

Optimization of DNA isolation and PCR - RAPD methods for molecular analysis of *Urginea indica* Kunth.

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Abstract

This is the first report on development of protocol for high purity DNA isolation from the bulb tissues of *Urginea indica* and optimization of conditions for RAPD-PCR analysis. Cell lyses was carried out in an extraction buffer supplemented with cetyltrimethylammonium bromide (CTAB) and sodium chloride. Two emulsification-washes with phenol: chloroform: isoamyl alcohol followed by re-precipitation with salt efficiently removed high protein and polysaccharide contaminations. Purity of the isolated DNA was confirmed by restriction digestion with *Bam* H I, *Alu* I, *Hind* III and *Eco* R I. The yield of the pure DNA ranged from 120-150ug per gram of bulb tissue. The RAPD profiling from the isolated DNA was optimized to produce scorable, clear amplicons in all populations studied.

Keywords: Bulb, DNA extraction, PCR-RAPD, polysaccharides, *Urginea indica*

INTRODUCTION

Urginea is one of the extremely interesting polytypic genera belonging to the family Liliaceae with about 100 species endemic to India, Africa and Mediterranean regions. In India, it is represented by nine species of which different populations of *Urginea indica*, commonly called as Indian squill or sea onion exhibit important medicinal properties (Shiva Kameshwari MN, 2006). Nearly all parts of the plant are used for therapeutic purposes such as expectorant, cardiac stimulant, asthma, rheumatism, dropsy, male sterility, dog bites, allergies, edema and gout (Khare CP, 2004). Two glycoproteins isolated from the bulbs have shown fungistatic effect (Deepak *et al.*, 2003; Sandhya R Shenoy *et al.*, 2006). Lately, the phytochemical constituents of the bulbs have received greater attention due to their reported anti-cancer properties (Khare CP, 2004).

However, presence of substantial variations in biochemical, morphological and cytological attributes in its different populations (Shiva Kameshwari MN, 2006) has rendered genetic improvement of *Urginea indica* difficult. Thus, there is a need for reliable characterization, both morphological and molecular, of different sub-populations of *Urginea indica* for authentic identification of this plant, before embarking on genetic improvement program.

The success of biotechnological tools such as molecular markers and genetic engineering are critically dependent on development of reliable protocol for isolating superior quality DNA and PCR analyses. Hence it has been widely reported in many plant species such as chickpea (Dipankar Chakraborti, 2006), *Lilium longiflorum* Thunb. var. *scabrum* Masam (Chia-Szu Wen and Ju-Ying Hsiao, 1999), *Pinus radiata* (Claudia Stange *et al.*, 1998), *Mucuna pruriens* (Padmesh P *et al.*, 2006), *Terminalia arjuna* (Maryam Sarwat *et al.*, 2006), *Parkia timoriana* (Robert Thangjam *et al.*, 2003), *Arachis hypogea* (Kiran K Sharma *et al.*, 2000) etc. The present study is the first report on

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Table 1: Variations tried out for the optimization of DNA extraction in *Urginea indica*

Method	Variation	Results
Phenol-chloroform method	Without any modification	Sheared bands on gel electrophoresis indicating higher protein and polysaccharide contaminations
	Salt concentration increased from 0.1 M to 1.4 M in the extraction buffer	Viscous DNA samples. Fire type bands on gel electrophoresis
	Additional phenol-chloroform extraction.	Insufficient protein removal
Doyle and Doyle CTAB method (1987)	Without any modification	Low fire type bands – lower proteins and polysaccharides, compared to phenol-chloroform method
	Tissue maceration using liquid nitrogen	Reduced shearing
	Increased salt concentrations in the extraction buffer ranging from 1.4M NaCl to 5M NaCl with proportional increase in CTAB concentration.	3M NaCl with 3% CTAB provided efficient removal of major polysaccharides
	Two phenol-chloroform extractions	Eliminated the protein contaminations along with CTAB-polysaccharide complexes
	Reprecipitation using salt	Elimination of residual polysaccharides

development of such protocols using bulb tissues in *Urginea indica*.

Table 2: List of primers used and the number of amplification products generated in the RAPD analysis of *Urginea indica*

Sl. No	Primers	Sequences (5'-3')	Number of generated bands
1	OPA-03	AGTCAGCCAC	12
2	OPA-04	AATCGGGCTG	14
3	OPA-05	AGGGGTCTTG	12
4	OPA-06	GGTCCCTGAC	12
5	OPA-09	GGGTAACGCC	11
6	OPA-10	GTGATCGCAG	13
7	OPA-15	TTCCGAACCC	10
8	OPA-16	AGCCAGCGAA	09
9	OPA-17	GACCGCTTGT	16
10	OPB-03	CATCCCCCTG	12
11	OPB-04	GGA CTGGAGT	11
12	OPAJ-02	TCGCACAGTC	13
13	OPAJ-04	GAATGCGACC	13
14	OPAK-03	GGTCTACCA	15
15	OPAK-04	AGGGTCGGTC	04

MATERIALS AND METHODS

Plant material

Bulbs of fourteen populations of *Urginea indica*, collected from different locations of Karnataka and neighboring states were stored at room temperature and used for the study. *Urginea indica* completes its entire reproductive cycle in just three months and stays in hibernation for remaining nine months in a year, offering critical difficulty for continual availability of plant material. Secondly, the leaf and other aerial parts, due to high mucilage content are unsuited for storage under low temperature even for short duration. On transferring to room temperature, they turn squishy and fragile rendering it very difficult to handle. Thus, the standardizations were carried out from the bulb tissue as a viable alternative.

DNA isolation

Two commonly used DNA isolation methods viz., Doyle & Doyle CTAB method (1987) and Phenol - chloroform method (Maryam Sarwat *et al.*, 2006) were tried in the beginning. Taking clue from the initial results, Doyle & Doyle CTAB method (1987) was modified (Table 1) as follows.

Reagents and solutions

- Extraction buffer: 3% (w/v) CTAB; 3 M sodium chloride; 100 mM Tris.HCl (pH 8.0); 20 mM EDTA (pH 8.0); 0.2% 2-mercaptoethanol.
- Chloroform:isoamyl alcohol (24:1)
- Phenol: chloroform: isoamyl alcohol (25:24:1)
- Absolute ethanol, -20°C
- 70% ethanol
- TE buffer (10 mM Tris.HCl +1 mM EDTA (pH 7.4))
- Autoclaved distilled water

DNA isolation protocol

- Preheat CTAB isolation buffer in a 30ml oakridge tube to 60°C in a water bath.
- Grind 1 g of fresh bulb tissue into fine powder by adding liquid nitrogen in chilled mortar & pestle and transfer it into 15 ml preheated extraction buffer.
- Incubate the sample at 60°C for 30 min with intermittent shaking. Cool and centrifuge at 6000 g (10000 rpm) to pellet the debris.
- Add equal volume of chloroform: isoamyl alcohol (24:1) to the supernatant & gently vortex for 10min. Subsequently centrifuge at 6000 g for 10 min.
- Extract the aqueous phase with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuge at 8000 g (12000 rpm) for 10 min. and collect the supernatant.
- Repeat the above step once. Extract the aqueous phase with equal volume of chloroform: isoamyl alcohol (24:1) to remove residual phenol from the solution. Centrifuge at 6000 g & collect the supernatant.
- Transfer the supernatant into 2.5 volumes of chilled ethanol to precipitate the DNA.
- Recover the DNA through spooling. Wash the recovered DNA with 70% ethanol twice. Air-dry and re-suspend in appropriate volume of TE buffer.
- Carry out RNase treatment at 37°C and remove the RNase using chloroform: isoamyl alcohol (24:1) extraction.
- Add 0.5 volumes of 5 M NaCl to the final DNA solution.
- Add the above mixture to 2.5 volumes of chilled ethanol for re-precipitation of DNA.
- Recover the DNA through spooling or centrifugation at 2700 g (4000 rpm) for 5min. Wash the recovered DNA with 70% ethanol twice.

- Air-dry & re-suspend in TE buffer and store at 4°C or -20°C for further use.

Restriction digestion

1 µg of genomic DNA was digested overnight with three units of each of the four restriction enzymes viz., *Alu* I, *Hind* III, *Eco* R I & *Bam* H I individually. The reaction was carried out in a buffered condition at 37°C following manufacturer's protocol (Bangalore Genei, India). All the four digested DNA were electrophoresed on 0.8% agarose gel with undigested genomic DNA used as control. Double digested (*Hind* III/ *Eco* R I) Lambda DNA (Bangalore Genei, India) was used as marker.

RAPD analysis

RAPD analysis was performed in a 0.025 cm³ reaction mixture containing 0.2 mM dNTP's (Fermentas), 10 mM Tris- HCl, 2.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 0.75 U *Taq* DNA polymerase (Bangalore Genei), 15 pmol primers (Operon Technologies, Alameda, USA) and 50 ng of genomic DNA. DNA amplification cycles were performed in a thermal cycler (PTC 200, MJ Research, USA). After initial cycle of denaturation at 92°C for 2 min., annealing at 48°C for 2 min. and an extension at 72°C for 2 min., 40 cycles of denaturation at 92°C for 1 min., annealing at 48°C for 1 min., and extension at 72°C for 2 min., were provided, followed by a final extension of 72°C for 7 min. Reaction mixture, wherein template DNA replaced with distilled water was used as negative control. A total of 15 primers were used to check the fidelity of amplification (Table 2). Amplified products were resolved on 1.8 % agarose gel (1x TAE) followed by ethidium bromide staining (5 µl/100 ml TAE). The results of RAPD were documented using Gel documentation system (BIORAD, USA).

RESULTS

Isolation of DNA from storage tissues like bulb is difficult due to high levels of polysaccharide and protein contents. In the present study, among the two protocols examined, Doyle and Doyle (1987) - CTAB method proved efficacious compared to phenol-chloroform method. On dissolving, DNA from the former resulted nearly clear solution, while the later formed partially

soluble gelatinous pellet. Different degrees of smeared and fire type bands, visualized upon staining the agarose gel with ethidium bromide (Fig. 1A) indicated high-levels of protein and polysaccharide impurities in the samples isolated from phenol-chloroform method compared to CTAB method. Puchooa D (2004) has used similar analysis for inferring the degree of protein and polysaccharide contamination in lychee (*Litchi chinensis* Sonn.).

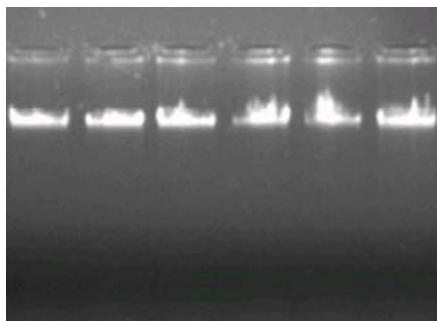


Figure 1A: Electrophoretic pattern showing fire type bands in the DNA samples isolated using Doyle & Doyle - CTAB method (1987).

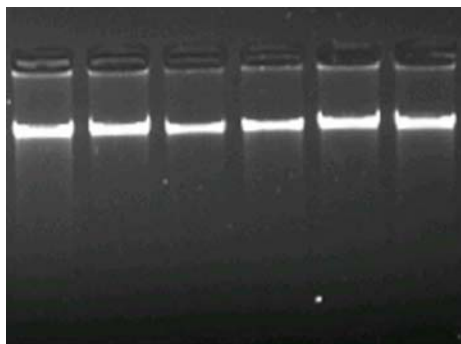


Figure 1B: Electrophoretic pattern of DNA samples showing sharp, distinct & clear DNA bands in the samples isolated using currently described method.

Several modifications were introduced to Doyle and Doyle (1987) - CTAB method for the removal of impurities (Table 1). Combination of high concentrations of CTAB (3% w/v) and sodium chloride (3 M) in the extraction buffer along with two successive washes with phenol: chloroform: isoamyl alcohol and final re-precipitation with salt proved very effective. While CTAB and sodium chloride helped removal of majority of the polysaccharides (Murray and Thompson, 1980; Paterson *et al.*, 1993; Suman PS Khanuja *et al.*, 1999), two consecutive washes with phenol: chloroform: isoamyl alcohol excluded protein impurities

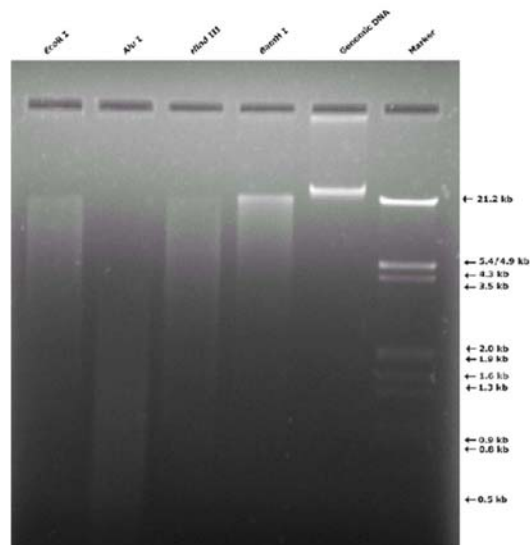


Figure 1C: Restriction digestion of DNA extracted from the bulbs of *Urginea indica* using the currently optimized protocol. Lane 1-4: DNA digested with restriction enzymes *Eco R I*, *Alu I*, *Hind III*, & *Bam H I* respectively; Lane 5: Undigested genomic DNA; Lane 6: Molecular weight marker, Lambda DNA double digest (*Eco R V Hind III*).

(Dipankar Chakraborti *et al.*, 2006). The phenol wash was also reported to help in removing the CTAB – polysaccharides complex formed early in the reaction (Dipankar Chakraborti *et al.*, 2006). However, in comparison with the protein, polysaccharide contamination was more difficult to remove as they remained in trace amount until the end and co-precipitated with the DNA sample in a complex way finally concentrating the nucleic acids to a semi-soluble gelatinous pellet, ineffective for any application. Tamsyn M Crowley *et al.*, (2003) has also reported similar observation in conifers. Complete removal of polysaccharides during DNA isolation assumes critical importance due to their well-established interference problems. These include failure of DNA amplifications during PCR due to inhibition of *Taq* polymerase activity (Fang GS *et al.*, 1992), inhibition of activity of DNA modifying enzymes and interference in the quantification process involving spectrophotometers (Wilkie *et al.*, 1993). Several methods on removal of polysaccharides from DNA have been extensively reviewed (Lodhi *et al.*, 1994; Maryam Sarwat *et al.*, 2006; Tamsyn M Crowley *et al.*, 2003) of which salt precipitation has been recommended to be most effective (Tamsyn M Crowley *et al.*, 2003; Lodhi *et al.*, 1994). Salts, when used in precipitation increase the solubility of polysaccharides in ethanol thus preventing its

Table 3: Optimization of RAPD-PCR reaction parameters for *Urginea indica*

PCR parameter	Tested quantity/ temp/ cycles	Optimum conditions	Results
Magnesium chloride (mM)	1.5 ,1.75, 2 & 2.5	2.5mM	Lower concentration of MgCl ₂ gave poor amplification due to inadequate <i>Taq</i> polymerase activity
Deoxynucleotide triphosphate (dNTPs) (mM)	0.05, , 0.1, 0.15 & 0.2	0.2mM	Reduced concentration of dNTPs, showed lack of reproducibility.
Primer concentration (pmol)	10 & 15	15 pmol	Lower concentration of primer failed to generate proper amplification products
<i>Taq</i> polymerase (units)	0.5, 0.75 & 1.0	0.75 unit	Higher concentration resulted in decreased specificity and background (smear) formation upon gel electrophoresis
Denaturation temperature (°C)	92, 93 & 94	92°C	Higher denaturation temperature resulted in poor recovery of amplified products
Initial denaturation time (min)	2, 3 & 4	2 min.	Higher time interval resulted in reduced amplification, loss of <i>Taq</i> polymerase activity & lack of reproducibility
Annealing temperature (°C)	35,36,40,42,44,46,48, 50,52 & 54	48°C	Lower / higher annealing temperature (from optimum) resulted in non-specific amplicons.
Amplification cycle profile	Double cycle & single cycle profiles	Double cycle profile	The resolution of amplified products was better in case of double cycle amplification profile
Number of cycles	25,30,35,39,40,41,42,43,44 & 45	40 cycles	Reduced number of cycles resulted in poor amplification and more than 40 cycles resulted in background formation.

coprecipitation with DNA (Lodhi *et al.*, 1994). In the present work, reprecipitation of dissolved DNA with salt at the end of the process, ensured complete removal of residual polysaccharide in the sample (Fig. 1B). This step proved very critical for the recovery of pure DNA in the entire isolation process. Traces of salt in the sample was subsequently removed by 2-3 washes with 70% ethanol. The purity of the extracted DNA was reconfirmed by subjecting the isolated DNA to restriction digestion with four restriction enzymes *Alu* I, *Hind* III, *Eco* R I & *Bam* H I. The characteristic restriction digestion pattern, akin to standard DNA samples (Fig. 1C), confirmed the suitability of the extracted DNA for molecular biology applications. The reproducibility of the protocol was tested in 14 different populations of *Urginea indica*, wherein the protocol resulted, in all the samples, distinct, sharp & clear DNA bands with yield ranging from 120-150 µg per gram of bulb tissue (Fig. 2).

The protocol for PCR-RAPD was optimized by introducing several modifications to the original Williams *et al.* (1990) protocol in both PCR-components such as template DNA, primer, magnesium chloride, *Taq* polymerase, dNTPs, as well as in amplification cycles including number of cycles, temperature and time intervals for denaturation, annealing and extension steps (Table-3). The optimized reaction conditions produced clear, scorable amplified products suitable for RAPD applications (Fig. 3) in all the 15 primers tested. The number of bands for each primer varied from 04 in OPAK-04 to 16 in OPA-17. Each primer produced amplification products in the size range 0.15 kb in OPA-17 to 3.0 kb in OPAJ-04. The 15 tested primers yielded totally 1275 scorable bands (number of propagules X number of screened markers) with an average of 12 bands per primer.

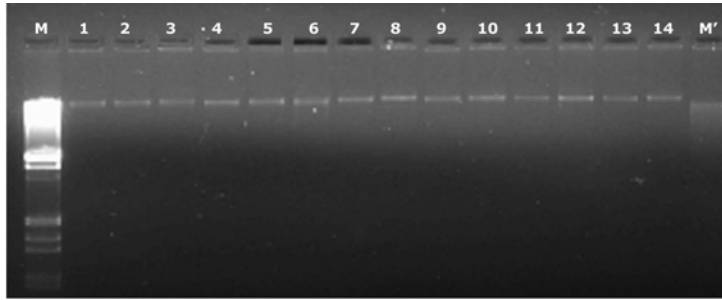


Figure 2: Electrophoretic pattern of DNA samples extracted from 14 different populations of *Urginea indica* using currently described method. Lane 1-14: DNA extracted from 14 populations of *Urginea indica*; M: Molecular weight marker Lambda DNA double digest (*Eco R I / Hind III*); M': Standard Lambda DNA (50ng/μl).

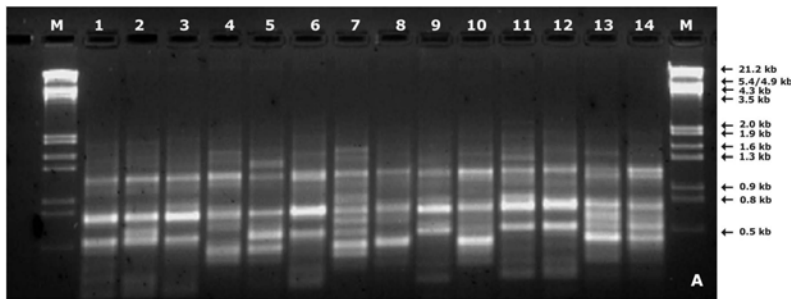


Fig. 3A

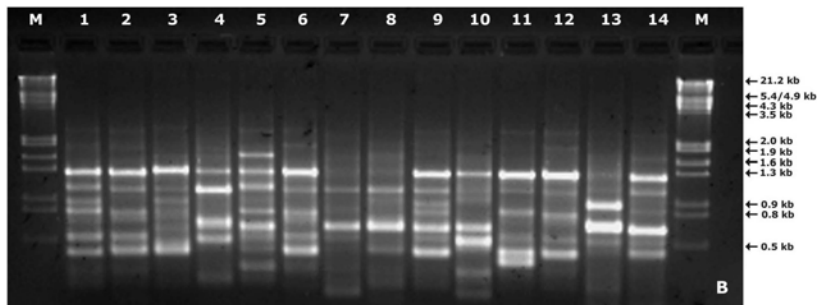


Fig. 3B

Figure 3: Electrophoretic pattern of RAPD products generated with primer OPA-15 (Fig 3A), OPA-16 (Fig 3B). M: Molecular weight marker Lambda DNA double digest (*Eco R I / Hind III*); Lane 1-14: Bulb DNA isolated from 14 different populations of *Urginea indica*.

The present study on development of protocol for isolation of high purity DNA and optimization of RAPD conditions is the first report in the species *U. indica*. This will form a strong beginning for future molecular characterization and genetic improvement works in this promising medicinal plant.

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