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TECHNICAL NOTE

A standardized protocol for genomic DNA isolation from *Terminalia arjuna* for genetic diversity analysis

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Abbreviations: AFLP: amplified fragment length polymorphism
RAPD: random amplification of polymorphic DNA
ISSR: inter simple sequence repeats

For studying genetic diversity in natural populations of *Terminalia*, a medicinal plant, our attempts to isolate high quality DNA using several previously reported protocols and even modifications were unsuccessful. We

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therefore combined CTAB based isolation, and column based purification step, to isolate DNA from *Terminalia arjuna*. The DNA isolated using this standardized protocol was high in quality and suitable for restriction digestion and generation of random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP).

Among the nearly 15,000 flowering plants documented, many of them are used as sources of medicine. In the developing nations, almost 80% people depend on these plants for medicine because of their easy availability and low cost of treatment. The modern allopathic system of medicine is known to produce serious side-effects and resistance against antibiotics which make these drugs non-potent (Pirttila et al. 2001). *Terminalia arjuna* is one of the plants which holds a reputed position in both Ayurvedic and Unani Systems of medicine. According to Ayurveda, it is alexteric, styptic, tonic, anthelmintic and useful in treatment of fractures, ulcers, heart diseases, biliousness, urinary discharges, asthma, tumours, leucoderma, anaemia, excessive perspiration etc. In Unani System, it is used both externally and internally in problems related to urinary discharges. It also serves as expectorant, aphrodisiac, tonic and diuretic. Its bark is reported to be useful in the treatment of coronary artery diseases, heart failure, hypercholestrolemia and anginal pain (Miller, 1998). The cardioprotective nature has also been described by Karthikeyan et al. (2003) and its antiviral activity (against HSV-2) by Cheng et al. (2002). The plant has potential antibacterial activity against *Escherichia coli, Klebsiella aerogenes, Proteus vulgaris, Pseudomonas aeruginosa* (Perumal et al. 1998), multi-drug resistant *Salmonella typhi* (Rani and Khullar, 2004), and also shows antimutagenic activity (Scassellati-Sforzolini et al. 1999).

There is no information available on the existing germplasm diversity in *Terminalia arjuna* and, therefore, cataloguing of natural genetic diversity becomes essential for its efficient and sustainable germplasm management. DNA based assays such as RAPD and AFLP are the most widely used tools for assessment of the genetic variation (Singh et al. 1999; Belaj et al. 2002; Shan et al. 2005). Isolation of purified DNA, for such an exercise, from medicinal plants is challenging because of secondary metabolites and other compounds. A large number of secondary metabolites such as tannins, alkaloids, phenolics and terpenes responsible for the valuable pharmacokinetic properties of medicinal plants interfere with the isolation process, tend to copurify with DNA and interact irreversibly with proteins and nucleic acids (Katterman and Shattuck, 1983). For characterization of germplasm of *Terminalia arjuna, T. bellerica and T. chebula,* isolation of purified DNA proved to be a major bottleneck, especially in case of *T. arjuna,* as has also been experienced with other species like *Theobroma cacao* (Haymes et al. 2004), *Vitis vinifera* (Hanania et al. 2004), *Pinus radiata* (Crowley et al. 2003), *Tagetes minuta* (Hills and van Staden, 2002) *Eucalyptus* spp., *Pinus* spp. and *Araucaria cunninghamii* (Shepherd et al. 2002), *Davidia involuctata* (Li et al. 2002) *Anthurium andreanum* (Buldewo and Jaufeerally-Fakim, 2002), *Drosera rotundifolia, Artemisia dracunculus* (Pirttila et al. 2001).

We attempted a dozen of previously reported and also modified protocols but the dirty yellow and highly viscous DNA obtained was not suitable for manipulation and analysis. The aim of this study was to establish a new protocol for DNA isolation from *Terminalia arjuna* plants to get high quality DNA that is suitable for generation of molecular markers, such as RAPD and AFLP.

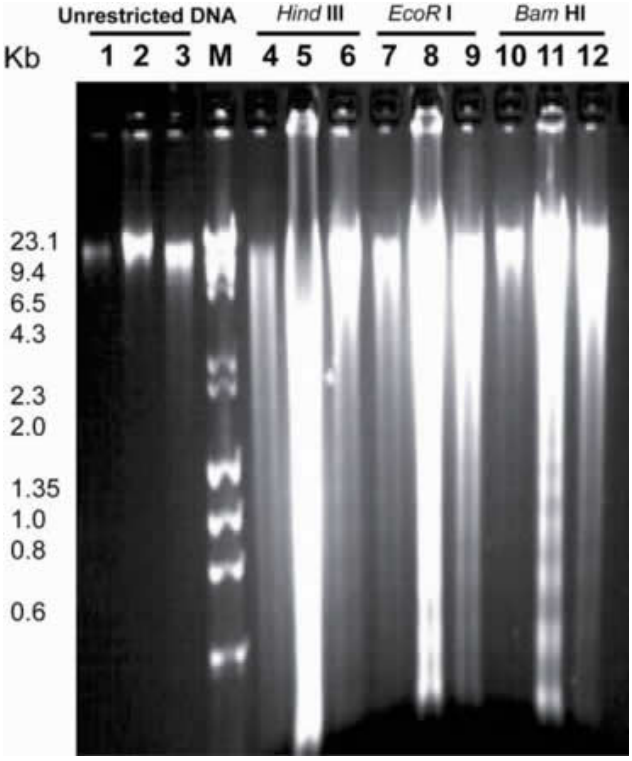

Figure 1. Restriction pattern of DNA isolated following modified protocol. Two µg of genomic DNA from *Terminalia arjuna, T. bellerica and T. chebula* were loaded either undigested (lanes 1, 2, 3, respectively) or after *Hind* III (lanes 4, 5, 6, respectively), *Eco* RI (lanes 7, 8, 9, respectively) and *Bam* HI (lanes 10, 11, 12, respectively) digestion. A mixture of λ DNA digested with *Hind* III and φX174 DNA digested with *Hae* III was loaded as marker (lane M).

MATERIALS AND METHODS

Material Collection: Juvenile leaves collected from different geographical regions of natural populations of *Terminalia arjuna* were stored on dry ice, lyophilized using standard procedure and stored at -20ºC till DNA isolation. DNA isolation was carried out within 7-10 days to avoid degradation.

DNA isolation

Only the successful procedure of DNA isolation along with

the modifications that were carried out and purification is reported here. The DNA isolated by CTAB method (Doyle and Doyle, 1990) was dissolved in 1 ml of sterile double distilled water (DDW) and purified using a column (Gen-elute plant genomic DNA extraction kit- G2N 70, Sigma Aldrich).

Solutions and solvents

- CTAB Buffer (1.4M NaCl, 100 mM Tris-HCl pH 8.0, 20mM EDTA pH 8.0, 2% CTAB; added β-mercaptoethanol before use).
- Chloroform: IsoAmyl alcohol (24:1).
- Isopropanol.
- Ethanol 70% and 96%.

The details of procedure are given below:

- Mix 700 µl of the binding solution provided in the kit with the dissolved DNA (1 ml).
- Take 700 µl of the above solution in a "red-o-ringed" column fitted in a 2 ml collection tube.
- Centrifuge at 12000 g for 1 min.
- Discard the flow-through liquid, add the remaining solution to the same column.
- Centrifuge at 12000 g for 1 min.
- Discard the flow-through liquid along with the collection tube and transfer the column to a new 2 ml collection tube.
- Add 500 µl of the Washing Buffer (diluted with ethanol) to the column and centrifuge as above.
- Again discard the flow-through liquid, repeat the washing and centrifugation steps.
- Discard the flow-through liquid as well as the collection tube and fix the column to a new 2 ml collection tube.
- Add 100 µl of Elution Buffer (pre-heated at 65°C) to the column. Centrifuge as above. This is first eluate of DNA. Transfer the column to a fresh collection tube and elute as above. This is second eluate.
- To test effectiveness and efficiency of this protocol, we restricted the DNA with commonly used restriction enzymes and also tried generation of RAPD and AFLP markers.

Restriction digestion

Two to three mg of genomic DNA was digested overnight with 10 units of restriction enzymes under optimal temperature and buffer following manufacturers recommendation (Bangalore Genei, India). The digested DNA was electrophoresed on 0.8% agarose at 5 V/cm along with undigested DNA as control.

RAPD analysis

RAPD analysis was performed in a 15 µl volume of reaction mixture containing 1 X *Taq* Polymerase Buffer (with 25 mM $MgCl_2$), 0.6 units of *Taq* DNA Polymerase (Bangalore Genei, India), 5 mM dNTPs (MBI Fermentas), 10 mM of random decamer primer (Finnzymes) and 15 ng of total genomic DNA. Amplifications were carried out using a DNA thermal cycler (Mastercycler gradient, Eppendorf) with the following parameters: One cycle at 94ºC for 2 min, 36ºC for 2 min and extension at 72ºC for 2 min; 29 cycles of denaturation at 94ºC for 1 min, primer annealing at 36ºC for 1 min and extension at 72ºC for 1 min; and final extension at 72ºC for 10 min. The reaction was stored at 8ºC till loaded on gel. The products were size fractionated on 1.2% agarose gel and visualized under UV light after ethidium bromide staining.

AFLP analysis

Genomic DNA (300-500 ng) was digested to completion with *Eco* RI + *Mse* I. After heat inactivation, an aliquot was ligated to *Eco* RI and *Mse* I specific adapters at 20ºC overnight. The adapter ligated DNA was preamplified using *Eco* RI and *Mse* I pre-amplification oligos with one-selective nucleotide. The pre-amplified library was diluted with sterile water in a ratio of 1:50. Selective amplification was carried out using γ-P^{32}-ATP labelled *Eco* RI oligo in combination with *Mse* I oligo, each with 3 selective nucleotides at the 3' end. Equal volume of formamide dye was added to the amplified products and electrophoresed on 6% PAGE under denaturing conditions and then autoradiographed (Sambrook et al. 2001).

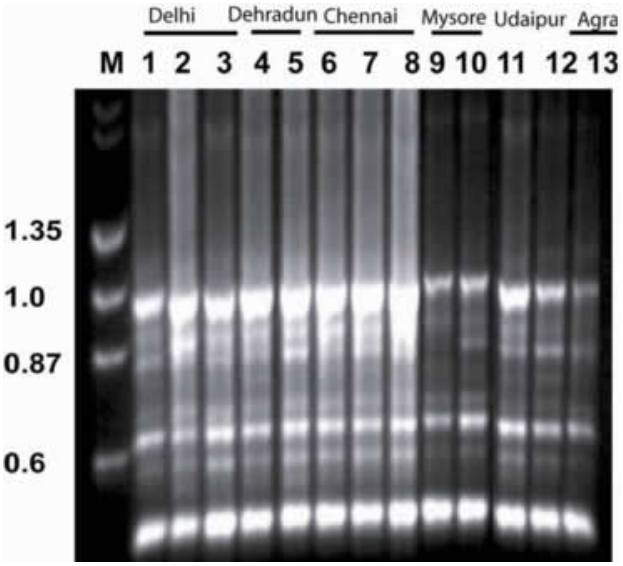

Figure 2. RAPD profile of *T. arjuna* generated by the primer OPA-02 (5'TGCCGAGCTG3'). The samples were collected from different parts of India (as indicated above the lanes). A mixture of λ DNA digested with *Hind* III and φX174 DNA digested with *Hae* III was loaded as marker (lane M).

RESULTS AND DISCUSSION

Published methods of DNA isolation including those of Doyle and Doyle (1990), Rogers and Benedich (1985) and Dellaporta et al. (1983) proved unsuccessful and unreliable

for *T. arjuna* as the DNA obtained was dirty yellow in appearance and with high viscosity. This may be due to high endogenous levels of polysaccharides, phenolics and other organic constituents that interfere with DNA isolation and purification. The isolated DNA could not be subjected to even agarose gel electrophoresis.

In higher plant tissues, particularly in medicinal plants, these secondary compounds generally get accumulated and the problem becomes severe as the material gets older. Polysaccharide contaminations are particularly problematic (Scott and Playford, 1996) as they can inhibit the activity of many commonly used molecular biological enzymes, such as polymerases (Fang et al. 1992), ligases and restriction endonucleases. This is because nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet and the embedded DNA is inaccessible to the enzymes (Sharma et al. 2002). Polyphenol contamination of DNA makes it resistant to restriction enzymes as also shown in other taxa where polyphenol copurify with DNA (Katterman and Shattuck, 1983) and interact irreversibly with proteins and nucleic acids (Loomis, 1974). This phenomenon is mainly due to the oxidation of polyphenols to quinones by polyphenol oxidase followed by covalent coupling or by oxidation of the proteins by the quinines. During homogenization, polyphenols are released from vacuoles and they then react rapidly with cytoplasmic enzymes. DNA isolation protocols generally use CTAB to avoid co-purifying polysaccharides from plant tissues. Keeping this in mind and the fact that *T. arjuna* samples carry high amounts of polysaccharides, we tried the standard CTAB method (Doyle and Doyle, 1990) and also several modifications including increase in the concentration of CTAB to 2.5% (Khanuja et al. 1999), β mercaptoethanol to 1% (Tel-zur et al. 1999), and NaCl to 2.2 M (Aljanabi et al. 1999). Other alterations tried include replacing CTAB with SDS (Keb Llanes et al. 2002) and sarcosyl (Sharma et al. 2002). Polyvinylpyrrolidone (PVP, MW 40,000) has also been successfully used to sequester or remove polyphenols. Since leaves of *Terminalia* are rich in polyphenols, we tried different percentages of PVP, 1, 2, and 4 as also proposed by others (1%, Khanuja et al. 1999; 2%, Dellaporta et al. 1993, and Csaikl et al. 1998; and 4%, Keb Llanes et al. 2002). These modifications were tried either alone or in combinations but the DNA yield remained unsatisfactory in terms of quality, restrictability and amplifiability. The only modification that proved successful was when CTAB based isolation was combined with column-based purification. The existing CTAB protocol for DNA isolation was further extended by incorporating an additional final step of purifiation based on Gen Elute (Sigma). Either of the protocols, CTAB or Gen-Elute did not succeed in overcoming the problem of low quality and quantity of DNA when used as a stand-alone procedure. In the CTAB-column based procedure, the DNA first binds to the column helping washing of the impurities in the flow-through liquid. The column bound DNA is washed twice with washing buffer and the pure DNA is then eluted with elution buffer. The total DNA yield was approximately 10-15 µg from 200 mg freeze-dried tissue.

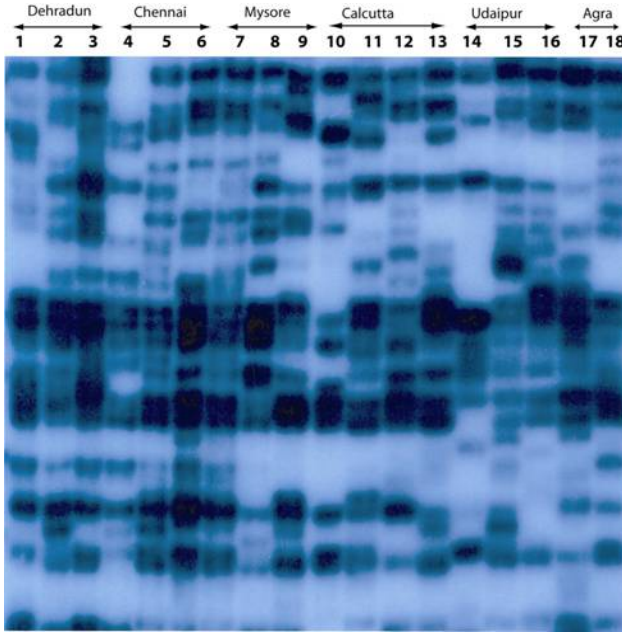

Figure 3. AFLP analysis of *T. arjuna* using selective primer combination E-AAG + M-CAG. The different lanes correspond to samples from Dehradun (1-3), Chennai (4-6), Mysore (7-9), Calcutta (10-13), Udaipur (14-16) and Agra (17-18).

The purity was evident when the DNA was restricted with *Hind* III, *Eco* RI and *Bam* HI (Figure 1). As a further test, we performed RAPD analysis using several primers. One such effort is shown in Figure 2. Primer A-02 amplified seven monomorphic bands but other primers have yielded polymorphic products both at inter-zonal and intra-zonal levels (data not shown). AFLP results are shown in Figure 3. Selective amplification of *Terminalia arjuna* DNA isolated following our modified protocol was carried out using a primer combination of *Eco* RI – AAG + *Mse* I – CAG. Several monomorphic and polymorphic products could be observed upon autoradiography (Figure 3). We are carrying out further work to generate data to calculate and catalogue the genetic diversity using both RAPD and AFLP.

We have thus established a reliable method for isolation of good quality DNA from *T. arjuna*, a problem tree material for DNA isolation, that is amenable to both restriction, and PCR based analyses such as RAPD and AFLP. The data demonstrate that the method devised by us yields highly purified DNA, which is transparent, non-sticky and has no visible RNA contamination when electrophoresed on agarose gel. The samples are amenable to restriction digestion by *Eco* RI, *Bam* HI and *Hind* III restriction enzymes.

In conclusion, extraction of DNA from *Terminalia arjuna* is difficult owing to the presence of large amount of secondary metabolites and polyphenols. We could

circumvent this by combining the CTAB method with a column-based purification process. This could be adopted as a standard method for the tree species, *T. arjuna*, *T. bellerica* and *T. chebula* and other trees for isolation of DNA for RAPD, ISSR and AFLP analyses.

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