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A novel and efficient protocol for the isolation of genomic DNA from mulberry (*Morus* L.)

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Abstract

The purity of DNA is one of the major factors affecting the success of Genomic studies. Nucleic acid isolation from polyphenol rich plants fail to produce good quality DNA or RNA as polyphenols adhere and interfere with DNA during isolation. An improvised, simple and inexpensive protocol has been developed for extracting genomic DNA from Mulberry (Morus spp.). The purity of the DNA as revealed by the ratios of absorbance at 260/280 nm (A 260/280) and 260/230 nm (A 260/230) was closer to 2.0. Genomic DNA analyzed for analytical applications like restriction digestion and PCR amplification with molecular markers viz., Inter Simple Sequence Repeats (ISSR), Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) primers further confirmed the purity of the DNA. A modified method of silver staining was employed for the resolution of SSR amplified products. Physiologically mature leaf was found more suitable for getting quality DNA in mulberry.

Key words: Mulberry, DNA, ISSR, RAPD, SSR, Silver staining

Introduction

Extraction of quality DNA is a major challenge for molecular biologists dealing with tree plants. Many contaminating agents like, higher contents of polyphenolic compounds, resins, latex, and polysaccharides, tannins present in the cell as secondary metabolites usually co precipitate with DNA and interfere with the activity of the DNA polymerase enzyme (Pandey et al., 1996). Cell lysis process followed by the polyphenols oxidation and co-precipitation causes browning of the DNA (Varma et al., 2007). Viscous DNA samples have been obtained as a result of the co-precipitation of the gelling polysaccharide making the samples viscous and hamper the proper loading of the samples on the gel for electrophoresis (Diadema et al., 2003). The procedures for the extraction of plant DNA are modified continuously to get quality DNA (Cheng et al., 1997; Kobayashi et al., 1999; Karakousis and Langridge, 2003; Vijayan, 2004). Mulberry (*Morus* spp.) is a perennial tree having high economic value and is the sole food plant for the silkworm, *Bombyx mori* L. (Lepidoptera). Leaves of mulberry contain large amount of polysaccharides, polyphenols, and many sticky and resinous materials, which are not fully characterized. Mulberry leaf extract contains 44.82% polyphenols (Chan et al., 2009). Leaves have comparatively high values of phenolic compounds which ranged from 8.33 to 11.79 mmol/100 g of dry weight of leaves in three species of mulberry *Morus nigra, Morus alba* and *Mours laevigata* (Memon et al., 2010). Attempts to extract DNA from leaf samples using many of the existing protocols (Cheng et al., 1997; Kobayashi et al., 1999; Karakousis and Langridge, 2003; Vijayan, 2004) have resulted in isolation of DNA with limited success. In most cases, the DNA extracted has not amplified successfully with Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) primers. Molecular work in mulberry with microsatellite primers (SSR) is very much limited. The setback experienced in Genomic research in mulberry can be attributed to the poor quality DNA. To overcome this problem, commercial DNA extraction kits (Qiagen, Valencia, CA, USA; Amersham Life science, England) are used to extract Polymerase Chain Reaction (PCR) grade DNA from mulberry leaves (Vijayan et al., 2006; Awasthi et al., 2004; Chatterjee et al., 2004).

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However usage of these commercial kits for routine extraction is economically difficult for large-scale genomic applications. In the present study, we report a simple, rapid and efficient method yielding appreciable levels of high quality DNA suitable for molecular studies including restriction digestion and PCR amplifications. PCR was carried out using molecular markers like RAPD, ISSR and SSR primers. A protocol of silver staining the Polyacrylamide Gel Electrophoresis (PAGE) gel for microsatellite analysis in mulberry is also established in the present study.

Materials and Methods

Collection of plant material

Five mulberry species namely *M. alba, M. rotundiloba, M. serrata, M. laevigata* and *M. nigra* grown in the germplasm of Central Sericultural Germplasm Resources Center (CSGRC), Hosur, Tamil Nadu, India were selected for the study. These plants were propagated through stem cuttings to maintain their genetic identity. Fresh leaf samples were collected from 90-day-old (after pruning) primary branches of these plants.

Isolation of genomic DNA

Protocol: The modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990) was followed in the present study. Sorvall RC-5C High-speed centrifuge was used for the extraction of the mulberry DNA.

Steps for the extraction of Mulberry DNA

1. 100 mg of the leaf lamina was homogenized in a pre-cooled mortar and pestle, adding approximately 15 ml of liquid nitrogen
2. The leaf powder was transferred to 15 ml Oakridge tube
3. Added 600 µl of preheated (60^{o}C) extraction buffer containing Sodium chloride (NaCl), Polyvinyl pyrrolidone (PVP), Cetyltrimethyl ammonium bromide (CTAB), L-ascorbic acid, Diethyldithocarbamic acid, Sodium metabisulfite, Sodium dodecyl sulfate (SDS), Tris-EDTA (TE) in the concentrations as detailed above.
4. Mixed the leaf powder in the extraction buffer thoroughly by vortexing
5. Added 33.3 µl β-mercaptoethanol and incubated at 60°C in a shaking water bath for ten minutes.
6. Centrifuged at 20000g for five minutes, and transferred the supernatant to a new 1.5 ml Eppendorf tube.
7. Added 600 µl chloroform: isoamyl alcohol in the ratio of 24:1 and mixed thoroughly for three minutes by vortexing to form an emulsion. Centrifuged at 20000g for five minutes.
8. Transferred the aqueous phase to a new1.5 ml Eppendorf tube.
9. Added 500 µl of ice cold 100% isopropanol and briefly vortexed the tube and kept on ice for ten minutes. Centrifuged at 20000g for two minutes and pipetted out the supernatant carefully into a waste jar without disturbing the DNA at the bottom of the tube.
10. Dried the DNA in SpeedVac Vaccum Concentrator and dissolved in 100 µl of Tris-EDTA (TE) buffer containing 0.2 µl of RNAse A and vortexed to enable uniform mixing.
11. Placed the DNA solution in a 37°C heat block and incubated for ten minutes.
12. Added 50 µl of 7.5M Ammonium Acetate and 200 µl 95% ethanol kept on ice for ten minutes and centrifuged at 20000g for five minutes.
13. Decanted the supernatant carefully and washed the DNA with 500 µl 95% ethanol and centrifuged at 12000g for five minutes.
14. Pipetted out the ethanol and repeated the washing step as in step 13.
15. Dried the DNA in the SpeedVac Vacuum Concentrator at 45°C (low heat setting) for ten minutes.
16. Dissolved the DNA in 50 µl of 10 mM Tris (pH 8.0). Tris is used instead of TE, as the EDTA can inhibit enzymatic reactions including PCR.
17. Stored at -20°C till further use.

Table 1. Buffers and solutions used for the present protocol.

Buffers/Solutions	Contents
Sodium chloride	0.98g, 1.4M
Polyvinyl Pyrrolidone (PVP) (40 kD)	0.24g, 2.0%
Cetyl Trimethyl Ammonium Bromide (CTAB)	0.24g, 2.0%
L-ascorbic acid	0.01g, 5 mM
Diethyldithocarbamic acid (DIECA)	0.008g, 4 mM
Sodium Meta Bisulfate	0.12g, 1.0%
Sodium Dodecyl Sulfite (SDS)	0.06g, 0.5%
Tris	2.4 ml, 200 mM, 1 M, pH 8.0
Ethylene Diamine Tetra Aceticacid (EDTA) Disodium salt	480 µl, 20 mM, 0.5 M, pH 8.0
Milli Q Water	8.5 ml, (18.2 MΩ)
Liquid Nitrogen	For quick freezing the plant material

Analysis of the genomic DNA

The resultant genomic DNA was electrophoresed for qualitative analysis on 0.8%. agarose gel containing ethidium bromide at a final concentration of 0.5 µg/ml. The DNA yield per gram of leaf tissue was measured by using a UV spectrophotometer (Shimadzu, Japan) at 260 nm. DNA purity was determined by calculating the absorbance $A_{260/280}$ ratio. Polyphenol contamination was assessed by calculating the absorbance ratio $A_{260/230.}$ The purity of DNA was further analyzed for its analytical applications like Polymerase chain reaction amplifications and restriction digestion.

PCR amplification of RAPD and ISSR primers

PCR amplification of the DNA with RAPD (Random Amplified Polymorphic DNA) primer was carried out as described by Chatterjee et al. (2004) on an MJ Research Thermal-Cycler, PTC-200 using 20 µl of reaction mixture containing 1 X PCR Buffer (MBI Fermentas) 200 µM each of dGTP, dATP, dCTP and dTTP; 2.0 µM $Mgcl_2$; 100pM primer; 10 ng genomic DNA and 1 U Taq polymerase. The PCR schedule followed was 93°C for 2 min followed by 35 cycles of 93°C for 1 min, 36°C for 1 min, 72° C for 2 min and a final incubation at 72°C for 15 min. The ISSR (Inter Simple Sequence Repeat) amplification was carried out as per Vijayan and Chatterjee (2003) using 20 µl reaction mixture containing 2.0 µl of 10 X PCR buffer (750 mM Tric-HCl pH 8.8; 200 mM $(NH_4)_2SO_4$; 0.1% Tween-20), 0.2 mM dNTP, 2 mM $MgCl_2$; 200nM Primer; 50 ng genomic DNA and 1 U Taq DNA polymerase (MBI Fermentas Inc, Hanover, MD-21076, USA). The PCR schedule included an initial cycle at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2 min and a final extension of 10 min at 72°C. The PCR products were separated in 1.5% agarose gel in 1 X Tris Boric Acid buffer (TBE) containing 0.5 µg/ml Ethidium Bromide as stain.

PCR amplification of microsatellite primers (SSR)

Five pairs of simple sequence repeat primers developed by Aggarwal et al., (2004) were used for the study. The PCR amplification was carried out in 20 µl reaction buffer containg 10 ng template DNA, 1 pmol of each primer, 2 mM of $Mgcl_2$, 100 µM of dNTPs, 1 X PCR buffer, 1 U DNA polymerase (Aggarwal et al., 2004) on an M.J Research Thermal-Cycler, PTC-200 (MJ Research Inc. Massachusetts, USA). The PCR schedule included initial denaturation at 94°C for 10 min followed by 35 cycles of 94°C for 1 min denaturation, primer-specific annealing temperature for 1 min and 72°C for 1.5 min extension followed by the final extension step of 72°C for 5 min.

Resolution of PCR-SSR amplification

The microsatellite amplicons was resolved in Polyacrylamide gels. The Polyacrylamide gel was prepared in a Pharmacia LKB Macrophor sequencing gel system. The gel casting plates were cleaned thoroughly with liquid soap and water, wiped with double distilled alcohol and allowed to dry. The smaller gel plate (33.3 x 39.4 cm) was treated with Bind Silane containing 20 µl of γ-methacryloxypropyl-trimethoxysilane (PlusOne Bind Silane, Amersham Pharmacia Biotech) in 1.5 ml of 10% acetic acid and 5ml of double distilled alcohol. The plate was kept for drying for 15 minutes. The larger plate (33.3 x 41.9 cm) was similarly cleaned with liquid soap and water and subsequently dried with alcohol. This plate was treated with 1ml of 2% Dimethyldichlorosilane in octamethyl cyclo- octasilane (Plus One Repel Silane, Amersham Pharmacia Biotech). This plate was also allowed to dry for 15 minutes. After proper drying, the gel plates were assembled using 0.75 mm spacer and clips. 6% polyacrylamide gel was prepared by dissolving 32.5g Urea, 4.25g acrylamide and 0.225g N, N'-methylene bisacrylamide in 75 ml 1X TBE buffer (89 mM Tris-base; 89 mM boric acid; 2 M EDTA). The solution was filtered through Whatman-1 filter paper. Just before casting the gel 600 µl of 10% freshly prepared Ammonium persulfate and 20 µl of Tetramethylethylenediamine (TEMED) were added to the solution. The gel solution was poured into the gel assembly slowly without trapping any air bubbles. The gel was allowed to polymerise for two to four hours. The gel was pre-run at 600 V for one hour at 45°C before loading the sample. The sample was prepared by mixing three parts of PCR product with one part of loading dye containing 95% Formamide (v/V), 10 mM NaOH, 20 mM EDTA, 0.05% (w/v) Bromophenol blue and 0.05% (w/v) Xylenecyanol. The sample was denatured, by heating at 95ºC for 5 minutes and keeping on ice immediately. About 6 µl of the sample was loaded to the gel and run it at 600 V for five hours at 45°C. Phage ØX174 DNA digested with *Hinf* I (MBI, Fermentas) was used as size marker.

Silver Staining of Polyacrylamide Gel

The staining of the gel was carried out using a modified protocol of Sanguinetti et al. (1994). Following electrophoresis, the gel bound to the gel

plate was fixed in 10% (v/v) Ethanol along with 0.5 ml/100ml acetic acid for 45 minutes. The gel was then quickly treated with 2% citric acid for 90 seconds. The gel was subsequently rinsed twice with double distilled water (ddH_2O) and transferred to 0.2% (w/v) Silver nitrate solution for 30 minutes with occasional shaking. The gel after a quick rinsing in double distilled H_2O was transferred to a solution containing 3.0% (w/v) Sodium hydroxide and 7.5 ml/L 37% formaldehyde. After appropriate development of the bands, the gel was transferred to Stop solution containing 1.5% (w/v) EDTA. The gel was photographed under white fluorescent light and data were scored directly from the gel on the basis of presence or absence of markers.

Endonuclease digestion

The suitability of the DNA for hybridization-based techniques was tested by subjecting 10 µg of genomic DNA with 30 U of EcoRI (Boehringer Mannheim, Germany) in the recommended buffer and incubated at 37ºC for 6 hours. The digestion mixture was subjected to electrophoresis in 1.0% agarose gel and observed under Ultra Violet (UV) transilluminator (Amersham Pharmacia Biotech).

Results and Discussion

The method described here could isolate good amount of high quality DNA, which was evident from spectrophotometric analysis**,** PCR amplification and endonuclease digestion of the DNA. The spectophotometric analysis at $A_{260/\ 280}$ gave a ratio ranging from 1.74 ± 0.14 to 1.95 ± 0.03, which indicates very little contamination from proteins, as proteins absorb at A_{280}. Likewise the $A_{260/230}$ ratio of near 2.0 indicates the absence of contaminants like carbohydrates, peptides and phenolic compounds in the DNA. Electrophoresis on agarose gel further revealed the absence of shearing in the DNA (Figure 1). The PCR amplification with ISSR, RAPD and SSR primers (Figures 2 to 4) and the complete digestion of the DNA with restriction endonuclease (Figure 5) further confirmed the absence of contaminants interfering PCR and restriction endonuclease digestion. It is a known fact that contaminants like polysaccharides and polyphenols are, in general, difficult to be removed from the DNA as they often adsorb and co-precipitate with the DNA (Murray and Thompson, 1980) and interfere with PCR and restriction endonuclease digestion. Polysaccharides also interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases (Shioda and Murakami, 1987; Richards, 1988).

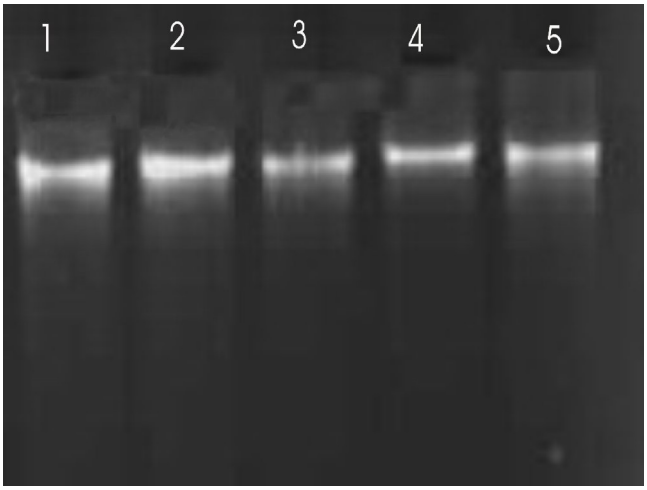

Figure 1. Genomic DNA extracted from five mulberry species. 1. ***M. alba***, 2. ***M. rotundiloba***, 3. ***M. serrata***, 4. ***M. laevigata***, 5. ***M. nigra***.

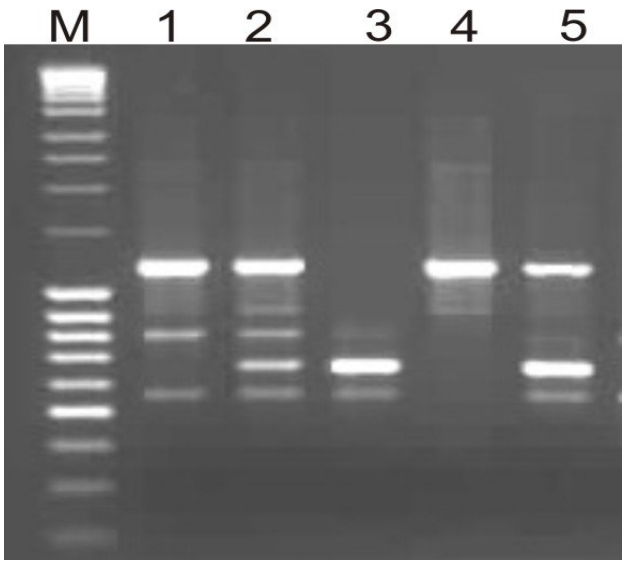

Figure 2. PCR amplification of the DNA with ISSR primer UBC-812. M-marker. 1. *M. alba*, 2. *M. rotundiloba*, 3. *M. serrata*, 4. *M. laevigata*, 5. *M. nigra.*

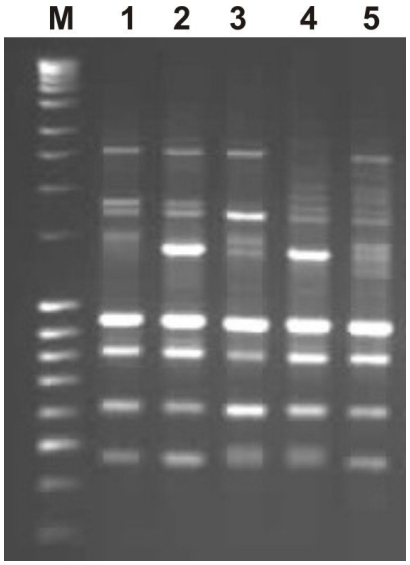

Figure 3. PCR amplification of the DNA with RAPD primer OPW-4. M-marker. 1. *M. alba*, 2. *M. rotundiloba*, 3. *M. serrata*, 4. *M. laevigata*, 5. *M. nigra*.

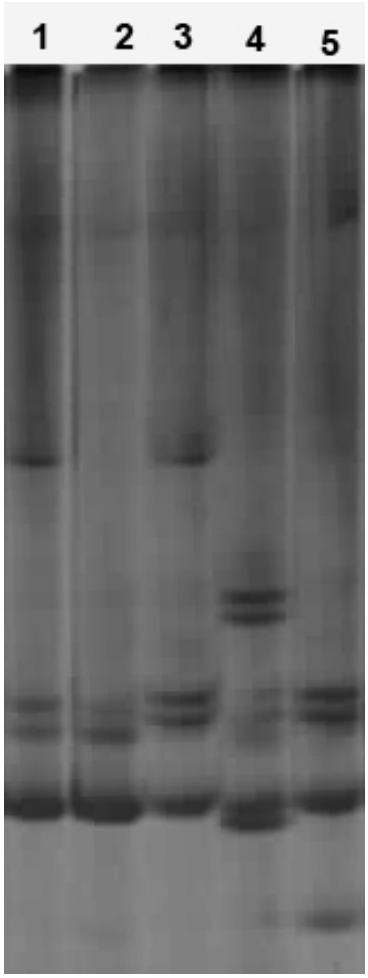

Figure 4. PCR amplification of the DNA with SSR primer MUL-5 and the gel was stained with silver nitrate. 1. *M. alba*, 2. *M. rotundiloba*, 3. *M. serrata*, 4. M. *laevigata*, 5. *M. nigra*

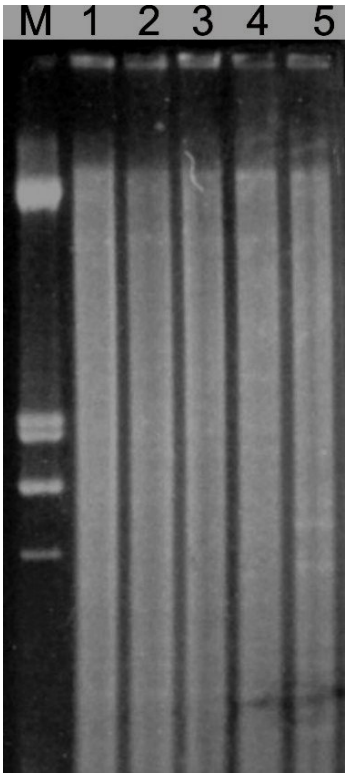

Figure 5. Endonuclease restriction digestion of the DNA with EcoRI. M. Marker. 1. *M. alba*, 2. *M. rotundiloba*, 3. *M. serrata*, 4. *M. laevigata*, 5. *M. nigra*.

The major modifications in the present study from the standard Cetyl Trimethyl Ammonium Bromide (CTAB) protocol (Doyle and Doyle, 1990) are the addition of polyvinyl pyrrolidone (PVP), L-ascorbic acid, diethyldithocarbamic acid (DIECA), sodium metabisulfite and sodium dodecyl sulfate in the extraction buffer. DIECA is a phenoloxidase inhibitor and it helps reduce brown colouring due to oxidation of polyphenols to quinons. Addition of L-ascorbic acid prevents oxidation of phenolic compounds and adsorption by DNA molecules. Similarly, PVP forms complex compounds by hydrogen bonding with phenolic compounds and co- precipitates along with cell debris thus preventing polyphenols from interacting with DNA. When the extract is centrifuged in the presence of chloroform, the PVP complexes accumulate at the interface between the organic and the aqueous phases (Kim et al., 1997; Barwell et al., 1998). CTAB is a cationic detergent, which solubilizes membranes and also binds to fructans and other polysaccharides to form complexes that are removed during subsequent chloroform extraction. The time taken for the extraction of DNA is very much less compared to the other protocols and the entire procedure could be completed within 2 hours. This method is a one-step extraction procedure, which includes the RNAase treatment for the removal of RNA. In this study the suitability of physiologically mature leaf (7th-9th leaf from the tip) over young leaf was observed. When samples from very young leaves were used**,** the DNA yield and quality was very poor. Hence, it is found better to use physiologically mature mulberry leaf for DNA extraction. This finding of low and poor quality of DNA from immature leaves is in complete agreement with the reports of Small et al. (2004) that in certain plants mature leaves are more suitable for DNA extraction. Another point needs mentioning here is the low ratio of $A_{260/280}$ observed in *M. serrata.* It is a known fact that the leaves of *M. serrata* are much leathery, feathery and thicker than the leaves of other species (Tikader and Dandin, 2001). However, the complete digestion of the DNA with restriction endocnuclease enzyme indicates that DNA is devoid of contaminants interfering digestion. Although silver staining was initially used for detection of proteins separated by Polyacrylamide gel electrophoresis (Merril et al., 1981) later its use in detection of nucleic acid on Polyacrylamide gel was realized (Sanguinetti et al., 1994). Using different modifications to the original protocols Bassam et al. (1991) enhanced the sensitivity of the staining to 1pg/mm² by different modifications such as inclusion of Sodium thiosulphate, pretreatment with Potassium dichromate and nitric acid. Sanguinetti et al. (1994) on the other hand used Sodium hydroxide to establish an alkaline environment for the silver ion to reduce into metallic silver in the presence of formaldehyde. However, all these protocols were developed for native or denaturing gels detached from the gel plates after electrophoresis. Since, in most of the SSR analysis, electrophoresis in long and thin gel is required for better separation of the alleles, separation of the gel from the glass plate is found difficult. In this report, we have developed a method for staining the thin Polyacrylamide gel still attached to the glass plate. In this protocol we have included an additional step of pretreatment of the gel plate with citric acid, which has enhanced the band visibility, by reducing the background staining considerably. Similarly, the use of higher formaldehyde concentration than the one used by Sanguinetti et al. (1994) resulted in darker bands with good contrast. Further, this modified staining protocol for large sequencing gels bound to glass has many advantages like it requires small amount of the sample, a large number of samples could be analyzed in a single run and most of the staining reagents can be reused without loss of quality.

Conclusion

The present protocol is suitable for isolating high quality DNA from leaves of tree plants containing higher amounts of polysaccharides, polyphenols, resins and other viscous contaminants. The successful endonuclease digestion and the amplifications of the different molecular markers confirm the purity of the DNA. This protocol is simple, rapid, inexpensive and easily amenable to other species.

References

Aggarwal, R. K., D. Udaykumar, P. S. Hendre, A. Sarkar and L. I. Singh. 2004. Isolation and characterization of six novel microsatellite markers for mulberry (*Morus indica*). Mol. Ecol. Notes 4:477-479.

Awasthi, A. K., G. M. Nagaraja, G. V. Naik, K. Sriramana, K. Thangavelu and J. Nagaraju. 2004. Genetic diversity in mulberry (genus *Morus*) as revealed by RAPD and ISSR marker assays. BMC genetics, 5, 1 (available from: http://www.biomedcentral.com/1471-2156/5/1).

Barwell, P., A. N. Blanchard, J. A. Bryant, N. Smirnoff and A. F. Weir. 1998. Isolation of DNA from highly mucilaginous succulent plant *Sedum telephium*, Plant Mol. Biol. Report 16:133-138.

Bassam, B. J., G. Caetano-Anolles and P. M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 196:80-243.

Chan, K. C., H. H. Ho, C. N. Huang, M. C. Lin, H. M. Chen and C. J. Wang. 2009. Mulberry leaf extract inhibits vascular smooth muscle cell migration involving a block of small GTPase and Akt/NF-kB signals. J. Agric. Food Chem. 57(19):9147-9153.

Chatterjee, S. N., G. M. Nagaraja, P. P. Srivastava and G. Naik. 2004. Morphological and molecular variation of *Morus laevigata* in India. Genetica 121:133-143.

Cheng, P. S., S. K. Brown and N. P. Weeden. 1997. A DNA extraction protocol from various tissues in woody species. Horticultural Science 32:921-922.

Doyle, J. J. and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Diadema, K., A. Baumel, M. Lebris and L. Affre. 2003. Genomic DNA isolation and amplification from callus culture in succulent plants, *Carpobrotus* species (Aizoaceae). Plant Mol. Biol. Report 21:173a-173e.

Karakousis, A. and P. Langridge. 2003. A high throughput plant DNA extraction method for marker analysis. Plant Mol. Biol. Report. 21:95a-95f.

Kim, C. S., C. H. Lee, J. S. Shin, Y. S. Chung and J. E. Hyung. 1997. A simple and rapid method of isolation of high quality genomic DNA from fruit trees and conifers using PVP. Nucleic Acid Res. 27:66-58.

Kobayashi, N., K. Tamura and T. Aotsuka. 1999. PCR error and molecular population genetics. Biochem. Genet. 3:318-321.

Memon, A. A., N. Memon, L. D. L. Luthria, M. L. Bhanger and A. A. Pitafi. 2010. Phenolic acids profiling and antioxidant potential of mulberry (*Morus laeviagata*., *Morus nigra* L., *Morus alba* L.) leaves and fruits grown in Pakistan. Pol. J. Food Nutr. Sci. 60(1):25-32.

Merril, C. R., D. Goldman, S. A. Sedman and M. H. Ebert. 1981. Ultrasensitive stain for protein in polyacrylamide gel shows regional variation in cerebrospinal fluid proteins. Science 211:1437-1438.

Murray, M. G. and W. F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res. 8:4321-4325.

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

Richards, E. 1988. Preparation of genomic DNA from plant tissue. In: F. M. Ausubel, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman and K. Struhl (Eds.). pp.2.3.2-2.3.3. Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.

Sanguinetti, C. J., E. D. Neto and A. J. G. Simpson. 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Bio Techniques 16:915-919.

Shioda, M. and K. Marakami-Muofushi. 1987. Selective inhibition of DNA polymerase by a

polysaccharide purified from slime of *Physarum polycephalum*. Biochem. Biophys. Res. Commun. 146:61-66.

Small, R. L., R. C. Cronn and J. F. Wendel. 2004. The use of nuclear genes for phylogeny reconstruction in plants. Aust. Syst. Bot. 17:145-170.

Tikader, A., S. B. Dandin. 2001. Breeding behaviour of some wild mulberry. Indian Silk 40:9-10.

Varma, A., H. Padh and N. Shrivastava. 2007. Plant genomic DNA isolation: an art or a science. Biotechnol. J. 2:386-392.

Vijayan, K. 2004. Genetic relationships of Japanese and Indian mulberry (*Morus* spp.) genotypes revealed by DNA fingerprinting. Plant Syst. Evol. 243:221-232.

Vijayan, K. and S. N. Chatterjee. 2003. ISSR profiling of Indian cultivars of mulberry (*Morus* spp.) and its relevance to breeding programs. Euphytica 131:53-63

Vijayan, K., C. V. Nair and S. N. Chatterjee. 2006. Molecular characterization of mulberry genetic resources indigenous to India. Genet. Resour. Crop Ev. 52:77-86.