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Extraction of High Quality Genomic DNA for Genetic Diversity and Molecular Marker Studies from Leaves of Makhana (*Euryale ferox*)

Nitish Kumar[1*], Hemant Kumar[1] and Swati Kumari[1]

[1]Centre for Biological Sciences (Biotechnology), School of Earth, Biological and Environmental Sciences, Central University of South Bihar, Patna 800 014, Bihar, India
[2]Centre for Education (Life Science), Central University of South Bihar, Gaya Campus, Gaya 823 001, Bihar, India

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ABSTRACT Genetic diversity study of plants through molecular markers depends on both quality and quantity of DNA. The standard protocols and commercially available kits failed to isolate high quality DNA from leaves tissue of Makhana (*Euryale ferox* Salisb.) due to the occurrence of the high amount of polysaccharides, polyphenols and proteins. A cetyl trimethyl ammonium bromide (CTAB) protocol has been optimized for isolation of high quality genomic DNA from the leaves of *E. ferox*. The optimized protocol yielded up to 4081 ng per gram leaves tissue of good quality DNA, which was free of polysaccharide, polyphenols and protein. The problems of DNA degradation, contamination, and low yield due to irreversible binding of phenolic compounds and co-precipitation of polysaccharides with DNA were avoided by this method. The isolated DNA has excellent spectral qualities, and e芯ciently digested by restriction endonucleases and it is also suitable for development of molecular markers such as RAPD and ISSR.

INTRODUCTION

Makhana (*Euryale ferox* Salisb.), a prickly floating plant of Nymphaeaceae family, is one of the most important food crops of north Bihar, India (Mandal et al. 2010). The commercial value of *E. ferox* seeds lies in their popped form. The starchy white puffs are very nutritious and tasty, and marketed as a premium dry fruit commodity of *E. ferox* (Nath and Chakraborty 1985). Seeds of *E. ferox* are also reported for its medicinal properties, and it is widely used in Ayurveda and Chinese preparations for the treatment of a number of human ailments involving respiratory, circulatory, digestive, excretory and reproductive systems (Kumar et al. 2016). The cultivation *E. ferox* is still neglected, probably because of lack of awareness among the rural masses and paucity of suitable improved varieties. In order to take effective measure to encourage genetic conservation and suitable use of *E. ferox* resources, the development and application of molecular markers in *E. ferox* are imperative. For molecular marker studies, there is need of high quality DNA. Many standard protocols are available for extracting plant genomic DNA, however, problems of DNA extraction is quit a vital concern in the area of plant molecular biology, as the quality of template DNA influences the accomplishment of subsequent downstream experiments (Sika et al. 2015). High levels of polysaccharides, many types of secondary metabolites; polyphenols and other organic compounds affect DNA purification, the quality of the DNA sample and also inhibit enzymatic reactions, such as polymerase chain reaction (PCR) while DNA is used as a template (Abdel Latif and Osman 2017). The *E. ferox* leaf is rich in pigments as the underside of the leaf is dark purplish. The leaf also contains intercalating ribs underside and upper surface is covered by sharp prickles. It seems leaves are rich in proteins, polysaccharides and polyphenolic compounds are the problem for DNA isolation.

[*]Address for correspondence:
Dr. Nitish Kumar
Centre of Biological Sciences, (Biotechnology),
School of Earth, Biological and Environmental Sciences,
Central University of Bihar, Patna 800014, Bihar, India
E-mail: nitish@cub.ac.in

Objective

The present investigation was, therefore undertaken to isolate a high quality genomic DNA which can be used in genetic diversity and molecular marker studies. The isolated DNA has proved amenable to PCR amplification and restriction digestion.

METHODOLOGY

DNA isolation method, developed by Doyle and Doyle (1990) was modified to recover good quality of DNA from leaves of *E. ferox*. The important modifications included in the present protocol are the use of (i) Different concentrations of NaCl in extraction buffer (Table 1), (ii) Different concentration of PVP in extraction buffer (Table 2) and, (iii) Tissue to buffer ratio (Table 3). To remove protein contaminations, the researchers used phenol: chloroform: isoamyl alcohol (25:24:1) followed by chloroform: isoamyl alcohol (24:1) extraction in extraction and purification phases. All the experiments were repeated three times to check reproducibility.

Table 1: Effect of different concentration of NaCl on quantity and purity index of extracted DNA from leaves of *E. ferox*

NaCl	A_{260}/A_{280}	*DNA quantity (ng/g tissue)*
1.5M	1.06± 0.017	2430± 21.60
2.0M	1.26± 0.20	2723± 18.87
2.5M	1.41± 0.018	2820± 16.83
3.0M	1.6± 0.021	1373± 22.33
3.5M	1.75± 0.041	4081± 15.12

Note: Values are the mean±SD

Table 2: Effect of different concentration of polyvinyl pyrrolidone (PVP) on quantity and purity index of extracted DNA from leaves of *E. ferox*

PVP (%)	A_{260}/A_{280}	*DNA yield(ng/g tissue)*
1.0	1.18± 0.13	2336± 13.60
1.5	1.25± 0.10	2424± 20.92
2.0	1.46± 0.069	2621± 17.45
2.5	1.67± 0.040	4003± 12.5
3.0	1.57± 0.055	2445± 15.29

Note: Values are the mean±SD

Extraction Buffer

The extraction buffer consisted of two percent cetyl trimethyl ammonium bromide (CTAB), 100 mM Tris-HCl pH 8.0, 20 mM Ethylene diamine tetraacetic acid (EDTA) pH 8.0, NaCl (1.5, 2.0, 2.5, 3.0 and 3.5 mM), polyvinyl pyrrolidone (PVP)(1.0, 1.5, 2.0, 2.5 and 3.0%) and 1.3 percent mercaptoethanol.

Table 3: Effect of different concentration of tissue buffer on quantity and purity index of extracted DNA from leaves of *E. ferox*

Tissue-buffer ratio	A_{260}/A_{280}	*DNA quantity (ng/g tissue)*
1:3	1.42± 0.17	2706± 9.70
1:4	1.61± 0.029	2744± 8.93
1:5	1.74± 0.063	3924±19.47
1:6	1.77± 0.023	4001±11.95
1:7	1.66± 0.051	2715±13.14

Note: Values are the mean±SD

Chemicals

Phenol: chloroform: isoamyl alcohol (25:24:1), Chloroform: isoamyl alcohol (24:1), seventy percent ethanol, 1M Tris-HCl (pH 8.0), 0.5M EDTA (pH 8.0), 5M NaCl, 3M Sodium acetate (pH 5.2) and TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) were used in present study. Solutions and buffers were autoclaved at 121°C at 15 psi pressure (Tomy autoclave, Japan). The stock solution of 10 mg/ml of Proteinase K (HiMedia, India) and 10 mg/ml of RNase (Sigma, USA) was prepared as per the user's manual.

DNA Isolation Protocol

Extraction Phase

Fresh leaves of *E. ferox* plants were rinsed with distilled water and blotted moderately with soft tissue paper. Hundred mg leaf tissue was ground in liquid nitrogen along with the different concentrations (1, 1.5, 2.0, 2.5 and 3.0%) of PVP. The powdered tissue was transferred into a 2.0 ml tubes containing preheated (65°C) extraction buffer in a 1:6 ratio (0.6 ml). 1.3 percent mercaptoethanol was then added and mixed well. The tube was incubated in water bath at 65°C for 60 min with intermittent shaking and cooled for 5 min. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the extract and mixed vigorously by inversion. The mixture was centrifuged at 10000 rpm for 10 min at room temperature (RT). Phenol: chloroform: isoamyl alcohol extraction step was repeated.

The upper aqueous phase was transferred to a fresh microfuge tubes, avoiding the interface. To the above solution, an equal volume of chilled isopropanol and moderately mix was added. The tubes were then incubated at -20°C for 1 h. After incubation, the mixture was centrifuged at 12000 rpm for 15 min. The subsequent white/translucent pellet was washed with ice-cold seventy percent ethanol. The DNA pellet was allowed to air dry for 30 min and suspended in 200 µl of TE buffer.

Purification Phase

The samples were then treated with 4 µl of 10 mg/ml RNase and incubated in water bath at 37°C for 60 min. 4 µl of 10 mg/ml proteinase K was added and incubate at 37°C for 30 min. After incubation add 200 µl of TE buffer and 400 µl of chloroform: isoamyl alcohol (24:1) mixed gently and centrifuge at 10000 rpm for 10 min at RT. Chloroform: isoamyl alcohol extraction step was repeated. The upper aqueous phase was transferred to a fresh microcentrifuge tubes. To the above solution half volumes of sodium acetate (pH 5.2) and two volumes of saturated cold ethanol was added, mixed gently and then incubated at -20°C for 1 h. The mixture was centrifuged at 12000 rpm for 15 min at RT to pellet the DNA. The pellet was then washed with seventy percent cold ethanol twice; air dried and finally suspended in 40-50 µl of TE buffer.

Quantification of DNA

DNA was quantified by spectrophotometer (Perkin Elmer Life and Analytical Sciences, Singapore) at 260 and 280 nm, and the A_{260}/A_{280} ratio was used to assess contamination. In order to validate DNA integrity, 3 µl of DNA were subjected to gel electrophoresis on 0.8 percent agarose gel, and visualized under a UV transilluminator and photographed by gel documentation system (BIO-RAD).

Post-extraction DNA Analysis

Restriction Digestion

The extracted genomic DNA of *E. ferox* was digested by incubating with *Eco* RI, *Bam* HI, *Hind* III and *Sau* III A restriction endonucleases (Thermo Scientific, USA) along with control (without adding enzyme) in the corresponding buffers at 37°C according to the user's manual. The digested DNA along with control was analysed by running the samples on one percent agarose gel.

RAPD Amplification

RAPD reactions were performed by decamer arbitrary primer OPK 16 (Xcelris genomics, India) with 25µl reaction mixture (Williams et al. 1990). Amplification mixture contains 1x PCR buffer (10mM Tris-HCl pH 8.8, 50 mM KCl, 0.1 percent Triton X-100, 10 mM $MgCl_2$), 0.2 mM each dNTP, 10 pmol of primer, 5 ng template and 1 unit *Taq* DNA polymerase (G-Biosciences, USA). PCR amplification was performed using ProFlex PCR System (Life Technologies, USA) with initial denaturation at 94°C for 5 min, followed by 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 37^0C, 1 min extension at 72^0C and final extension at 72^0C for 5 min. PCR products were separated on 1.5 percent agarose gel.

ISSR Amplification

ISSR amplification reactions were performed by primer UBC 807 (Xcelris genomics, India) with 25µl reaction mixture containing 10 ng template DNA, 1x PCR buffer, 0.2 mM each dNTP, 1 unit *Taq* DNA polymerase (G-Biosciences, USA) and 10 pmol of primer (Xcelris genomics, India). The amplification reaction consisted of an initial denaturation step at 94^0C for 5 min, followed by 35 cycles of 1 min at 94^0C (denaturation), 1 min annealing at 52^0C , and 2 min extension at 72^0C followed by a final extension step at 72^0C for 5 min. Amplification products were electrophoresed in 1.5 percent agarose gel.

RESULTS

DNA Extraction and Purification

In the present study, different concentrations of NaCl and PVP were used in the extraction buffer. The mean DNA yield per gram of leaf tissue was 4081ng and ratio 1.75 at A_{260}/A_{280} was obtained by the use of 3.5 M NaCl. 2.5 percent of PVP gives the good quality DNA with A_{260}/A_{280} of 1.67 and the DNA yield mean per gram leaf tissue was 4003 ng. Polyphenols contamination decreased when increasing the concentration of PVP as shown in agarose gel electro-

phoresis (Fig. 1). DNA showed as fire type bands in lane 1 and 2 due to the presence of polyphenolic compounds. DNA showed as sharp bands in lane 3 and 4, and amount of DNA is more while using 2.5 percent PVP shown in lane 4 (Fig. 1). The DNA concentration of per gram of leaf tissue 2445 ng and A_{260}/A_{280} ratio 1.57 was found while using three percent of PVP. The DNA concentration and A_{260}/A_{280} have been decreased by using three percent PVP in comparison to the use of 2.5 percent PVP in extraction buffer. The protein hydrolysing enzyme like proteinase K was not sufficient to remove proteins. Thus, the use of high concentration NaCl (3.5M) in the extraction buffer along with two extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) in the extraction and purification phase, could successfully remove protein impurities without affecting DNA yield. It was also observed that tissue to buffer ratio was a vital factors for obtaining higher yields of DNA in the case of *E. ferox*. The tissue to buffer ratio (1:6) gave the best results with 4001 ng of DNA per gram of tissue with A_{260}/A_{280} ratio of 1:77.

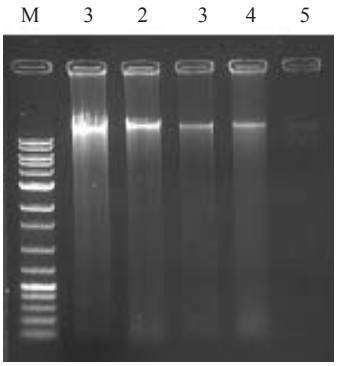

Fig. 1. Genomic DNA isolated using different concentration of polyvinyl pyrrolidone (PVP) from leaves of *E. ferox*. M:1 Kb standard marker; Lane 1-5 represent DNA isolated with 1%, 1.5 %, 2%, 2.5%, and 3% PVP respectively.

Restriction Digestion Analysis

Genomic DNA was digested by with *Eco* RI, *Bam* HI, *Hind* III and *Sau* III A restriction endonucleases at 37°C. The restricted DNA produced smear on one percent agarose gel, indicating complete digestion of DNA samples indicating the purity of DNA (Fig.2).

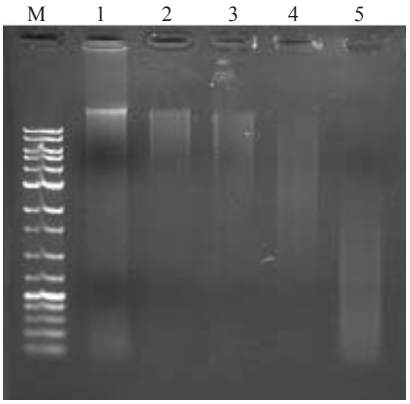

Fig. 2. Restriction digested genomic DNA isolated from leaves of *E. ferox*. Lane M: 1 Kb standard marker; Lane 1- undigested genomic DNA; Lane 2-5 represent DNA digested with *Eco* RI, *Bam* HI, *Hind* III, III A respectively.

RAPD and ISSR Analysis

Genomic DNA obtained could be amplified using RAPD and ISSR primers and good amplifications were observed in all 16 *E.ferox* germplasm which was collected from different regions of Bihar, India (Figs. 3 and 4).

DISCUSSION

The isolation of high quality genomic DNA is essential for several molecular biology applications and it is a difficult task for the plant having high amount of polysaccharides, polyphenols, and proteins. The protein and polysaccharides contamination in DNA was confirmed by electrophoresis as it gave fire type of bands, uneven migration and often remained in the wells (Abdel Latif and Osman 2017). The occurrence of polysaccharides has been shown to inhibit *Taq* polymerase activity (Sajib et al. 2017) and restriction enzyme activity (Martinez 2017). The occurrence of polysaccharides in the DNA sample is characterized by the formation of a highly viscous solution (Abdel Latif and Osman 2017). In the present investigation, DNA was isolated from leaf tissue of *E. ferox* by the modified CTAB protocol (Doyle and Doyle 1990) and commercial kit yielded very low quantity of DNA with polysaccharide, protein and polyphenols contamination. DNA isolated from modified protocol yield high quality genomic DNA which was free from different types of contam-

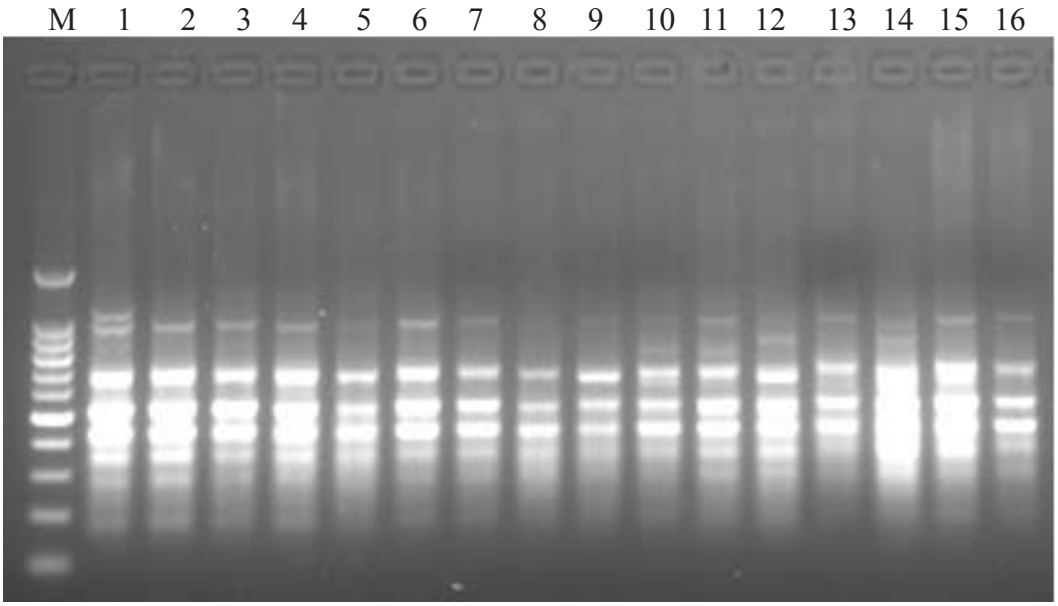

Fig. 3. RAPD profile of DNA from 16 *E. ferox* germplasm using OPK 16 primer. Lane M represent 100bp standard marker; Lane 1-16, represent different 16 *E. ferox* germplasm.

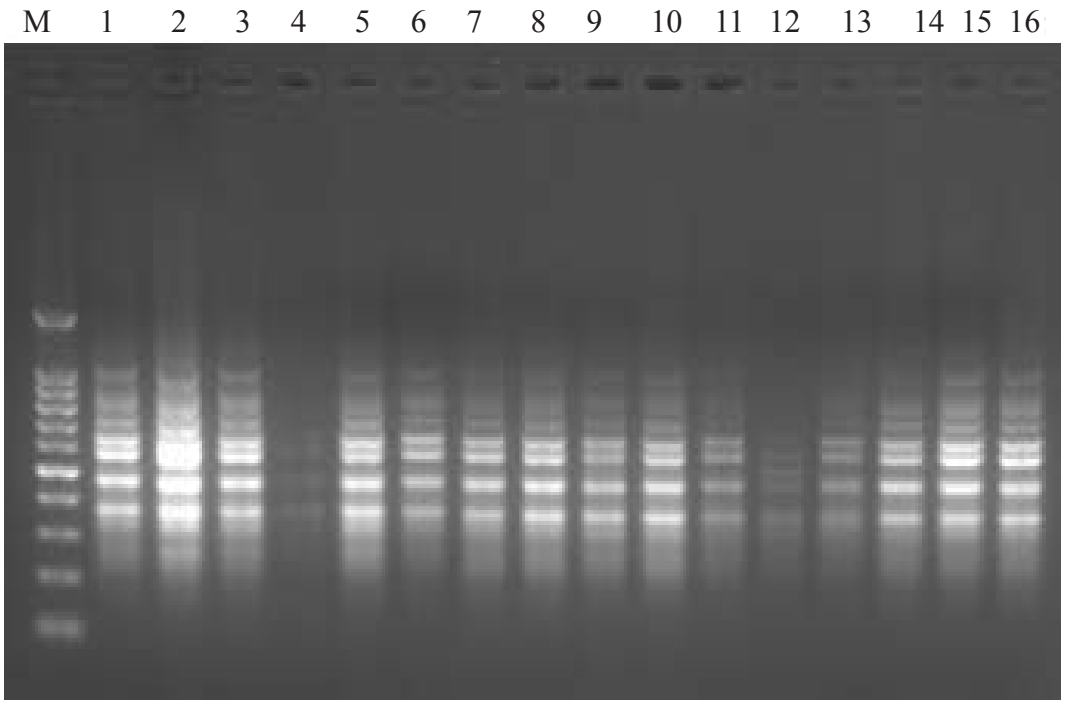

Fig. 4. ISSR profile of DNA from 16 *E. ferox* germplasm using UBC 807 primer. Lane M represent 100bp standard marker; Lane 1-16, represent different 16 *E. ferox* germplasm.

ination and the quality of DNA was evaluated by its physical appearance, spectrophotometry, gel electrophoresis, and PCR amplification. The physical appearance of the extracted samples by the modified methods performed preeminent and resulted in absolutely transparent DNA solutions, while the DNA obtained from original methods showed yellowish and dark contaminations mainly due to the fast oxidation of the extracts. In the present study, modified CTAB protocol enhanced the yield and quality of DNA. PVP helps in forming complex hydrogen bonds with phenolic compounds and co-precipitates with cell debris upon lysis. When the extract is centrifuged in the presence of chloroform, the PVP complexes accumulate at the interface between the organic and the aqueous phases (Arruda et al. 2017). The present protocol yielded high molecular weight DNA and A_{260}/A_{280} was closest to 1.8 after using 3.5M NaCl, 2.5 percent PVP and 1:6 tissues to buffer ratio. In other samples A_{260}/A_{280} was less than 1.8 which indicated compromised DNA quality. This may be due to the differences in levels of polysaccharides, proteins and polyphenols associated anti-quality features in these plant species. The purity of DNA sample was confirmed by its A_{260}/A_{280} ratio (1.8) and digestion with restriction enzyme *Eco* RI, *Bam* HI, *Hind* III and *Sau* III A (Fig. 2). The suitability of isolated DNA from the present protocol in molecular techniques was assessed by RAPD and ISSR analysis and good amplification was observed in case of all *E.ferox* plants collected from different regions of Bihar, India, which are useful to evaluate the genetic diversity and phylogenetic relationship.

CONCLUSION

DNA isolation from *E. ferox* has used the modified CTAB method using different concentration of PVP and NaCl. Taking into consideration of the presence of high amount of phenolic, an effective procedure has therefore been developed for DNA extraction from *E. ferox*. The protocol described here is efficient, inexpensive, and yields clean genomic DNA, amplifiable by PCR, as indicated by the results of the RAPD and ISSR technique. This method is very simple and reliable for plant species like *Euryale*.

RECOMMENDATIONS

The leaf of *E. ferox* contains intercalating ribs and spines which contains high amount of pigment and polysaccharides. So, during extraction of genomic DNA, ribs and spines must be removed for pure DNA isolation.

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