OPTIMIZING PROCEDURES TO OBTAIN RELIABLE DNA FINGERPRINTING DATA FOR USE IN SYSTEMATIC, ECOLOGICAL AND EVOLUTIONARY STUDIES.

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ABSTRACT

The use of PCR based fingerprinting using short primers is very sensitive to variation in procedures. Strict laboratory procedures, exceeding general molecular biology standards, must be followed. Many laboratories have experienced difficulties in obtaining reproducible banding. Using a stock solution greatly minimizes pipetting small quantities, but the stability of a *Taq* stock is not well known. An investigation of the stability of a RAPDs stock (*Taq*, $MgCl_2$, 10X buffer, dNTPs) revealed the stock to be very stable (for 4 d, and for 60 d at 22°C). Interim storage of DNA at 4°C was found to be a significant source of variability in banding. Variability due to different amounts of polysaccharide inhibitors in the DNA is a significant source of variation in banding. Experiments show that diluting the DNA to about 0.25 ng/rxn (15 µl) dilutes the effects of inhibitors, yielding stable banding. Thermocycler temperature variation between runs can be a problem and methods for monitoring are presented. Other sources of variability are discussed and remedies suggested.

KEY WORDS: PCR, RAPDs, DNA fingerprinting, lab procedures

DNA fingerprinting methods (i.e., producing a bar-code of DNA bands) that utilize inverted repeats include RAPD (Random Amplified Polymorphic DNA), ISSR (Inter Simple Sequence Repeats) and SSR (Simple Sequence Repeats, when using a single primer). Most of these methods do not require sequence knowledge and are widely utilized in gene mapping, populational studies, infraspecific variation, cultivar identification, etc. Studies concerning higher levels of relationships (between genera, families, etc.) almost exclusively utilize DNA sequencing. A search of PubMed and SciFinder gave the following frequencies of citations : RFLP (PubMed 30,621; SciFinder 32,764), RAPD (4,343; 12,560), AFLP (1,597; 5,253), SSR (3,862; 12,659); ISSR (208, 807) and microsatellites (23,612; 7,961). Although RFLP has been the method of choice for gene mapping, other PCR based methods are finding considerable utilization.

RAPDs (Random Amplified Polymorphic DNAs) is a PCR technique that generates DNA fingerprints using a single oligonucleotide primer. The polymorphisms observed may result from point mutations, insertions, deletions and inversions (Williams, et al., 1990). RAPDs are usually dominant markers and are inherited in a simple Mendelian fashion. In comparison with RFLP, the procedure is less expensive, faster, requires a smaller amount of DNA (0.1-0.5 ng), does not involve the use of radioisotopes or fluorescent labels and requires less skill to operate. Because of these advantages RAPDs have proven useful in genotype identification and gene mapping as well as evolutionary studies (Demeke and Adams, 1994).

However, all PCR DNA banding methods rely on clean DNA, reproducible thermocycling temperatures, cycle times and stable, active *Taq* polymerase (or other DNA polymerase), exact pipetting of homogenous solutions and exceptional laboratory methods. Although RAPDs will be the focus of much of this paper, the results apply to other PCR based, fingerprinting methods. Obtaining reproducible RAPD patterns can present a problem in many labs. In fact, Penner et al. (1993) have investigated reproducibility in RAPDs using the same target DNA and primers in different laboratories. They found considerable differences between labs. They concluded that "if the overall temperature profiles (especially the annealing temperature)

inside the tubes are identical among the laboratories, then RAPD fragments are likely to be reproducible."

Benter et al. (1995) concluded that "a slow heating/ ramping from the annealing to the extension temperature increased the number of amplified bands and enhanced reproducibility". Yu and Pauls (1992) explored various PCR programming details to optimize the reactions for RAPDs production. Levi, Rowland and Hartung (1993) evaluated a range of concentrations of Triton X-100, gelatin, dNTPs, primer, template DNA, *Taq*, $MgCl_2$, as well as various times for annealing, elongation and denaturation. Bielawski, Noack and Pumo (1995) examined changes in protocols to obtain reproducible RAPD markers in vertebrate DNA (striped bass, plus a few other vertebrate species). In general, they found that using 30 sec denaturing and 30 sec. annealing times, coupled with the addition of a single-strand binding protein, Gp32, to the reaction mixture prevented nonspecific primer annealing during preparation of the reaction.

Han et al. (2003) examined the stability of RAPDs for genotyping *Helicobacter pylori* and reported that the method was very useful, however, "it seems unstable in amplification of low yield fragments, especially those that do not appear as visible bands on the agarose gel stained with EB, since the primer is partially matched to the template." Of course, these very low intensity bands would never be scored in systematic or evolutionary studies.

A recent examination (Adams, unpublished) of *Styrax texanas* revealed that there is essentially no variation among 30 individuals from three populations (Figure 1). Lanes 1-10, 11-20 and 21-30 are individuals from 3 geographically distinct populations. There is uniformity in the number and intensity of bands. Unless one has extremely good laboratory procedures, it is very difficult to obtain these kinds of results.

Adams, Flournoy and Pandey (1998) examined several sources of errors that cause difficulty in obtaining reproducible PCR amplification, even when using a single PCR machine. Non-uniform mixing of *Taq* was found to lead to considerable variation between duplicate runs. Because glycerol is included in the *Taq*, the material is

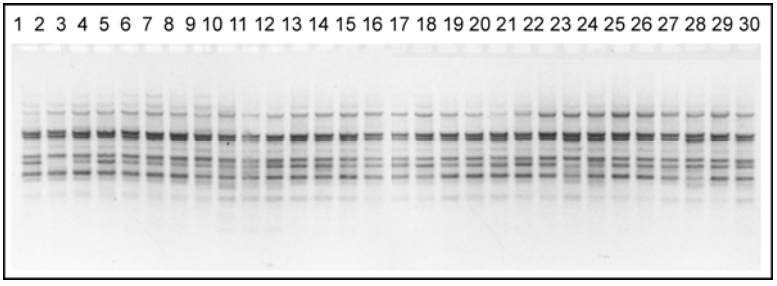

Figure 1. RAPD profiles for 30 individuals of *Styrax texanus* from 3 different populations (1-10, 11-20, 21-30) using UBC primer 431. Note the uniformity of the banding patterns.

difficult to mix and tends to settle to the bottom of the tube. Pipetting of small amounts is a significant source of errors. Therefore, stock solutions that include *Taq* should be prepared and aliquoted. In this paper, we expand on factors that cause difficulty and present some solutions to these problems involved in PCR for RAPDs.

MATERIALS AND METHODS

Plant material

Leaves were obtained from native plants (species, collection number): *Juniperus ashei* Buch. (Adams 7433, 7473), *J. flaccida* Schlecht. var. *flaccida* (Adams 6892), and *Prosopis glandulosa* Torr. (Adams 7375, 7401). Vouchers are on deposit at the Baylor University Herbarium (BAYLU). DNA was extracted using the hot CTAB protocol (Doyle and Doyle, 1987) (note: we recently began using the Qiagen DNeasy plant mini kit and have found it to be superior to the classical CTAB extraction for most species).

RAPD analysis

PCR was performed in a volume of 15 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 2 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton x-100, 0.2 mM of each dNTPs, 0.36 µM primer, 0.3 ng of DNA (except as noted below), and 0.6 unit of Promega *Taq* DNA polymerase. The primers used in this study were (5'-3'): IBC 237: CGA CCA GAG C; #250 CGA CAG TCC C; #327 ATA CGG CGT C; #431 CTG CGG GTC A from the University of British Columbia.

Amplification was performed in a MJ Research Programmable Thermal Cycler. The thermal cycling was: 94°C (1.5 min) for initial strand separation, then 40 cycles at 38°C (2 min) for annealing, 72°C (2 min) for extension, 91°C (1 min). Two additional steps were used: 38°C (2 min) and 72°C(5 min) for final extension. Amplification products were analyzes by electrophoresis in 1.5% agarose (Sigma) gels and detected by staining with ethidium bromide.

Stability of RAPD stock

Because it is desirable to prepare a large volume of the RAPD stock, it is important to understand the stability of *Taq* and how long the stock can be stored. Preliminary tests, storing the *Taq* at 4ºC, did not find any changes after 2 months. To hasten the changes, a new RAPD stock (ddwater, *Taq*, $MgCl_2$, 10X buffer, dNTPs and *Taq*) was made and stored at 22ºC, then utilized after 4 days, 2 weeks, and after 60 days. In addition, a complete PCR RAPD mixture (stock + DNA + primer) was made and stored at 22°C for 4 days, 2 weeks, and 2 months before running PCRs.

Stability of DNA stored at 4ºC

Often DNA is diluted to a uniform concentration (0.05 ng/µl). During the course of running many PCR reactions, DNA stocks are often stored at 4ºC to save time in thawing them and avoid possible freeze-thaw problems. After a few months of DNA storage at 4ºC, the loss of many bands was observed. When a new dilute DNA stock was made from DNA stored at -20ºC, the original banding pattern was restored. To investigate the stability of diluted DNA, samples were stored at 4ºC and -20ºC, and the DNA analyzed after 1, 3, 5 and 8 months of storage. To investigate the effects of freeze/thaw cycles on DNA for PCR, frozen DNA (-20ºC) was thawed every day, an aliquot of DNA taken, and a PCR run, then the DNA sample was re-frozen each day for 60 days.

Dilution of inhibitors by diluting genomic DNA

Many plant species produce considerable amounts of polysaccharides that are inhibitory to RAPDs PCR (Pandey, et al. 1996). *Juniperus flaccida* is such a species. PCR was performed using primer UBC 237 with various amounts of *J. flaccida* DNA: 1 ng/rnx., 0.5 ng/ rxn., 0.25 ng/rxn. and 0.125 ng/rxn. to examine these effects.

RESULTS AND DISCUSSION

Because errors in pipetting very small quantities are frequent, these errors could be minimized by preparing a large quantity of RAPD stock (ddwater, $MgCl_2$, 10x buffer, dNTPs and *Taq*). However, *Taq*, being an enzyme, might lose some activity when stored in solution. Preliminary storage of RAPD stock at 4ºC did not reveal any differences after 2 months. In order to increase the rate of chemical reactions (degradations), the stock was stored at 22ºC for various periods. Figure 2 shows that the RAPD stock is unaffected after 4 days at 22ºC. It was also unaffected after 2 weeks (data not shown). However, after 60 days at 22ºC, there is a noticeable decrease in band brightness, but the pattern and relative intensity of the bands is very similar to the control. Apparently the RAPD stock is very stable at 22ºC and, also extremely stable when stored at 4ºC (data not shown). However, when the primer and target DNA were included in the

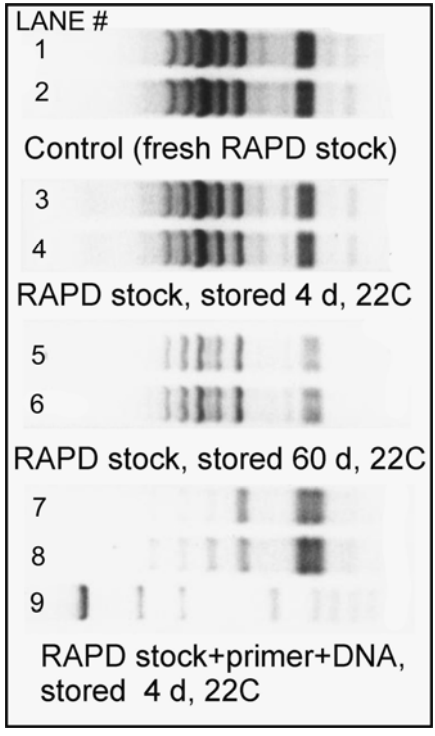

Figure2. PCR using primer 250 and *P. glandulosa* DNA (7401): Lanes 1,2 - fresh RAPD stock; Lanes 3,4 - RAPD stock stored 4 days at 22°C; Lanes 5,6 - RAPD stock stored 60 days at 22°C; Lanes 7,8 - PCR using RAPD stock plus primer 250, plus *P. glandulosa* DNA(7401) stored for 4 days at 22°C; Lane 9 - pGEM markers.

mixture, it was not stable after 4 days at 22ºC (Figure 2). To reduce variation among analyses, it is recommended that an entire tube of *Taq* be used to make RAPD stock (ddwater, $MgCl_2$, 10x buffer, dNTPs and *Taq*) and the stock be stored at 4ºC. Note that the loss of high molecular weight bands is an indication that the stock is degraded.

Due to the reproducibility problems encountered when storing DNA at 4ºC (for ease of utilization), a systematic investigation of DNA storage temperature vs. PCR-RAPDs was performed. Figure 3 shows the triplicate analyses of DNAs from *Prosopis glandulosa* and *Juniperus ashei*, stored frozen (-20ºC) vs. refrigerated (4ºC). Notice that in every case, the frozen DNA yielded reproducible bands, whereas the refrigerated DNAs (stored for 8 mos.) showed considerable

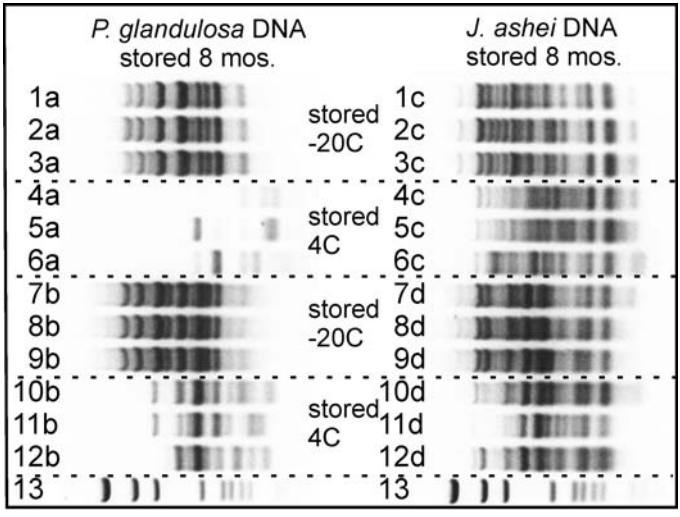

Figure 3. Comparisons of RAPDs (primer 327) obtained from frozen DNA vs. refrigerated DNA (4°C). Panel A: Lanes 1-6, *P. glandulosa* DNA (7401), lanes 1-3 - frozen DNA, lanes 4-6 - DNA stored for 8 mos. at 4°C; Lanes 7-12, *P. glandulosa* DNA (7375), lanes 7-9 - frozen DNA, lanes 10-12 - DNA stored for 8 mos. at 4°C. Panel B: Lanes 1-6, *J. ashei* DNA (7473), lanes 1-3 - frozen DNA, lanes 4-6 - DNA stored at 4°C for 5 mos.; lanes 7-12, *J. ashei* DNA (7433), lanes 7-9 - frozen DNA, lanes 10-12 - DNA stored at 4°C for 8 mos.

variability among the bands and a general loss of the higher molecular weight bands. Storage of DNA at 4ºC for 2 weeks and one month did not reveal problems. Clearly, aliquots of genomic DNA can be stored at 4ºC for several weeks. However, do several freeze-thaw cycles also affect the PCR-RAPDs? A sample of DNA was subject to 60 cycles (days) of freeze-thawing. Analysis revealed there were no effects on the RAPD pattern (data not shown). It seems likely that storing diluted for a few days at 4ºC will not affect PCR. However, the loss of higher molecular weight bands is a clear indicator that the DNA is degraded and should be replenished with new DNA.

Polysaccharides have been shown to inhibit PCR and RAPD banding (Pandey, et al. 1995). It is likely that other inhibitors such as proteins and pigments may be in the DNA extract. Although several methods have been proposed to eliminate inhibitors, a general method is unlikely to be found that will work on all plants (or organisms). *Juniperus flaccida* is a species that produces PCR inhibitors. The potential of using dilution of the DNA to reduce the effects of inhibitors was examined. It is clear that 1 ng of DNA/rxn. is completely inhibitory for primer 237 (Figure 4). At 0.5 ng/rxn. the banding appears, but the highest molecular weight band is missing (the loss of high molecular weight bands is an excellent indicator of the presence of inhibitors). The banding is fully restored at 0.25 ng/rxn and 0.125 ng/rxn. (Figure 4). If high molecular weight bands are not obtained, one should dilute the DNA and re-run the PCR.

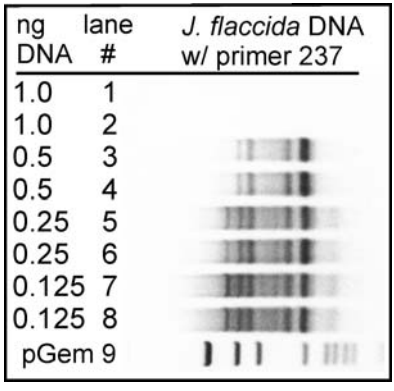

Figure 4. Effects of dilution of DNA from *Juniperus flaccida* (6892) on inhibition of PCR (primer 237). Lanes 1,2 - 1 ng/rxn., lanes 3,4 - 0.5 ng/rxn., lanes 5,6 - 0.25 ng/rxn., lanes 7,8 - 0.125 ng/rxn., lane 9 - pGEM markers.

Optimizing procedures to obtain reliable DNA fingerprinting data in systematic and evolutionary studies

There are several procedures that seem very important in obtaining reproducible RAPD banding and other PCR based fingerprinting. It is very important to vortex all reagents and DNA that have been frozen. This is critical for the Mg stock, as it tends to precipitate upon freezing/thawing, but this seems to be a potential problem with all components (e.g., primers, DNA, dNTPs, *Taq*, Mg, 10X buffer, etc.).

It is best to prepare an entire tube of *Taq* for the RAPD stock (but don't add a primer or DNA). The RAPD stock is stable at 4°C for several weeks. Freeze the RAPD stock if it will not be used for more than one month. Centrifuge the *Taq* tube before adding any components to get the *Taq* near the bottom of the tube. Centrifuge the tube again after all components (ddwater, $MgCl_2$, 10X buffer, dNTPs and *Taq*) have been added. Vortex to get the components well mixed, then centrifuge (but only for a pulse). Then repeat the vortex and centrifuge step. Mixing is very critical and this can be a major problem in training new students. Using a large RAPD stock solution will reduce errors and decrease variability in RAPD analyses

It is important to make up working stocks of DNAs (ex. 0.1 ng/µl), but these stocks should be stored at least at -20°C. It is recommended to return the working DNA stocks back to the freezer after thawing the working DNA stocks prior to making up the PCR reactions. Diluting the DNA in 1mM Tris (pH 8.5) is an effective way to prevent DNA degradation (data not shown).

Perform a concentration test on your DNAs with a reliable, proven primer. Run your DNA at the lowest concentration possible where you still get good, bright bands. For *Juniperus*, 0.3 ng of DNA / 15 µl PCR reaction has effectively eliminated problems with indigenous inhibitors. However, it was necessary to use 0.15 ng of DNA / 15 µl PCR reaction for *Prosopis* analyses.

Proteinase is not often included in most plant DNA extraction protocols, but we have found it to be essential for the extraction of alcohol-preserved specimens (Flournoy, et al. 1996). If extracts of alcohol-preserved specimens are not incubated in proteinase, the histones that have been precipitated onto the DNA will result in the loss of the DNA during extraction. Although Proteinase K is listed for

many protocols, one can substitute Pronase E and it costs only about 5% the cost of Proteinase K.

Try different methods for grinding materials. For most plants, grinding fresh leaves in hot CTAB (60°C) or extraction buffer, resulted in higher yields and higher molecular weight DNA than grinding the leaves in liquid nitrogen and then placing the ground material in hot CTAB (or extraction buffer). However, Adams, Zhong and Fei (1999) reported that for *Vetiveria*, a tropical grass (and all other grasses examined), the DNA yield was very small and it was almost completely degraded when either fresh or dried leaves were ground in hot CTAB (or an extraction buffer). However, grinding the leaves in ethanol resulted in good yields and high quality DNA. We now grind all plant samples in as small amount of ethanol as possible, then dilute with extraction buffer before treating with proteinase (if too much ethanol is present, proteinase will be inhibited). The Qiagen mini-plant extraction kit is also excellent for most applications, but it is very difficult to use on ethanol preserved materials.

The maximum temperature (ex. 94ºC) and minimum temperature (ex. 40ºC) for each well of the Thermocycler should be checked. You may find some cool spots around the margin of the heating block. If so, do not use these areas of the plate.

The temperature pattern for each PCR run should be monitored with a linear strip chart recorder (e.g. Cole Parmer 201 chart recorder coupled to a Omega Engineering, CJ cold joint temperature compensator). This will generate an exact record of each temperature cycle for every run (Figure 5). If there is a deviation in temperature (maximum, minimum or cycle width), it will be very evident. If

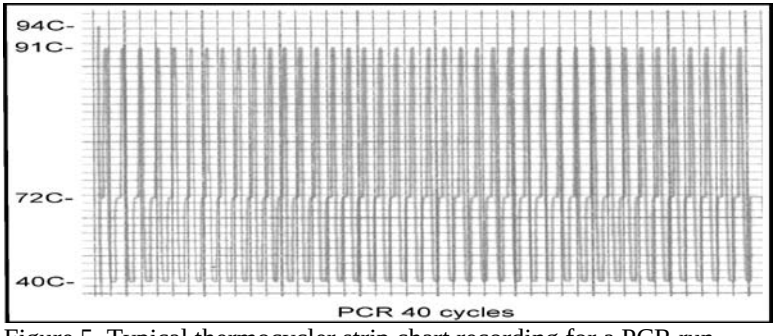

Figure 5. Typical thermocycler strip chart recording for a PCR run.

deviations occur, the PCR thermocycler must be repaired and re-calibrated. A copy of the temperature profile can be placed in the lab book with each PCR analysis.

One of the best safeguards is to run two very closely (or identical) individuals for each taxon or population. Figure 6 shows 5 taxa of *Juniperus* with 2 individuals run from each taxon. Two similar individuals that were growing near each other were intentionally sampled and used to represent the taxon. Notice that each of the pairs is very similar in their banding pattern (Figure 6). It is often the case that one of these will not have the larger band or the larger bands will be very faint. One should re-run DNA in triplicate from the poor performing individual. If it still fails to be similar to the other individual, after diluting the DNA, then new DNA needs to be extracted. Variation in banding between near-identical individuals (or sibs) is an excellent method by which to obtain constant feedback in every analysis.

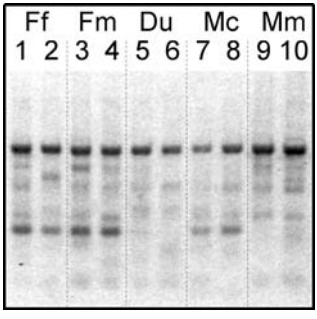

Figure 6. DNA banding for pairs of individuals from five taxa of *Juniperus*. Note the similarity between individuals.

After loading all components into a PCR tube for a RAPD run, it is important to make sure all the mixture is in the bottom of the tube (pulse centrifuge in a mini-centrifuge), then vortex 10 sec to make a uniform mixture. The *Taq* tends to settle on the bottom and it is critical that the *Taq* be well dispersed in the solution. A quick pulse centrifuge of the mixture is required to get all the solution to the bottom of the tube. Then check to see if any air bubbles are in the tube. If there are bubbles in the tube, tap it on a table until they are removed, then centrifuge with a quick pulse. This is such a critical procedure that we do this twice to make very sure that components are mixed.

If you are using a PCR tube format, add a drop of oil to the holes in the PCR machine. When you place the PCR tube in a hole, a little oil should come out around the tube. Be sure tubes fit down securely in the holes in the PCR machine and that the PCR tube lids are completely closed.

Evaluate PCR tubes from several suppliers, using the same DNA, primer(s), *Taq*, etc. Use the PCR tubes that work best for your PCR machine and lab conditions. There are considerable differences among suppliers.

Screen lots of primers. Screening of 600 primers from the University of British Columbia has revealed (Adams, et al. 1998) that about 25% of the primers give no bands, about 50% give a few bands and about 25% produce 5 or more bands. Of the best 25%, about 1/4 will be really excellent. Out of 100 primers, one should expect to find 5 to 10 really good primers. Generally, these primers are useful for all species we have studied (ranging from gymnosperms to monocots and dicots, as well as fish). It is not time-efficient to attempt to optimize the PCR (Mg concentration is particularly sensitive) for every possible primer.

Some primers are more useful at the specific level, whereas other primers are more useful to characterize differences between individuals. Screen until primers are found that will give the necessary resolution.

In addition, it should be emphasized that multivariate statistical methods have the capability of accounting for error variance and are highly desirable for analysis. The use of parsimony tree building methods is not appropriate for PCR based methods because there is no provision to allow for error variance. However, chemosystematists, who have worked many years with secondary compound data, are well aware of error variance and the need to factor data to remove (and account for) error variance. Multivariate methods that are compatible with PCR banding data include PCO (Principal Coordinate Ordination), PCA (Principal Components Analysis), and CVA (Canonical Variate Analysis). Minimum spanning networks and neighbor joining methods can be used with some cautions.

In conclusion, obtaining reproducible PCR banding can be difficult. It demands very strict lab conditions and attention to detail. However, PCR banding can reproducible if exacting laboratory procedures are followed and appropriate analyses methods are utilized.

ACKNOWLEDGEMENTS

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LITERATURE CITED

Adams R. P. , L. E. Flournoy and R. N. Pandey. 1998. Obtaining reproducible patterns from random polymorphic DNA amplification (RAPDs). In: Adams RP and Adams, JE (eds.) Conservation of Plant Genes III: Conservation and utilization of African plants, pp. 229-236, Missouri Botanical Garden, St. Louis.

Benter T., S. Papadopoulos, M. Pape, M. Manns and H. Poliwoda. 1995. Optimization and reproducibility of random amplified polymorphic DNA in human. Anal. Biochem. 230: 92-00.

Bielawski J. P., K. Noack and D. E. Pumo. 1995. Reproducible amplification of RAPD markers from vertebrate DNA. BioTechniques 18: 856-859.

Demeke T. and R. P. Adams. 1994. The use of PCR-RAPD analysis in plant taxonomy and evolution. In: Griffin H.G. and Griffin A. M. (eds.), PCR Technology: Current Innovations, pp. 179-192, CRC Press, Boca Raton, FL.

Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15.

Flournoy L. E., R. P. Adams and R. N. Pandey. 1996. Interim and archival preservation of plant specimens in alcohols for DNA studies. BioTechniques 20: 657-660.

Han F. C., H. C. Ng, and B. Ho. 2003. Stability of randomly amplified polymorphic DNA fingerprinting in genotyping clinical isolates of *Helicobacter pylori*. World J. Gastronentrol. 9: 2021-2024.

Levi A., L. J. Rowland and J. S. Hartung. 1993. Production of reliable randomly amplified polymorphic DNA (RAPD) markers from DNA of woody plants. HortScience 28: 1188-1190.

Pandey R. N., R. P. Adams and L. E. Flournoy. 1996. Inhibition of random amplified polymorphic DNAs (RAPDs) by plant polysaccharides. Plant Mol. Biol. Reptr. 14: 17-22.

Penner G. A., A. Bush, R. Wise, W, Kim, L. Domier, K. Kasha, A. Laroche, G. Scoles, S. Molnar and G. Fedak. 1993. Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. PCR Methods and Applications, 2: 341-345.

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Yu K. and K. P. Pauls. 1992. Optimization of the PCR program for RAPD analysis. Nucleic Acids Res. 20: 2606-2608.