 1986). These techniques demanded prior knowledge of DNA sequence information or clones and/or characterized probes, and often required extensive experimentation (Caetano-Anollés 1996). Since then, many techniques (see review, Caetano-Anollés, 1996; Caetano-Anollés and Trigiano, 1997) have been developed that use relatively short (5 to 20 nucleotides) arbitrary oligodeoxynucleotide primers to direct DNA polymerase-mediated amplification of discrete, but anonymous, segments of DNA. Among these techniques are random amplified polymorphic DNA (RAPD) analysis (Williams et al., 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anollés et al., 1991; Caetano-Anollés and Gresshoff, 1994). Both methods produce information that characterizes a genome somewhere between the level of the primary DNA sequence and chromosomes.

Arbitrary oligonucleotide primers amplify multiple genomic regions (amplicons), many of which are variant (polymorphic) and represent allelic differences that can be traced in inheritance studies or can be treated as characters that can be used in population or phylogenetic analyses. The amplification reaction occurs, much like the polymerase chain reaction (PCR), through the succession of temperature cycles. Under low strigency conditions (low annealing temperature and ionic environment), the primer with arbitrarily (user)-defined sequence binds to many sites distributed in the genomic DNA template. The bound primers prime or initiate DNA synthesis by a thermostable DNA polymerase, even in those cases where there is substantial mismatching between primer and template base sequences. Despite perfect or imperfect priming, the DNA polymerase initiates the amplification process by the successive addition of template-complementary bases to the 3' terminus of the primer. Strand elongation is increased by raising the reaction temperature to an optimum temperature (usually ≈72 °C) and generally ends when the temperature is high enough to allow for the disassociation (denaturation) of the template DNA and the newly copied DNA strand. The separated strands

now serve as template DNA when the reaction temperature is decreased to a point where primer annealing is permitted again (usually <60 °C). Following this initial amplification cycle, successive changes in temperature result in the selective amplification of genomic regions bordered by primer annealing sites occurring in opposite strands and separated by no more than a few thousand nucleotides (bases). The outcome of the amplification reaction is primarily determined by a competition process in which amplicons that are the most stable (efficient) primer annealing sites adjoining the easily amplifiable sequences prevail over those that are inefficiently amplified. A model to explain the amplification of DNA with arbitrary primers was proposed by Caetano-Anollés et al. (1992) and later discussed in detail by Caetano-Anollés (1993) and is based on the competitive effects of primer-template as well as other interaction established primarily in the first few cycles of the process. Essentially, the rare but stable primer-template duplexes are transformed into accumulating amplification products. The final outcome is the selection of only a small subset (5 to 100) of possible amplification products.

Within the sample DNA sequences, polymorphisms arise from nucleotide substitutions that create, abolish, or modify particular primer annealing sites, which may alter the efficiency of amplification or priming. The resultant polymorphic RAPD or DAF fragments are useful DNA markers in general fingerprinting or mapping applications. These markers have been profusely applied in the study of many prokaryotic and eukaryotic organisms.

Q-Card 1

Sources for laboratory equipment and materials

Supplier	Product	Address	Telephone
•Barnstead/Thermo-lyne Corp.	Nanopure water	2555 Kerpar Blvd. PO Box 797, Dubuque, IA 52004	800.553.0039
•Bio-Rad	Electrophoresis supplies; tips	2000 Alfred Nobel Dr., Hercules, CA 94547	800.424.6723
•BioVentures, Inc. 800.235.8938	Biomarkers	P.O. Box 2561, Murfreesburo, TN	37133-2561
•Electron Microscopy Sciences	16% Formaldehyde	321 Morris Rd., Fort Washington, PA 19034	800.523.5874
•Ericomp, Inc.	Thermocycler	6044 Cornerstone Ct. W., Suite E, San Diego, CA 92121	800.541.8471
•Exeter Software	NTSYS-pc, version 1.8	100 N. Country Road, Sedtauket, NY 11733	516.689.7838
•FMC Bioproducts	Gelbond	101 Thomaston St., Rockland, ME 04841	800.341.1574
•Gentra Systems, Inc.	DNA isolation kit	15200 25th Ave. N., Suite 104, Minneapolis, MN 55447	800.866.3039
•Midwest Scientific	Flat loading tips	280 Vance Rd., Valley Park, MO 63088	800.227.9997
•MJ Research	Thermocycler	149 Grove St., Watertown, MA 02172	800.729.2165
•Perkin-Elmer-Applied Biosystems	DNA polymerase	850 Lincoln Center Drive, Foster City, CA 94404	800.327.3002
•Pharmacia Biotech, Inc.	Mini-Fluorometer; plates	800 Contennial Ave., Piscataway, NJ 08855-1327	800.526.3593
•Rainin	Pipettes and tips	Mack Road, Box 4026, Woburn, MA 01888-4026	Fax: 617.938.1152
•Sierra-Lablogix, Inc.	Staining trays	1180-C Day Rd., Gilroy, CA 95020	800.522.5624
•US Biochemical	dNTPs	P.O. Box 22400, Cleveland, OH 44122	800.321.9322

Q-Card 2

Troubleshooting gels: Some common imperfections in gels and their causes

- •Bands in some lanes but not in others: DNA template missing or degraded in lanes with weak or no products.
- •No amplification—all lanes blank: Missing ingredient in master mix, degraded primer, or less likely, all DNA templates degraded.
- •Dark streaks in lanes: Old loading buffer or dust particle on the bottom surface of the well.
- •Lightly staining products in center of gel: Developer poured directly on gel; glass plates warped creating thickened gel in center; developer <8 °C.
- •Individual bands not straight but jagged: Bottom surface of well damaged.
- •Lanes not straight, but deflected: Air bubble under support film.
- •Bubbles in gel: Aspirated air from syringe or air bubble adhering to a dirty glass plate.
- •Black smudges in gel: Incomplete removal of silver stain solution before adding developer.

Although RAPD and DAF analyses produce similar types of information, there are some differences between the two techniques that should be noted.

- DAF uses very short primers, usually 7 or 8 nucleotides in length, whereas RAPD typically uses 10 nucleotide primers.
- DAF products are resolved using 5% to 10% polyacrylamide gel electrophoresis and silver staining (Bassam et al., 1991), whereas RAPD products are typically separated electrophoretically in agarose gels and visualized with ethidium bromide staining under UV light.
- The reaction mixture or cocktail in DAF contains higher primer-to-template ratios than RAPD analysis and produces relatively complex banding profiles containing 30 to 40 products that are fewer than 700 base pairs in length. In turn, RAPD usually generates more simple patterns of 5 to 10 bands.
- DAF polyacrylamide gels are backed using polyester films and are amenable to permanent storage, whereas RAPD agarose gels are difficult to store and a photograph serves as the permanent record.

One could argue the relative merits of each fingerprinting technique, but from our experience, DAF is easier for students and instructors to learn and use and is very tolerant, almost forgiving, of some errors typically made by novices, such as inaccurate pipetting. The data are permanently recorded in the form of a gel instead of a photograph, which is very gratifying to students and facilitates research by allowing repeated and close scrutiny of data.

The intention of these exercises is not to describe and explore fully the theoretical aspects of DNA fingerprinting, which may be otherwise obtained by reading the literature cited throughout this paper. The educational objectives of the laboratory exercises are to acquaint students with the general concepts, techniques, and uses of DNA fingerprinting and to remove some of the perceived mystique underlying molecular genetics.

Several products are mentioned throughout the laboratory exercises. Complete information is provided in Q-Card 1 should an instructor wish to order from any company mentioned in this paper. These are simply what we normally use and do not constitute product endorsements by either the authors or the University of Tennessee, nor implied criticism of those products not mentioned. There are equally suitable, if not alternative, products and equipment that may be substituted for those listed herein.

Before beginning the exercises, a few essential generalities applicable to all laboratories are listed below.

- All pipette tips, eppendorf centrifuge tubes, water, and reagents used to extract DNA and assemble the amplification reactions should be sterilized by autoclaving.
- Participants should wear latex gloves to avoid hazardous materials (acrylamide and silver nitrate) and protect samples from DNAses found on the skin (Dragon, 1993).
- Where possible, use only American Chemical Society (ACS)-certified pure chemicals and double distilled or nanopure water [<16 MΩ/cm: Barnstead/Thermolyne Corp., Q-Card 1), hereafter referred to as pure. It is not necessary to use high-performance liquid chromatography-grade water.

Characterizing genomes using DAF, as well as any other of the arbi-

Q-Card 3

An example of matrix definitions and binary data for carnation cultivars to be analyzed using the NTSYS-pc program

This data set (rectangular = 1) includes 154 character loci from 7 cultivars (initals) and contains missing data (1) represented by a 9. Rows with all 1s are monomorphic; a row containing a single 1 identifies a unique marker for a cultivar.

1	154	7L	1	9		
NL	TA	TO	ET	KI	HO	WG
0	1	0	0	0	1	1
1	1	1	1	1	1	1
1	1	1	1	1	9	1
0	0	1	0	0	0	0
.	.	.	.	.	.	.
1	1	1	1	1	1	1

Q-Card 4

An example of a similarity matrix generated using Jaccard coefficients for seven carnation cultivars

Cultivars	NL	TG	ET	TR	KI	HO	WG
NL	1.000						
TG	0.760	1.000					
ET	0.687	0.693	1.000				
TR	0.697	0.703	0.988	1.000			
KI	0.737	0.760	0.702	0.695	1.000		
HO	0.796	0.800	0.723	0.716	0.798	1.000	
WG	0.730	0.752	0.647	0.657	0.711	0.768	1.000

trary primer-based techniques, always consists of at least four independent phases, including DNA isolation, DNA amplification, electrophoresis and visualization of amplified products, and data collection and analysis. Each of these steps requires 4 to 6 h to complete. If class and laboratory time is of a premium, the instructor may opt to complete one or more of the laboratory sessions for the students. However, students will derive the most benefit by fully participating in each of the laboratory sessions. This paper emphasizes DNA amplification, DAF product separation, and visualization, and to a lesser degree DNA isolation and data analyses. We recommend that these laboratory exercises be completed by advanced undergraduate or graduate students working in teams of four or less.

Exercise 1: DNA preparation

DNA ISOLATION. Following an examination of a few journal articles concerning some aspect of DNA analysis, it is evident that there are many methods to isolate genomic DNA from plant cells, all of them more or less suitable for the purposes of this laboratory exercise. Fortunately, DAF reactions do not require the high quality or large quantity of DNA necessary, for example, in restriction fragment length polymorphisms (RFLP) analysis, a classical DNA profiling technique. In our lab, we use a DNA isolation kit especially formulated for plants (Puregene; Gentra Systems, Inc., Q-Card 1). Unlike earlier methods for DNA isolation, most commercially available kits avoid the use of highly toxic materials, such as phenols. Regardless of the technique or kit used to isolate DNA, use only young, not fully expanded, light-green leaf tissue collected early in the morning before starch accumulation. Tissue should be stored at –75 °C until needed. Just about any group of cultivars or varieties of a single plant species may be used in the exercises. For instance, we have successfully used DAF to identify cultivars of chrysanthemum (Scott et al., 1996), petunia (Cerny et al., 1995), bermudagrass (Caetano-Anollés et al., 1995), and flowering dogwood (unpublished data). Similarly, isolates of the fungus that causes dogwood anthracnose have been characterized using slight modification of the methods described herein (Trigiano et al., 1995). These laboratory exercises will be illustrated using seven carnation (*Dianthus caryophyllus* L.) cultivars, including six red-flowered ['Nelson' (NL), 'Tanga' (TG), 'Tornado' (TR), 'Etna' (ET), 'Killer' (KI), and 'Homero' (HO)] and one having white flowers ['White Giant' (WG)], obtained from Yoder Brothers, Inc. (Barberton, Ohio).

For each cultivar, place ≤25 mg of leaf tissue with ≈25 mg polyvinylpolyprrolidone (PPVP), which sequesters plant phenols, into a sterile mortar and pestle, add liquid nitrogen, grind frozen to a powder, and add extraction buffer. Freeze and thaw at least twice. After the tissue slurry melts and comes to room temperature, load ≈300 to 400 µL into each of two sterile, prelabelled, 1.5-mL eppendorf centrifuge tubes and centrifuge at maximum rotation (14,000 rpm) to deposit (pellet) debris. Complete the rest of the manufacturer instructions except for the RNAse step, unless the DNA concentration will be determined using a spectrophotometer (Procedure Card 1). When precipitating DNA with alcohol, incubate the tubes at –20 °C for 30 min. At the end of the isolation procedure, do not redissolve the DNA in TE buffer, instead use 50 µL of sterile, pure water. Heat the contents of the tubes in a 65 °C water bath for ≈2 min to help dissolve the DNA and refrigerate (4 °C) overnight. The next morning, centrifuge for 2 min to pellet any undissolved particulate material, then carefully pipette the supernatant containing the DNA into a new, sterile, 0.65-µL centrifuge tube and store at 4 °C.

DETERMINING DNA CONCENTRATION. DAF is exceptionally tolerant of the quality (purity) and quantity of DNA used in the reaction mixture. Optimum concentrations of DNA range from 0.02 to 2.0 ng·µL^{-1} in a 20-µL reaction mixture, but we recommend using a final concentration of 0.1 ng·µL^{-1} in the amplification cocktail. DNA concentration is determined spectrophotometrically with a dedicated fluorometer (e.g., Mini-Fluorometer; Pharmacia Biotech, Q-Card

Procedure Card 1

Spectrophotometeric determination of DNA concentration

Step 1	Zero spectrophotometer by pipetting 1 mL of distilled water into sample and reference quartz cuvettes.
Step 2	Pipette and mix by gentle inversion 2 µL of DNA into 1 mL of distilled water in the sample cuvette.
Step 3	Read absorbance at 260 nm (e.g., 0.012).
Step 4	Since an O.D. of 1.0 = 50 µg·mL^{-1} DNA, then the sample contains 50 µg·mL^{-1} × 0.012 = 0.6 µg or 600 ng·mL^{-1}.
Step 5	The amount of DNA in each µL = 600 ng/2 µL or 300 ng·µL^{-1}.

Procedure Card 2

Dilution of isolated DNA to make template DNA stock

Step 1	Determine amount of DNA in isolation (see Procedure Card 1), e.g., 79 ng·µL^{-1}
Step 2	Make a 10-ng·µL^{-1} solution using the following formula: $C_1 \times V_1 = C_2 \times V_2$, where C = ng·µL^{-1} and V = µL. $79 \times 20 = 10 \times V_2$ V_2 = 158 µL = total volume
Step 3	Pipette 20 µL of the original DNA solution into a sterile 0.65-mL tube and add 138 µL (158–20) of sterile, pure water. Mix throughly by vortexing and centrifuge (14,000 rpm for 5 s) to remove air bubbles.
Step 4	Make a 0.5 ng·µL^{-1} solution using 20 µL of the 10 ng·µL^{-1} stock. Using the formula provided in Step 2, the calculated total volume = 400 µL.
Step 5	Pipette 20 µL of the 10-ng·µL^{-1} stock into a sterile 0.65-mL tube and add 380 µL (400–20) of sterile, pure water. Mix throughly and centrifuge as in Step 3.
Step 6	Store all DNA stocks at 4 °C.

Procedure Card 3

Preparation of primer stocks

Step 1	Prepare 300 µM primer stock, e.g., 159 nmol provided by supplier. 159 nmoles primer/x µL = 300 µM x = 532 µL Add 532 µL of sterile, pure water to the manufacturer's tube containing primer. Mix thoroughly and centrifuge.
Step 2	Prepare 30 µM primer stock. Pipette 30 µL of 300 µM into an sterile eppendorf tube and add 270 µL of sterile, pure water. Mix thoroughly and centrifuge. This is the stock to use in the amplification cocktail.
Step 3	Store all stocks at –20 °C.

Procedure Card 4

Preparation of master mixes for each primer (sufficient for eight reactions)

Step 1	Pipette 65.6 µL (8.2 × 8) of sterile, pure water into a sterile 0.65-mL tube.
Step 2	Add 16 µL (2 × 8) of 10× stoffel buffer provided by the manufacture (final concentration[z] = 1×).
Step 3	Add 16 µL (2 × 8) of 2 mM dNTPs (final concentration = 200 µM).
Step 4	Add 16 µL (2 × 8) of 30 µM primer stock (final concentration = 3 µM).
Step 5	Add 9.6 µL (1.2 × 8) of 25 mM $MgCl_2$ provided by manufacture (final concentration = 1.5 mM).
Step 6	Add 4.8 µL (0.6 × 8) of DNA polymerase provided by manufacture.
Step 7	Mix thoroughly by vortexing and centrifuge briefly at 14,000 rpm to eliminate air bubbles.
Step 8	Dispense 16 µL of the master mix into each of seven, sterile 0.65-mL tubes.

[z]Final concentrations of reagents are based on 20-µL reaction volumes after the addition of 4 µL of template DNA.

1) invariably set at 365 nm. Instructions for using the fluorometer are included with the instrument; dye and calf thymus DNA for standard concentrations of DNA may be purchased directly from Pharmacia Biotech (Q-Card 1). Since most isolations will usually yield DNA concentrations between 100 and 250 ng·µL^{-1}, we recommend preparing and calibrating the fluorometer with standards of similar concentration.

If a dedicated fluorometer is not available, DNA content can be determined directly using a spectrophotometer (Procedure Card 1). The 260/280 ratio of a pure double-stranded DNA preparation should be 1.65 to 1.85. While this ratio depends on the fractional GC content, higher ratios are often due to RNA contamination and lower values to protein or phenol contamination. Thus, if determining DNA concentration using this method, it is imperative that the RNA in the sample be eliminated with RNAse step in the isolation procedure.

Adjusting DNA concentration. Typically, the concentration of DNA from most isolations is too high to be used directly as the template in the amplification stage of DAF. Therefore, the DNA must be diluted with sterile, pure water to a more functional concentration, such as 0.5 ng·µL^{-1}. The dilution is easily accomplished by a two-step operation (Procedure Card 2). We have stored isolated DNA and dilutions at 4 °C for >5 years without apparent degradation.

Exercise 2: DNA amplification

This laboratory exercise requires careful experimental design and planning and involves handling many liquid reagents. By completing this, students will gain experience and confidence with routine procedures in the molecular biology laboratory.

To begin, a few helpful hints. Plan ahead and write everything down! It is very easy to forget what has and needs to be done; record keeping is an integral part of good laboratory practices. When in doubt, change sterile pipette tips. Don't risk cross-contamination of solutions and templates or introduction of DNAses to save a pipette tip. Mix, by vortexing, all stock solutions, except DNA polymerase, and centrifuge tubes before opening to remove large bubbles from the liquid and to avoid aerosols. Look at the pipette tip to ensure that an appropriate amount of fluid has been taken into the lumen. Finally, always wear gloves to protect stock solutions and DNA.

Preparation of solutions and reagents. For this section, a number of supplies and solutions must be either purchased or prepared. AmpiTaq Stoffel fragment DNA polymerase can be purchased from Perkin-Elmer, Applied Biosystems Division (Q-Card 1). This component is required to complete the laboratory exercise and contains 1000 units (100 µL) of DNA polymerase, 10× Stoffel buffer (100 mM Tris, 100 mM KCl, pH 8.3) and 10× $MgCl_2$ (25 mM) in separate tubes. Primers can be synthesized by several companies (Integrated DNA Technologies, Inc, Q-Card 1) and primer stock preparation is described in Procedure Card 3. In these laboratory exercises, we have used four primers with the sequences (5' to 3') GAGCCTGT (8.6A), CCTGTGAG (8.6B), CTAACGCC (8.6G), and CCGAGCTG (8.7A). The first number in the primer codes denotes oligonucleotide length and the second represents the approximate fractional GC content. These primers generate clear and informative fingerprints with carnation. However, any octamer sequence with an approximate GC content of 60% to 70% may be used. Deoxynucleoside triphosphates (dNTPs) may be purchased from U.S. Biochemical (Q-Card 1) and are supplied as a set of four ampoules containing 25 µmol of each dNTP in 250 µL of water (100 mM). Simply combine the four ampoules (1 mL) in a sterile container and add 11.5 mL of sterile, pure water to produce a 2-mM solution containing all the necessary dNTPs. Dispense 250 µL into fifty 0.65-mL sterile centrifuge tubes and store at –20 °C. This is the working dNTP concentration for the amplification mixture.

Planning the experiment. DNA from the seven cultivars of carnation

should be amplified with four primers. Twenty-eight 0.65-mL sterile centrifuge tubes are needed. Label the tubes 1 to 28 according to the scheme depicted in Fig. 1 and record in a laboratory notebook.

DNA AMPLIFICATION. The assembly of the amplification cocktail is the heart of the DAF technique. Each group of students should make master mixes (one or more) containing all the ingredients common in each amplification reaction cocktail. The only two variables in the carnation experiment are the DNA templates from the individual cultivars and the primers. Master mixes should, therefore, contain sterile pure water, stoffel buffer, dNTPs, stoffel magnesium chloride, stoffel enzyme, and a single primer; assembly should be in a sterile centrifuge tube labelled with the primer code (e.g., 8.6A). DNA template will be added to individual reaction tubes later. Procedure Card 4 details how to make a master mix and provides a list of reagents and their final concentration in 20 µL of the mixture. Gloves should be worn by all persons involved in making the master mixes.

Start by removing the ingredients from the freezer and allowing them to warm to room temperature. In the interim, label four 1.5-mL sterile centrifuge tubes for assembling master mixes and place to the left of the four rows of seven reaction tubes (in a plastic flipper rack) labelled 1 to 7, 8 to 14, 15 to 21, and 22 to 28, as shown in Fig. 1. Vortex the constituents of the master mix after thawing and centrifuge briefly. At this time, remove the 0.5-ng·µL^{-1} DNA stocks from the refrigerator, vortex, and briefly centrifuge at high speed. Place the DNA stocks at the top of the flipper rack (NL–WG) above those reaction tubes 1, 2, 3, 4, 5, 6, and 7, which correspond to the cultivars to be analyzed. In the experimental setup depicted in Fig. 1, volumes used to prepare the master mixes are for eight tubes (Procedure Card 4). Calculate the volumes of reagents needed to prepare sufficient amplification cocktail. The extra volume will compensate for minor assemblage errors, especially in pipetting. Following the example in Procedure Card 4 and, using a sterile tip, pipette 65.6 µL of sterile water into each master mix centrifuge tube and place a check mark next to 65.6 in the laboratory notebook. This will ensure that all

Procedure Card 5

Composition of 10× TBE buffer and 10% polyacrylamide stock

10× TBE Buffer

Step 1 Dissolve 121.1 g Tris base, 51.4 g boric acid and 3.7 g $Na_2EDTA{\cdot}2H_2O$ in 800 mL of pure water.

Step 2 Bring the final volume to 1 L with pure water; pH = 8.3. Store at room temperature.

10% Polyacrylamide Stock (wear protective clothing, gloves and particle mask)

Step 1 Dissolve 19.6 g acrylamide, 0.4 g PDA, and 20.0 g urea in 130 mL of pure water.

Step 2 Add 20.0 mL of 10× TBE buffer and 10.0 mL glycerol to the acrylamide solution.

Step 3 Bring the final volume to 200 mL with pure water. Store at 4 °C in a brown bottle and discard unused portion after 6 weeks.

Procedure Card 6

Composition of anticracking solution and DNA loading buffer

Anticracking Solution[z]

Step 1 Under a fume hood, add 100 mL of glacial acetic acid and 370 mL of 95% ethanol to 520 ml of pure water.

Step 2 Add 10 mL of glycerol.

DNA Loading Buffer

Step 1 Add 12 g urea and 2 mL of xylene cyanol stock solution[y] to 8 mL of pure water.

[z]Solution may be used repeatedly without discarding.

[y]4 mg xylene cyanol/mL of pure water.

Procedure Card 7

Compostion of silver stain and developer solutions

Silver Stain

Step 1 For 2 gels, dissolve 0.15 g of ACS certified silver nitrate in 150 mL of pure water.

Step 2 A few minutes before use, add either 750 µL of 16% or 325 µL of 37% formaldehyde.

Developer

Step 1 For 2 gels, dissolve 4.5 g of ACS certified sodium carbonate ($NaCO_3$) in 150 mL of pure water and chill to 2–4 °C.

Step 2 Add 75 µL of sodium thiosulfate solution (0.2 g/50 mL)

Step 3 Before use, warm the solution to 8 °C and add either 600 µL of 16% or 260 µL of 37% formaldehyde.

Procedure Card 8

Timetable for fixing, staining, and developing gels

Step 1 Fix gels in 7.5% acetic acid for 10 min on a rotary shaker (60 rpm).

Step 2 Rinse gels with pure water 3 times each for 2 min on a rotary shaker (60 rpm).

Step 3 Soak gels in silver stain for 20–30 min on a rotary shaker (30 rpm).

Step 4 Rinse gels in pure water for 10–30 s.

Step 5 Soak gels in developer for 5–8 min (or until bands are dark) with manual shaking.

Step 6 Fix gels in cold (4 °C) 7.5% acetic acid for 5 min on a rotary shaker (60 rpm).

Step 7 Soak gels in pure water 2 times each for 5 min on a rotary shaker (60 rpm).

Step 8 Soak gels in anticracking solution for 5 min on a rotary shaker (60 rpm).

Step 9 Hang gels overnight to dry.

reagents are included in the master mix. Each time a tip is immersed in a fluid contained in another tube, change the tip. After all the constituents have been added to the master mix, vortex, and briefly centrifuge at high speed. Dispense 16 µL of each primer master mix into seven tubes using a single pipette tip (Fig. 1). For example, master mix for primer 8.6A can be distributed to tubes 1 to 7 without changing tips. However, a different tip should be used for each of the four master mixes since they contain different primers.

Next, pipette 4 µL of DNA template from each of the 0.5-ng·µL^{-1} stocks into the corresponding tubes (Fig. 1). For example, pipette 4 µL from NL into tube 1, then close the reaction tube and discard the tip. Repeat the sequence for tubes 8, 15, and 22, then close stock tube NL. Repeat the procedure for cultivars TG through WG. Each tube now contains 20 µL and is ready to be amplified. Mix the contents of the tubes by vortexing and centrifuging briefly at high speed. Open the tubes and add a drop of heavy white mineral oil to each to prevent evaporation and condensation during amplification.

A good alternative experiment is to include one or more of the cultivars anonymously as X1 and X2 in the design. By comparing the fingerprints of the known cultivars to the unknown, the identity of X1 and X2 can be established. If this modification is desired, add the two unknowns in the design between cultivars TR and KL, and renumber the tubes. Adding the unknown cultivars in the positions indicated places their fingerprints in the middle of the gel, which will facilitate comparison to the known cultivars. Lastly, remember to increase the multiplier for the master mixes from 8 to 10. For example, the water for each master mix should now be 8.2 µL×10, or 82 µL.

Place the tubes in a thermocycler for amplification of the DNA. Thermocyclers are programmed to establish annealing (30 to 55 °C), extension (72 °C) and denaturing (95 °C) temperatures for prescribed times. This set of temperature regimens constitutes a cycle, which is repeated 30 to 40 times. However, since there may be significant differences in ramping times between thermocyclers, proceed with caution when searching for a suitable cycle. Ramping time can be thought of as the time necessary for the amplification mixture or cocktail to go from one designated temperature to the next (e.g., from annealing to extension temperature). The Easy Twin Block System (Ericomp Inc., Q-Card 1) has relatively slow ramping times compared to the DNA Engine PTC-200 (MJ Research, Q-Card 1). Reproducible, clear profiles can be generated with the Ericomp machine using a cycle of 10 s at 95 and 30 °C, without an extension step. However, for the DNA Engine, which has faster ramping times, the cycle of 1 min at 95 and 30 °C with an extension step at 72 °C for 30 s works well. A general rule is to increase the annealing, extension, and denaturing times when the thermocycler has short ramping times. The entire amplification process takes between 2 and 6 h depending on the thermocycler.

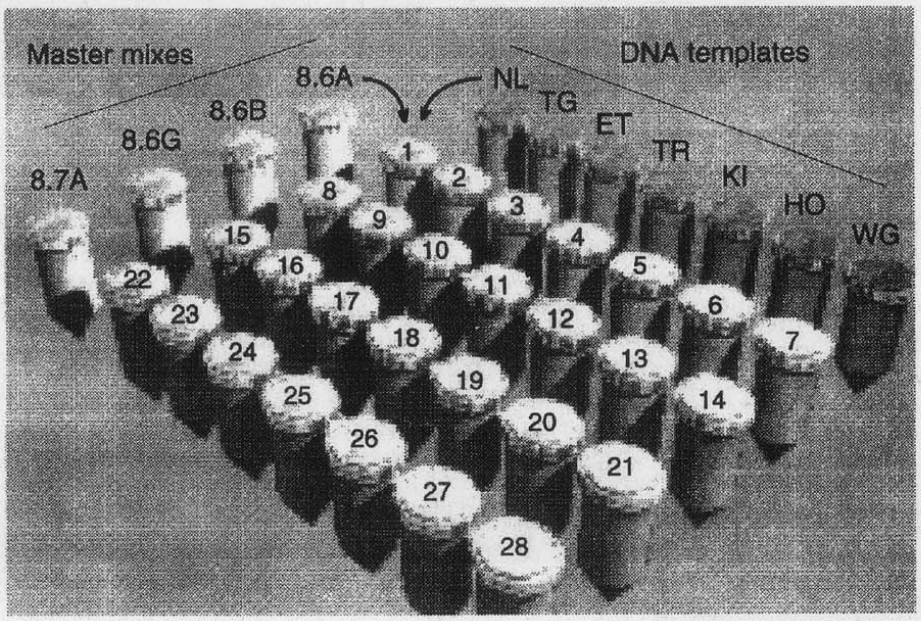
Recovery and storage of amplification products. Once the amplification program is completed, the reaction products are removed from beneath the oil. Label 28 sterile 0.65-mL centrifuge tubes (Fig. 1) by designating the experiment (e.g., 1) and tube number (1.1, 1.2, . . . 1.28). Before removing the products, prepare several tubes containing 20 µL of blue water [i.e., a drop of xylene cyanol stock (Procedure Card 6) in 20 mL of water] under a drop of mineral oil. Students can easily see the blue color and practice pipetting the water without drawing any oil into the tip. A P-100 (Rainin, Q-Card 1) pipette or equivalent set on 22 or 23 µL with a sterile Prot/Elec tip (Bio-Rad, Q-Card 1) can be used. The operation should be performed quickly and the contents of the tip squirted into a new sterile, prelabelled 0.65-mL centrifuge tube without touching the sides with the tip. When working with the reaction tubes, be sure to change pipette tips between each sample. Samples may be stored at 4 °C as is or diluted 1:1 to 1:5 with sterile, pure water.

Exercise 3: Electrophoresis and staining of DAF products

This laboratory exercise focuses on the electrophoretic separation of amplification products and should expose students to one of several electrophoretic techniques routinely used to characterize biological molecules. Experience with this technique should facilitate understanding and performance by students of similar procedures such as protein electrophoresis.

Assembling the gel apparatus. While wearing gloves, assemble two

Fig. 1. Scheme for dispensing master mixes and DNA templates. Pipette 16 µL of master mix into each row of seven sterile 0.65-mL reaction centrifuge tubes. For example, master mix containing primer 8.6A would be distributed to tubes 1 to 7. Pipette 4 µL of DNA template into each reaction tube. For example, dispense stock NL (0.5 ng·µL^{-1}) into tubes 1, 8, 15, and 22. Change tips between reaction tubes.



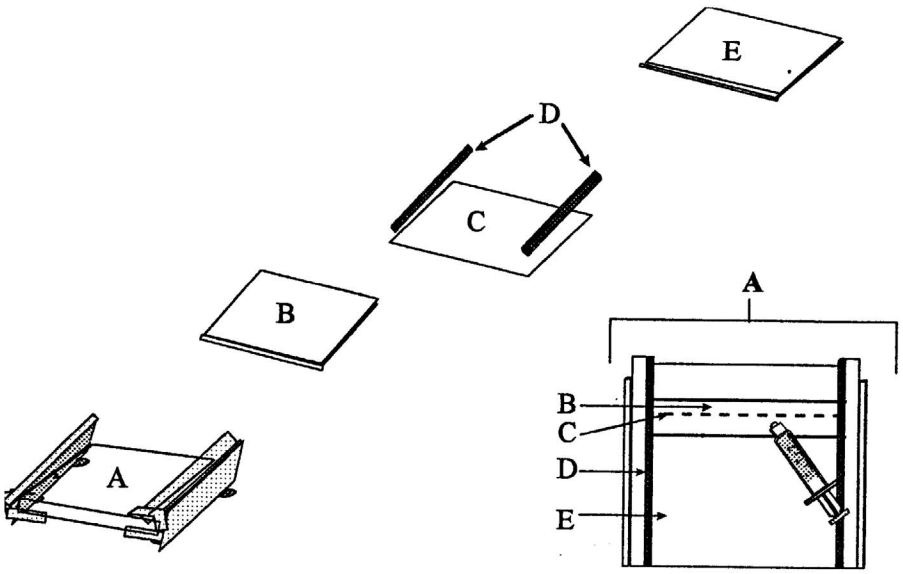


Fig. 2. Assembling gel rigs. Wash all parts of the apparatus with distilled water. Slide large glass plate B into the holder (A) and flood with distilled water. Place the support film (C) hydrophobic side on the large glass plate and rub with index finger to remove air. Set spacers (D) in place and slide the small glass plate (E) into position. Level the apparatus, flush all components of the apparatus, and tighten the knobs on the holder to secure the apparatus.

Protean II electrophoresis cells (Bio-Rad, Q-Card 1) as illustrated in Fig. 2 1 day before the DAF products are to be separated electrophoretically. The units are supplied with 0.75-mm spacers, but we recommend using 0.45-mm spacers that can be purchased separately. GelBond flexible backing supports (sheets) can be purchased from FMC Bioproducts (Q-Card 1). Here are a few helpful hints in assembling the rigs. Meticulously clean the glass plates with running distilled water to remove any dust and acrylamide from previous experiments. Assemble the rig under running distilled water. Place the hydrophobic surface (the side that water beads on) of the backing film on and toward the large glass plate and rub the hydrophilic surface until all trapped air is evacuated. All gel rig components should be flush at the bottom. Lastly, **do not overtighten** the knobs—the glass plates will bow and produce a thickened center portion of the gel, which will not stain properly. The assembled apparatus should be examined carefully to ascertain that the glass plates, spacers, and backing film are flush with each other on the bottom. Run a fingernail across the bottom of the apparatus. If it does not feel smooth or if the fingernail gets hung up, the level of the glass plates, spacers, or support film needs to be adjusted. The gel rigs should be allowed to dry overnight in a place that is dark and dust free. Alternatively, the apparatus may be dried in a 30 to 50 °C oven for a few hours.

CASTING THE GELS. Wear gloves! Mount the gel rigs onto the casting stand using the gray rubber gaskets on the bottom. We usually place several equal length and width strips of parafilm wrap under the gasket to ensure a good seal with the glass plates. A very distinct snap should be heard as the rigs are set into place on the casting stand. Place the casting stand onto a large piece of aluminum foil on which a 10-mL syringe and 0.45-µm filter can be laid. Pipette 10 mL of the 10% polyacrylamide stock (Procedure Card 5) into a 20-mL beaker containing a small magnetic stir bar. [Caution: wear protective particle mask, gloves, eyeglasses and clothing—unpolymerized acrylamide is a potent neurotoxin; skin contact and accidental inhalation of the compound should be avoided.] See Procedure Card 5 for instructions for making the acrylamide stock solution. Do not substitute BIS (*N,N'*-methylene bis acrylamide) for PDA (piperazine diacrylamide) as it affects staining quality. Place the pipette tips containing 15 µL of TEMED and 150 µL of 10% ammonium persulfate (100 $mg \cdot mL^{-1}$ pure water) solutions into the stirring acrylamide solution and dispense. Ammonium persulfate may be made in bulk, dispensed into 1.5-mL centrifuge tubes, and frozen at –20 °C. Dispose of the tips in a safe location and wait ≈10 s. The following steps in casting the gel should be completed as quickly as possible (usually <2 min). Carefully draw the gel solution into a syringe avoiding introducing air into the barrel. If air bubbles are present, hold the syringe upside-down at 70° away from the body; the air should rise to the top. Slowly depress the plunger until the air is expelled. Mount a nonsterile 0.45-µm filter on the open end of the syringe. Slowly express a small amount of acrylamide to wet the filter and release any trapped air. Place the tip filter in the middle of the ledge formed by the small plate and quickly dispense the acrylamide solution into the space between the glass plates. Rotate the casting stand 180° and fill the second rig with acrylamide. If there are bubbles trapped in the gel, gently tap the inner glass plate and, with luck, they will rise to the top. Position the 15-well combs about half way (level) in each of the rigs and examine for small bubbles residing on the bottom surface of the teeth. If bubbles are present, remove and reposition the combs. Allow the acrylamide to polymerize for at least 20 min. If desired, the gels may be cast the day before the laboratory exercise and stored overnight lying flat on the bottom of a plastic container that is lined with wet paper towels. Be careful not to disturb the combs and store in the dark.

PREPARING SAMPLES FOR ELECTROPHORESIS. Amplification products can be prepared for electrophoresis while the acrylamide is polymerizing. First, carefully pipette 3 µL of loading buffer (Procedure Card 6) into the number of wells in a 6 × 10 microtitre plate (Pharmacia Biotech, Inc., Q-Card 1). In the case provided in Fig. 3, each row in the plate would have eight wells filled, seven for the samples and one for the molecular weight marker. However, we encourage teams of students to use separate plates for their DNA. Map the order of the samples in the gel in the laboratory notebook. For instance (from left to right), sample 1.1, 1.2... 1.7, and M. Now, in the order that the samples will be placed in the gel, pipette 3 µL of each into their respective wells and mix by repipetting the solution several times. Change tips

between samples. The last well is the molecular weight marker (M) consisting of a 50 to 1000 base-pair ladder (Biomarker Low, BioVentures, Inc., Q-Card 1). The working solution is made by mixing 10 µL of biomarker with 90 µL of sterile, pure water. As with the other samples, 3 µL of biomarker are used per well.

PRERUNNING GELS AND PREPARING STAIN AND DEVELOPER SOLUTIONS. Make 1 L of 1× TBE buffer by mixing 100 mL of 10× TBE (Procedure Card 5) and 900 mL in a 1-L graduated cylinder. Dismount the two gel rigs from the casting stand and gently remove any polymerized acrylamide from the bottom of the plates with a laboratory tissue. Rotate the rigs 180° and snap into the central stand. Be careful not to touch or disturb the combs. When both rigs are mounted, the small plates of the rigs will be toward the interior, facing each other, and the outer plates will form the top buffer reservoir. About 800 mL of 1× TBE will be required to fill the inner or central and the outer reservoirs. Carefully remove the combs from the gels by gently pulling straight up with equal pressure on both sides; do not damage the wells. Fill a tuberculin syringe equipped with a 1.5-inch long, 25-gauge needle with buffer from the central well. Gently insert the needle tip about one-fourth of the way into the top portion of a well and gently force the buffer into the well. This will flush the accumulated urea and errant bits of acrylamide from the wells. Repeat the process so that all wells of both gels have been cleaned. Connect the apparatus to the power supply and set to a constant 180 V for 15 to 20 min.

While the gel is prerunning, there will be time to prepare the silver stain and carbonate developer solutions. Both solutions may be prepared in bulk, including every constituent except formaldehyde. Silver nitrate solution is light sensitive and should be stored in a brown bottle. The sodium thiosulfate solution should be prepared weekly and stored in the refrigerator. If preparing developing and staining solutions for daily use, then plan on 75 mL for each gel (Procedure Card 7). Do not add formaldehyde [10-mL ampoules of 16%; Electron Microscopy Sciences, Q-Card 1) to either of the solutions.

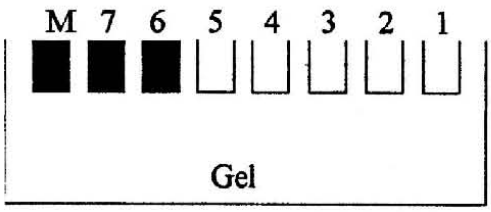
LOADING SAMPLES AND RUNNING THE GEL. After prerunning the gels, clean the wells in one gel as described previously. With a P10 pipette or equivalent adjusted to deliver 6.5 µL, load the samples into the wells using flat tips (Midwest Scientific, Q-Card 1). Since the gels were rotated 180°, load the gels in reverse order or from right to left as indicated in Fig. 3. Draw the far-right sample in the microtitre plate into a flat pipette tip. Keeping the flat tip parallel to the glass plate, guide it partially into the third well from the left side with the left index finger and gently dispense the sample into the well. Be careful not to damage the well. Eject the tip into a beaker of sterile water and load the next sample with a new flat tip. Load all the samples for this gel and repeat the procedure, including cleaning the wells, for the other gel. Reconnect the power supply and run at a constant 180 V for ≈1 h or until the blue tracking dye reaches the level of the bottom electrode. Flat tips can be cleaned by first drawing water and then 95% ethanol through them with a vacuum. These tips do not need to be autoclaved.

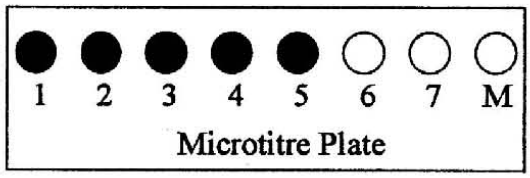
STAINING AND DEVELOPING GELS. Turn off the power and disconnect the gel apparatus from the power supply. Wearing gloves, disassemble the gels under water by first loosening the four knobs and gently removing the glass plate sandwich from the apparatus. Holding the sandwich with the large glass plate contacting the palm of the left hand and in a stream of distilled water, insert the fingernail of the right index finger under the top corner of the small glass plate and gently pry it upward. Let the water stream do most of the work. The backing film and the gel may now be separated from the large plate and placed in a staining tray (Sierra-Lablogix, Inc., Q-Card 1) or in lids from pipette boxes. Follow the staining and developing procedures in Procedure Card 8. Add formaldehyde to silver stain and developer solutions (Procedure Card 7) just before use. After silver staining is completed, quickly and thoroughly rinse the gels to remove all excess silver nitrate solution. Do not pour the cold developing solution directly on the gels; instead introduce the solution onto the bottom of the staining dish and immediately begin to shake in one direction for 20 s and then at a right angle for 20 s. Continue shaking until the bands in the marker and sample lanes are dark and sharp or until the margins and the background of the gel starts to discolor (overdeveloped). After the gels are treated with anticracking solution (Procedure Card 6), they may be hung to dry in a dust-free environment. Label the gels with the experiment number and gel identification number (e.g., 1-A) with a permanent marker after the gels are dry in 12 to 24 h. Gels may be stored indefinitely in photo albums. Q-Card 2 describes some of the more common gel imperfections, their causes, and remedies.

Exercise 4: Data collection and analyses

DAF data will be analyzed using

Fig. 3. Loading DNA samples into the gel. Since the gel apparatus was rotated 180° when mounted on the central stand, the samples must be loaded in reverse order or from right to left in the gel. For example, load sample 8 from the microtitre plate into the third well from the left in the gel.





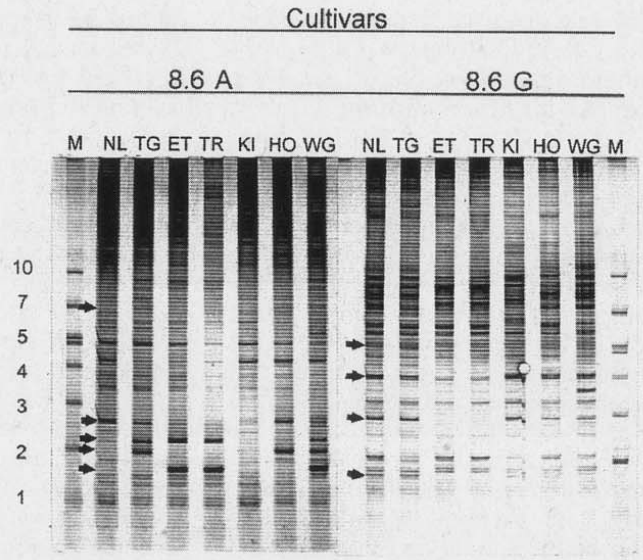


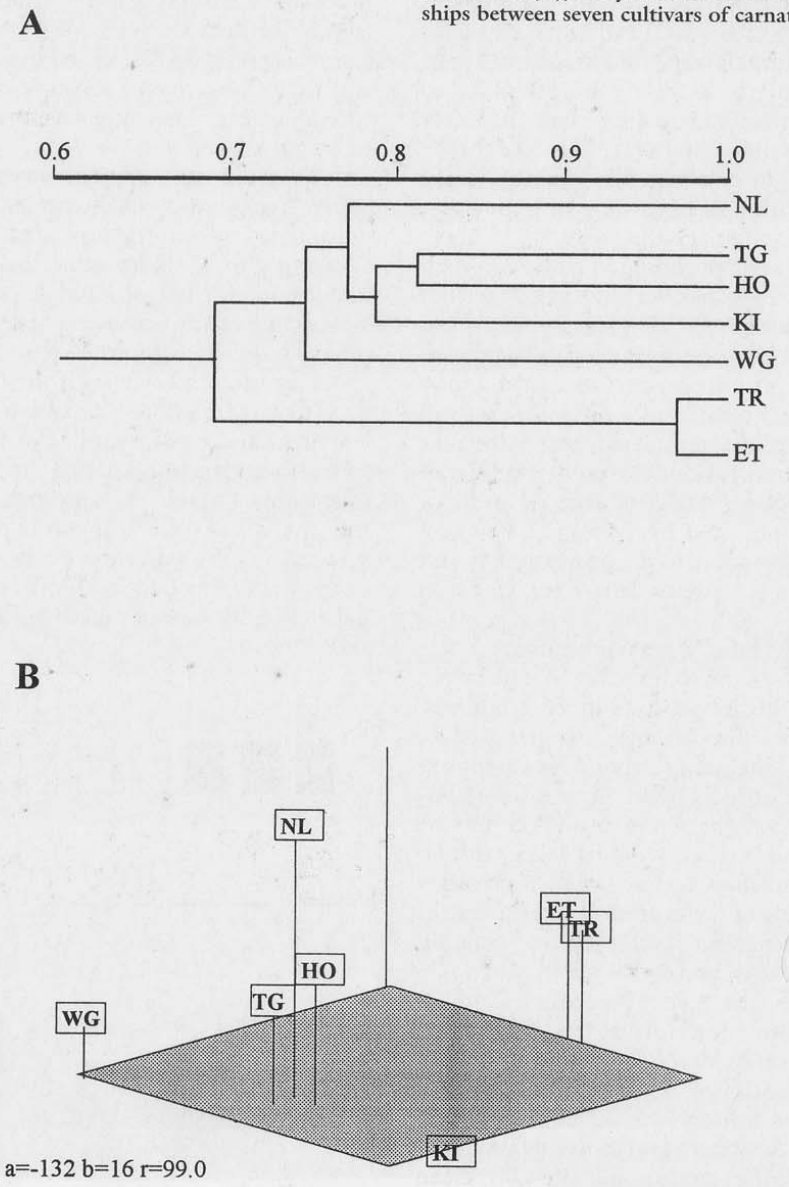
Fig. 4. Actual DNA profiles of carnation cultivars produced by OHLD students. Note the many polymorphisms indicated by arrows when using primers 8.6 A and 8.6 G (not all labelled). Hole in lane KI was caused by an air bubble (dirty plate) and streaking in lane WG of the 8.6G gel was caused by old buffer or dust particles on the bottom surface of the well. Numbers on left equal molecular weight in base pairs × 100.

the DOS-based Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) program, version 1.8 [Exeter Software, Q-Card 1). The analyses are easily understood and provide estimates of genetic distances and relationships (relatedness) between cultivars.

DATA COLLECTION. View the dried gels on a light box (transilluminator) and cover with a clear frosted, glass photographic copy plate. Beginning at ≈700 bp, align common bands in the different sample lanes with a straight edge and enter the binary data: 1 = product present, 0 = product absent, and 9 = missing data. For example, in Fig. 4 (primer 8.6A) at ≈700 bp, a prominent band appears in sample lanes 2, 6, and 7 and not in lanes 1, 3, 4, and 5. The data for this character locus would be the following: 0 1 0 0 0 1 1. Continue to record data for the entire gel. If unknowns are also included in the exercise, note the appearance and approximate weight of unique bands or bands that can either identify or eliminate specific cultivars (Fig. 4). Combine the data from the groups and enter in the computer as shown in Q-Card 3. Be careful to include a hard return after each line of the data set. The first line of the data set should begin with the number 1 followed by the number of lines (character loci) in the data set e.g., 154, then the number of samples (7L) and if there were any missing values (yes, use 1 then 9; if not, then use 0). The next line contains the abbreviations for the samples—NL, TA, etc. for 'Nelson', 'Tanga', etc. (Q-Card 3). Save the data set as an ASCII file.

DATA ANALYSES. Select the NTSYS program and choose Similarity Measures and then Qualitative. Enter the ASCII file name and select Jaccard coefficient. Name the output file as a:carnsim and select show listing to yes with the space bar. Press the F2 key and the similarity matrix should appear (Q-Card 4). If the program warns you of an error, such as unable to read line xxx, return to the original data matrix and check if a hard return appears after each line. If no errors are found, recount the number of character loci in the data set. The values in the similarity matrix provide a estimated measure of relatedness of the cultivars. To perform cluster analysis, select

Fig. 5. Diagrammatic representation of A, UPMGA or cluster, and B, principal coordinate (B), analyses of the relationships between seven cultivars of carnations.



SAHN Clustering and enter the input file name a:carnsim and a:carnclus as the output file. Select UPGMA (unweighted pair group cluster analysis using arithmetic means) and run by pressing F2. To print the tree (Fig. 5A), select Tree Display, use A:carnclus as the input file, and choose phenogram. Follow the remaining directions. To complete principal component analysis (PCO), transform the a:carnsim data matrix using the Double Center option and record the output as a:carndc. Now select Eigenvectors, use a:carndc as the input file, and label the output as a:carneign. The plot of PCO is completed by selecting 3-D Model and inputing the file a:carneign. The direction to plot the analysis is by columns. Run the program with F2 and view the graph and print with ALT P (Fig. 5B). Compare the output of the cluster analysis with that of PCO—they should be similar.

Concluding comments

These laboratory exercises or similar ones have been conducted successfully on the first attempt by several classes of beginning graduate students as well as research personnel. DAF is not a difficult technique and with a little planning should provide an enjoyable learning experience depicting a practical application of a molecular technique for solving horticultural problems and needs. Additional help in the form of photographs illustrating various techniques and operations are available on the home page of the Department Ornamental Horticulture and Landscape Design under the author's names at http://funnelweb.utcc.utk.edu./~uthort.

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Practical Training in Horticultural Undergraduate Courses: Understanding Job Opportunities and Availability through a Study Tour of Alumni

Daniel J. Cantliffe[1] and Stephen R. Kostewicz[2]

Additional index words. vegetable crops, teaching, students

Summary. For many years, the former Vegetable Crops Department, now the Horticultural Sciences Department, at the University of Florida offered a vegetable crop industries course. This one-credit course is offered each year as a 3- to 5-day field trip into vegetable production areas of Florida in the spring semester during spring break. The intent of the course is to give undergraduate students an extensive on-site evaluation of the application of scientific principles learned in lectures related to Florida's commercial vegetable industry. A new, innovative approach to structuring this course was initiated recently wherein only alumni of the department interacted with the students on all phases of commercial vegetable agriculture in Florida. These alumni had obtained degrees at the BS, MS, or PhD level and represented many professional backgrounds related to

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