From Field to Film: Rapid Sequencing Methods for Field-Collected Plant Species

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ABSTRACT

Methods that have proven effective in the rapid extraction of plant DNA, PCR amplification and sequencing of ribosomal and chloroplast genes are presented. Techniques that can be used under field conditions to preserve DNA for subsequent extraction are reviewed. Tissues that are fresh, heat-desiccated, silica gel-desiccated and cetyltrimethylammonium bromide (CTAB) buffer preserved are compared for DNA quality and quantity. The "delayed CTAB" method yields high molecular weight DNA, thereby providing an alternative to preservation using silica gel. Optimized methodologies for PCR amplification and double-stranded sequencing of the product are detailed including gel purification of PCR products using DEAE membranes.

INTRODUCTION

With the growing interest in plant molecular phylogenetic studies and the advent of the polymerase chain reaction (PCR) (20), an increasing number of workers are using DNA sequencing as opposed to other approaches. This report describes a series of methods that have proven effective in the extraction of DNA, PCR amplification and sequencing of plant ribosomal and chloroplast genes. Given an interest in obtaining sequence information from a large number of plants from taxonomically diverse groups, methods have been selected that do not require long optimizations for any one species. Although not all of the methodologies compiled here are novel, each has been selected for convenience, speed and simplicity without sacrificing the final sequence quality. A review of the methods used to obtain DNA from plant samples that have been collected in remote locations and preserved in different ways, and a liquid preservation procedure—the "delayed hot CTAB" method—are discussed. The use of gel purification and direct sequencing of double-stranded PCR products is also presented.

PLANT DNA PRESERVATION AND EXTRACTION

The eventual use of the DNA ultimately determines one's requirements for amount and purity. Fresh or liquid nitrogen frozen tissues are obviously ideal; however, these options may not be available when collecting plants in remote areas (4,18,27). For this reason, a number of workers have experimented with alternate methods to preserve plant tissues prior to initiating DNA extractions. The limiting factors associated with obtaining good quantity and quality DNA from plants are tissue type, tissue amount (8,26), presence of secondary compounds such as polyphenolics (28), polysaccharides (6, 10), and extent of DNA degradation (when using other than fresh tissues). One of the first studies to examine such factors was by Rogers and Bendich (26) who tested the effect of the preservation method (fresh, frozen, dried, chemically fixed, etc.), tissue type (leaves, seeds, cotyledons, pollen, etc.) and tissue amount (ranging from milligram to gram amounts) on genomic DNA yield. Similarly, the study by Pyle and Adams (24) tested dried leaves as well as tissues stored in a number of chemical preservatives such as ethanol, Chlorox®, NaCl and formaldehyde. None of the solutions preserved DNA for at least seven days. Rogstad (27) had better success using a saturated NaCl–cetyltrimethylammonium bromide) (NaCl–CTAB) solution, at least for some of the plant species used.

One of the most effective and widely used methods to preserve plant tissue for subsequent DNA extraction is rapid drying at ambient temperatures using desiccants such as silica gel or $CaSO_4$ (W.A. Hammond Drierite, Xenia, OH, USA). Experiments using silica gel showed that this method often results in high-quality DNA that can be used for restriction endonuclease

digestions, PCR and other applications (4,18). The most appealing aspect of this preservation method is that field-collected plant material can be quickly and easily preserved and shipped at ambient temperatures for later DNA extraction. It should be noted that extreme drying, e.g., over phosphorus pentoxide, removes structurally important water molecules from the DNA double helix, which makes bases more prone to damage (17).

Plant samples dried as herbarium specimens have also been used as sources of DNA. Such samples may be suitable for PCR amplification; however, the quantity and quality are often not sufficient for restriction fragment analyses. The factors that are important in obtaining DNA from traditionally dried samples are age of the specimen, drying temperature (gentle heat is optimal) and the taxonomic group. The use of ethanol or formaldehyde to combat fungal growth on specimens collected in moist, tropical areas adversely affects DNA quality and should be avoided if the samples are to be used for molecular work. Successful DNA extractions from herbarium material were obtained from several families of parasitic plants such as an 8-year-old specimen of *Ginalloa* (Viscaceae) and a number of genera of African Loranthaceae (e.g., *Oncocalyx*, Figure 1, lane 4). The latter are interesting since members of this family typically contain large amounts of secondary compounds that make DNA extractions difficult even with fresh tissue. Since the leaf samples were not discolored (darkened), it is assumed that they were dried slowly and with low heat.

Since silica gel drying may result in DNA degradation, an alternative method was developed called the "delayed hot CTAB" method. This procedure, a modification of that described by Rogstad (27), has also been used successfully to obtain DNA from 20 different genera of parasitic plants from Costa Rica and Australia. This method assumes no refrigeration is available and all of the following items can be packed into a small suitcase (carry-on size for most airlines): homogenizer (e.g., Polytron®; Brinkman Instruments, Westbury, NY, USA), stirring hot plate and stir bar, miniature top-loading balance, thermometer, wash bottle, glass test tubes (25 × 200 mm), cheesecloth, funnels, as many 50-mL sterile polypropylene centrifuge tubes with caps (e.g., Corning 25330; Corning, NY, USA) as the number of samples to be collected, scissors, beakers, 500 mL plastic bottle and the dry CTAB buffer components. The modified 2× CTAB buffer (8) is composed of 100 mM Tris-HCl, 1.4 M NaCl, 30 mM EDTA, 2% wt/vol CTAB, 5 mM ascorbic acid, 4 mM diethyldithiocarbamic acid (DIECA) and 2% wt/vol polyvinylpyrrolidone (PVP-40), the latter two ingredients added just before use.

The best DNA extracts come from young, recently collected material. For extraction, the buffer (ca. 25 mL for every 2–3 g of plant tissue) is heated to 95°C in a water bath. The sample is cut into small pieces, placed in the glass test tube and homogenized in the hot CTAB buffer. Homogenization should use only short bursts at low speeds to reduce shearing of DNA and minimize foaming. The extract is then strained through cheesecloth into the sterile 50-mL tube and incubated in a warm (70°–80°C) water bath for about 30 min with occasional swirling. The parasitic plant samples collected in Australia were processed to this point and stored at ambient temperature for four weeks prior to completing the extraction.

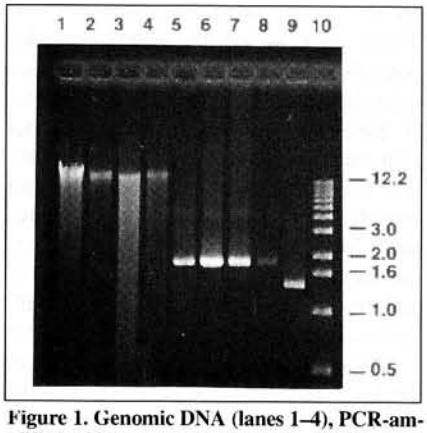

Figure 1. Genomic DNA (lanes 1–4), PCR-amplified 18S rRNA (lanes 5–8), PCR-amplified *rbc*L (lane 9) and 1-kb ladder (lane 10). Lane 1, fresh extracted *Viscum album* (Nickrent No. 2145). Lane 2, from silica gel dried *Thesium impeditum* (No. 2845). Lane 3, delayed (4 weeks) CTAB-extracted *Notothixos subaureus* (No. 2790). Lane 4, from herbarium specimen of *Oncocalyx sulfurens* (No. 2850). Lanes 5–8, 1.8-kb PCR products amplified from genomic DNA shown in lanes 1–4. Lane 9, 1.4-kb PCR product amplified from delayed CTAB-extracted genomic DNA of *Opilia amentacea* (No. 2816).

After returning to the laboratory, the samples are briefly centrifuged, and 20 mL of the supernatant are transferred to a clean 50-mL centrifuge tube. Chloroform:isoamyl alcohol (24:1) (0.7 volume) is added and the tubes are mixed for 5 min. They are then centrifuged at 8500 rpm for 15 min, and the aqueous phase is removed with a wide-bore pipet and placed in a clean centrifuge tube. The DNA is precipitated by the addition of a 0.7 volume of ice-cold isopropanol and incubated at -20°C for at least 1 h. The DNA pellet is collected by centrifugation for 20 min at 10000 rpm. The pellet is allowed to dry at ambient temperature for 30 min and then resuspended in 3.0 mL of TE (1 mM Tris, 0.1 mM EDTA, pH 7.5) and 2.0 mL of 4.0 M ammonium acetate. The solution is extracted with an equal volume of Tris-saturated (pH 8.0) phenol and chloroform (1:1), and 2 volumes of ethanol are added to the aqueous phase. The tube is incubated at -20°C for at least 30 min, and the DNA is collected by centrifugation, dried and resuspended in TE. An RNase digestion can be performed at this time if required.

The above method differs from that of Rogstad (27) in that the buffer is not saturated in CTAB and NaCl, and it contains additional ingredients. Also, instead of simply cutting the leaf tissue and placing in the CTAB buffer, this method quickly homogenizes the tissue in buffer, which is similar to grinding on liquid nitrogen (the standard method when starting in the laboratory). Homogenization and heating following extraction may be important in allowing the buffer components to quickly begin binding polyphenolics and denaturing DNases. Figure 1 shows the results of genomic DNA extractions from fresh plant tissue or tissue preserved using silica gel, the delayed CTAB method and traditional drying (herbarium specimen). Although some DNA degradation is seen, the delayed hot CTAB method results in a sufficient quantity of high molecular weight DNA for PCR amplification and other procedures (Figure 1, *Notothixos*, lane 3).

PCR AMPLIFICATION OF PLANT GENES

Increasing attention has recently been focused on the direct sequencing of double-stranded PCR products. This method is preferred over asymmetric PCR (11) since it eliminates the second round of amplification (thereby saving time) and further reduces base incorporation errors inherent in the PCR process. It also overcomes the difficulty sometimes encountered when attempting amplification from an existing PCR product and allows determination of sequence from both strands of the same product.

In plant phylogenetic studies, the chloroplast gene *rbc*L has been the focus of a number of studies (1,22); however, nuclear 18S (and 26S) ribosomal RNA is now gaining increasing attention (12,21). PCR amplifications were performed in 0.5-mL microcentrifuge tubes in a Perkin-Elmer DNA Thermal Cycler. The reaction mixture contained (final concentrations in a 100-µL reaction): 50 mM KCl, 10 mM Tris-HCl pH 8.8, 0.1% Triton® X-100, 2.5 mM $MgCl_2$, 1.25 mM of each deoxyribonucleoside triphosphate (dNTP), 1 µL of each primer at 125 µg/mL (ca. 20 pmol), 2.5 units of *Taq* DNA Polymerase (M166; Promega, Madison, WI, USA), and about 10–30 ng of genomic DNA. The tubes are overlaid with mineral oil, centrifuged briefly and placed in the thermal cycler. An initial incubation (time delay) at 94°C for 3 min is linked to 35 cycles, each of which consists of 1 min at 94°C, 1 min at 50°C, and 2.5 min at 72°C. Two seconds are added to each extension step as the cycling progresses using the autoextension function. Figure 1 shows the results of PCR amplification of a 1.8-kb portion of 18S rDNA obtained from plant genomic DNAs (fresh and preserved in silica gel, CTAB buffer and an herbarium specimen) and a 1.4-kb product containing *rbc*L amplified from delayed CTAB-preserved *Opilia*.

In the PCR mixture, the optimal $MgCl_2$ concentration for the particular organism, primer combination, etc., must be determined experimentally and ranges from 1.0 to 3.0 mM (often on the high end of the range for plants). Oxidation of the dNTPs occurs following numerous rounds of freezing and thawing. This is circumvented by aliquoting small volumes of stock solution and storage at -70°C. High concentrations of RNA are known to suppress PCR amplifications (23). This is especially the case when large amounts of rRNA exist and one is attempting to amplify rDNA genes. This problem can be overcome by digesting the genomic DNA with RNase (e.g., RNase A, Type I-AS; Sigma Chemical, St. Louis, MO, USA) or by agarose gel purification (above). If amplifications are still not successful, other impurities (such as secondary compounds) should be suspected. Moderately impure DNA can often be amplified by further dilution; however, a rapid and reliable method involves purification of the genomic DNA on low melting temperature agarose (e.g., NuSieve®; FMC BioProducts, Rockland, ME, USA) containing ethidium bromide. A 1% gel is prepared in TBE or Tris-acetate. Genomic DNA (20 µL; ca. 5 µg) is mixed with 2 µL of Ficoll® loading dye (Pharmacia Biotech, Piscataway, NJ, USA) (25% Type 400 Ficoll, 40 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.05% bromophenol blue), loaded into the gel and electrophoresed. The genomic DNA band is visualized with long wavelength UV light, excised and excess agarose is removed. The band is then diluted 1:1 (by weight) with sterile water. For use in PCRs, the NuSieve band is heated to 65°C and about 1.0 µL used per 100 µL PCR. This method is more rapid and straightforward than purification using CsCl and has allowed amplification of numerous recalitrant plants.

GEL PURIFICATION OF PCR PRODUCTS USING DEAE MEMBRANES

Potential contaminants in a PCR mixture include remaining primers, *Taq* DNA Polymerase, unincorporated nucleotides, compounds introduced with the template DNA and mineral oil (if this was used as an overlay). These must be removed before proceeding to sequencing reactions. A number of methods to purify PCR products for sequencing are currently in use such as glass powder (GeneClean®; Bio 101, La Jolla, CA, USA) (25) or direct precipitation using ethanol or polyethylene glycol. Ultrafiltration devices, such as Centricon®-100 (Amicon, Beverly, MA, USA), are also quite effective in the removal of primers, mineral oil, and so on, from PCR products (15). Many of these methods, however, do not allow the isolation of a single band when multiple PCR products are obtained. Direct sequencing from PCR products excised from low melting point agarose gels has been reported (14), or the DNA in bands excised from gels may also be removed from the agarose using chaotropic agents combined with microconcentrator filter units (e.g., Spin-Bind™; FMC BioProducts). Although not tested, direct elution from an agarose gel, such as the method described by Hansen et al. (13), likely would produce similarly high-quality PCR product templates for sequencing.

The procedure described below involves agarose gel purification and recovery of the PCR product by binding onto a DEAE membrane (Grade NA45, No. 23400; Schleicher & Schuell, Keene, NH, USA). Similar recovery protocols from agarose and acrylamide gels have been reported that utilize DEAE-cellulose (9), but the former appears to be more durable, hence easier to use. This simple method of recovering nucleic acids up to 7 kb in length has been known for some time (19); however, its application to the purification of PCR products has not been widely reported. Template concentration, homogeneity and purity appear to be critical in obtaining good sequence from PCR products. We have found that higher yields are obtained using DEAE membranes than with spin columns or glass bead methods, possibly owing to retention of DNA on spin-column membranes or glass surfaces. The DEAE membrane method yields DNA of very high purity (29). Primers and nucleotides are eliminated owing to their faster migration rate on the agarose gel. Remnant mineral oil floats to the top of the gel buffer upon loading and is subsequently not carried over with the purified product.

The amount of PCR product to be made and purified depends on the amount required for subsequent sequencing reactions. As a starting point, we have found that 100 µL of product

is usually sufficient for four primer/template combinations. For purifying several 100-µL samples, a 20- × 6- × 1.2-cm gel with a 16-place comb is used. The comb teeth are 10 × 12 × 1.5 mm which produce wells that each hold 100 µL of sample. A 1% Tris-acetate agarose gel (15 mM Tris-HCl, 33 mM acetate, pH to 8.3 with glacial acetic acid) with ethidium bromide (10 µg/100 mL gel) is prepared. The membranes are cut to fit the gel lanes (7.5 × 11 mm) and washed for 10 min in 10 mM EDTA, then 5 min in 0.5 M NaOH. The membranes are rinsed several times in distilled water and stored in water at 4°C until needed (up to several weeks). One hundred microliters of sample are mixed with 10 µL of Ficoll loading dye, loaded onto the gel and electrophoresed. When the band has migrated the desired distance (check progress with long wave UV light), the gel is removed from the electrode chamber, and an incision is made just ahead of the band of interest. The prepared DEAE membrane is placed in the incision, the gel is returned to the electrode chamber and electrophoresis is continued. After approximately 10 min, the DNA is bound to the membrane.

To elute the DNA from the membranes, they are removed from the gel and placed in a microcentrifuge tube. One milliliter of NET buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl pH 8.0) is added, the tube is vortex mixed briefly to remove residual agarose and the buffer is eluted. For 2–3 membranes, 350 µL of high-salt NET buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl pH 8.0) are added and the tubes incubated at 65°C for 30 min with occasional vortex mixing. The buffer is transferred to a clean tube. The membranes are washed with 50–75 µL of high-salt NET buffer and incubated at 65°C for 10 min, and then the buffers are pooled. The buffer is then extracted with 3 volumes of water-saturated *n*-butanol (BT-105; Sigma Chemical), vortex mixed, centrifuged for 5 min and the top phase removed. Two and one-half volumes of 100% ethanol are added, mixed thoroughly and precipitated at -70°C for at least 1 h. The pellet is collected by centrifugation for 20 min at 4°C and then rinsed in 70% ethanol. The ethanol is decanted, and the pellet is dried and resuspended in sterile, distilled water. The intensity of the bands in the gel should be used to determine the appropriate dilution, but usually 30 µL are used for every 100 µL of double-stranded DNA product loaded on the gel. Optionally, one can determine the DNA concentration spectrophotometrically, ideally using 0.5–1.0 µg per sequencing reaction set.

SEQUENCING REACTIONS

Many articles have appeared in recent years describing various methods for sequencing double-stranded DNA produced by PCR (3,7,16,25). The factors that appear most important in double-stranded sequencing are homogeneity and purity of the PCR product (to reduce false priming, seen as doublets on the sequencing ladder), efficiency of the primer/template annealing, and proper ratios of template DNA, nucleotides and primer. The template can be alkaline-denatured using sodium hydroxide (5,30) or denatured with heat. Since the two strands of the template DNA have a much higher binding affinity to each other than to the primer, it is essential to set the conditions so that primer annealing is favored. Rapid cooling at -70°C produces better results (judged by the clarity of sequencing ladders) than gradual annealing at warmer temperatures, even on molecules with a high degree of secondary structure (such as rRNA). The following protocol appears to work as well as alkaline denaturation and requires less time.

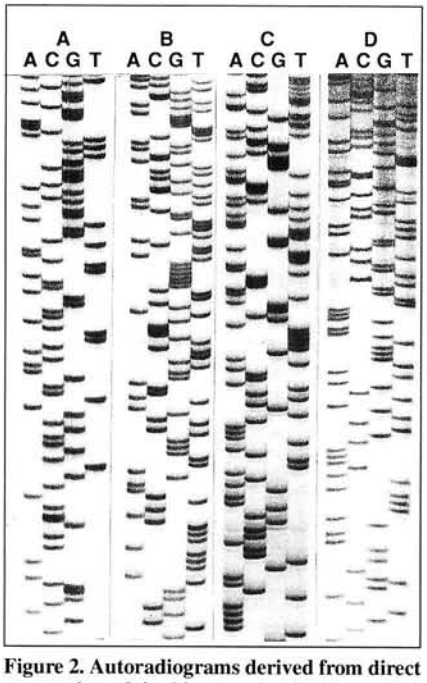

Figure 2. Autoradiograms derived from direct sequencing of double-stranded DNA purified using DEAE membranes. Set A, fresh extracted *Saruma henryi* 18S rDNA (1830 reverse primer). Set B, delayed CTAB-extracted *Opilia amentacea rbc*L (Z-967 forward primer). Set C, silica gel-preserved *Myzodendron* sp. *rbc*L (Z-674 reverse primer). Set D, *E. coli fur*, amplified directly from lysed cells (36 forward primer).

The double-stranded DNA (7 µL or ca. 0.5–1.0 pmol) is mixed with 1 µL of primer (125 µg/mL or 20 pmol) and the tube cap secured with lid locks. The primer/DNA mixture is then placed in a boiling water bath for 1 to 2 min, snap-cooled for 1–2 min in a -70°C ethanol bath and centrifuged briefly. Two microliters of 5× Sequenase® reaction buffer (United States Biochemical, Cleveland, OH, USA) are then added. The reaction mixture is allowed to anneal at 37°C for 20 min. To the annealed mixure, the following are added (per set): 1 µL of Mn buffer, 1 µL of 0.1 M dithiothreitol (DTT), 2 µL Sequenase labeling mixture (diluted 1:5 in dH_2O), 0.5 µL [^{35}S]dATP and 2 µL of Sequenase (diluted 1:8 in enzyme dilution buffer). The reaction is mixed and incubated at room temperature for 5 min. Labeling mixture (3.5 µL) is added to each termination tube (A, C, G, T) containing 2.5 µL of the appropriate Sequenase termination mixture (see below). These are mixed and incubated at 37°C for 5 min. The reaction is terminated by the addition of 4 µL of stop solution and stored at -20°C. For electrophoresis, the reaction tubes are heated to 85°C for 2 min, and 4 µL are loaded per lane in a standard (Tris-boric acid, EDTA) 6% or 8% acrylamide gel. Typical electrophoresis and autoradiography are then performed. Routinely, an 8% gel is used to read bases near the primer (2 h at 37 W), and a second loading on a 6% gel (7 h at 37 W) is used to resolve bases farther from the primer.

Experiments with primer concentrations in the range recommended in the Sequenase protocol manual (0.5–1.0 pmol) produced a very light sequence.

Higher concentrations (e.g., 20 pmol) are required for double-stranded sequencing to optimize primer annealing. As previously noted (2,3), double-stranded sequencing is especially sensitive to the molar ratio of template DNA to nucleotides in the labeling reaction. Sufficient DNA template must be present to provide enough terminations close to the primer to visualize small products. When sequencing double-stranded PCR products, a high concentration of template is not a problem since this can be controlled by dilution, whereas insufficient DNA is more likely the cause of light bands on autoradiograms. To realize long reads using double-stranded sequencing, the extending mixtures described in the Sequenase protocol manual should be used. By using the 2.5× relative extension, long reads can be obtained without sacrificing fragments near the primer. Resolution of these small fragments is also increased by routinely using the Mn buffer, which does not appear to adversely affect long gels run from the same reaction. Also, by diluting the dGTP labeling mixture by 3.75 (11 µL dH_2O + 4 µL dGTP labeling mixture) instead of the recommended 5×, the readable sequence is maximized on the long gels. For sequencing rRNA genes where secondary structure may result in band compressions, inositol and deaza nucleotides (dITP or 7-deaza-dGTP) can be used in parallel with the dGTPs. With these modifications, 300–350 bp can routinely be read per sequencing reaction.

Figure 2 shows results of double-stranded sequencing of PCR products obtained by amplification of two different plant genes (18S rRNA and *rbc*L) and a bacterial gene (*fur*). These results demonstrate that the DEAE membrane purification method, combined with heat denaturation, can result in superior double-stranded sequencing reactions using a variety of organisms, genes and primer combinations.

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REFERENCES

1. **Albert, V.A., S.E. Williams and M.W. Chase.** 1992. Carnivorous plants: phylogeny and structural evolution. Science *257*:1491-1495.
2. **Anderson, R.D., C.-Y. Bao, D.T. Minnick, M. Veigl and W. D. Sedwick.** 1992. Optimization of double-stranded sequencing for polymerase chain reaction products. US Biochem. Comments *19*:39-58.
3. **Casanova, J.-L., C. Pannetier, C. Javlin and P. Kourilisky.** 1990. Optimal conditions for directly sequencing double-stranded PCR products with Sequenase®. Nucleic Acids Res.*18*:4028.
4. **Chase, M.W. and H.H. Hills.** 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. Taxon *40*:215-220.
5. **Chi, H.-C., J.-C. Hsieh, C.-F. Hui and M.F. Tam.** 1988. Modified method for double-stranded DNA sequencing and synthetic oligonucleotide purification. Nucleic Acids Res. *16*:10382.
6. **Do, N. and R.P. Adams.** 1991. A simple technique for removing plant polysaccharide contaminants from plant DNA. BioTechniques *10*:162-166.
7. **Douglas, A.M., A.M. Georgalis and B.A. Atchison.** 1993. Direct sequencing of double-stranded PCR products incorporating a chemiluminescent detection procedure. BioTechniques *14*:824-828.
8. **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.
9. **Dretzen, G., M. Bellard, P. Sassone-Corsi and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. *112*:295-298.
10. **Fang, G., S. Hammar and R. Grumet.** 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques *13*:52-54.
11. **Gyllensten, U.B. and H.A. Erlich.** 1988. Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the *HLA-DQA* locus. Proc. Natl. Acad. Sci. USA *85*:7652-7656.
12. **Hamby, K.R. and E.A. Zimmer.** 1992. Ribosomal RNA as a phylogenetic tool in plant systematics, p. 50-91. *In* P.S. Soltis, D.E. Soltis and J.J. Doyle (Eds.), Molecular Systematics of Plants. Chapman and Hall, New York.
13. **Hansen, H., H. Lemke and U. Bodner.** 1993. Rapid and simple purification of PCR products by direct band elution during agarose gel electrophoresis. BioTechniques *14*:28-29.
14. **Kretz, K.A., G.S. Carson and J.S. O'Brien.** 1989. Direct sequencing from low-melt agarose with Sequenase®. Nucleic Acids Res. *17*:5864.
15. **Krowczynska, A.M. and M.B. Henderson.** 1992. Efficient purification of PCR products using ultrafiltration. BioTechniques *13*:286-289.
16. **Kusukawa, N., T. Uemori, K. Asada and I. Kato.** 1990. Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. BioTechniques *9*:66-72.
17. **Lindahl, T.** 1993. Instability and decay of the primary structure of DNA. Nature *362*:709-715.
18. **Liston, A., L.H. Riesberg, R.P. Adams and Z. Ge-lin.** 1990. A method for collecting dried plant specimens for DNA and isozyme analyses, and the results of a field test in Xinjiang, China. Ann. Missouri Bot. Gard. *77*: 859-863.
19. **Lizardi, P.M., R. Binder and S.A. Shart.** 1984. Preparative isolation of DNA and biologically active mRNA from diethylaminoethyl membrane. Gene Anal. Tech. *1*:33-39.
20. **Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn and H. Erlich.** 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb. Symp. Quant. Biol. *51*:263-273.
21. **Nickrent D.L. and C.R. Franchina.** 1990. Phylogenetic relationships of the Santalales and relatives. J. Mol. Evol. *31*:294-301.
22. **Olmstead R.G., H.J. Michaels, K.M. Scott and J.D. Palmer.** 1992. Monophyly of the Asteridae and identification of its major lineages inferred from *rbc*L sequences. Ann. Missouri Bot. Gard. *79*:249-265.
23. **Pikaart, M.J. and B. Villeponteau.** 1993. Suppression of PCR amplification by high levels of RNA. BioTechniques *14*:24-25.
24. **Pyle, M.M. and R.P. Adams.** 1989. *In situ* preservation of DNA in plant specimens. Taxon *38*:576-581.
25. **Reichardt, J.K.V. and S.L.C. Woo.** 1990. Direct double-stranded sequencing of PCR products. US Biochem. Comments *17*:21-22.
26. **Rogers, S.O. and A.J. Bendich.** 1985. Extraction of DNA from milligram amounts of fresh, herbarium, and mummified plant tissues. Plant Mol. Biol. *5*:69-76.
27. **Rogstad, S.H.** 1992. Saturated NaCl-CTAB solution as a means of field preservation of leaves for DNA analysis. Taxon *41*:701-708.
28. **Rowland, L.J. and B. Nguyen.** 1993. Use of polyethylene glycol for purification of DNA from leaf tissue of woody plants. BioTechniques *14*:735-736.
29. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. **Zhang, H., R. Scholl, J. Browse and C. Somerville.** 1988. Double stranded DNA sequencing as a choice for DNA sequencing. Nucleic Acids Res. *16*:1220.

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