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Optimization of genomic DNA isolation method from mature leaves of *Dalbergia latifolia*

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ABSTRACT

Germplasm conservation of *Dalbergia latifolia* is very important as it is listed in vulnerable category of IUCN Red list. To reduce the efforts, cost, land and labour in conservation of germplasm, it is needed to assess genetic diversity present in the species. The success of genetic improvement program also depends on genetic diversity present in the breeding material. The molecular markers are widely utilized for the assessment of genetic diversity these days. High quality and quantity of genomic DNA is prerequisite for utilizing molecular markers. Hence, the protocol for the isolation of genomic DNA of black shisham (*D. latifolia*) has been optimised under the present study. The optimized protocol yielded 500-9000 ng/µl genomic DNA with an average of 2500 ng/µl DNA from 89 samples of *Dalbergia latifolia*. A very low level of RNA, protein, phenolics and polysaccharide contaminants were recorded (A_{260}/A_{280} ratio ranges from 1.56-2.06) from the isolated DNA. The optimized protocol was yielded genomic DNA of good quality and quantity from both, juvenile leaf and matured leaf samples so, the protocol can be utilized for isolation of high-quality DNA for genetic improvement programs and genetic diversity assessment.

Key Words - Vulnerable, *Dalbergia latifolia*, molecular markers, genomic DNA, genetic improvement

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INTRODUCTION

Dalbergia latifolia is an important timber species belongs to the family Fabaceae and distributed naturally in southern and Eastern India. It is commonly known as Indian rosewood, black shisham, rosewood and gives premier quality timber. It is listed as vulnerable (A1cd ver 3.1) in the IUCN Red list of threatened species (Lakhey *et al.,* 2020). It is a slow growing species, grows up to 20-40 meters in height and about 1.5-2.00 meters in girth (Prasad *et al.* 1993). Its wood is used for decorative, carving, ornamental ply-boards purposes, for making wheels of gun carriages and ammunition boxes. The ply-boards made up of Indian rosewood utilized in aircrafts.

In Eastern India, its population is sporadically distributed and mainly found as plantations and sparsely distributed in forest areas. The species is also listed in vulnerable category of IUCN red list; therefore, it is needed to conserve its germplasm. Germplasm conservation is laborious, time taking, needs lot of space and to reduce these, accommodate maximum diversity of the species, the genetic diversity present in the species needs to be estimated (Kumar and Das, 2013). Genetic diversity estimation can be performed by using conventional methods but it takes lot of time and space. The modern molecular tools viz. molecular markers provide information in lesser time and utilized extensively by researchers in genetic diversity estimation. To utilize molecular markers,

the genomic DNA of high quality and quantity is needed and as such there was no protocol available for the isolation of genomic DNA of the *D. latifolia*. In the present study the genomic DNA isolation protocol from the mature leaves of the species has been optimized by modifying the CTAB method.

MATERIAL AND METHODS

The genomic DNA isolation protocol was optimized by using mature leaves of *Dalbergia latifolia* planted at the field of Institute of Forest Productivity, Ranchi (Jharkhand).

Table 1- Reagents, Chemicals and Laboratory materials

Sl. No.	Stock solution	100 ml working solution
1.	10% CTAB (Hexadecyl trimethyl-ammonium bromide)	20 ml
2.	1 M Tris HCL pH 8.0	**10 ml**
3.	0.5 M EDTA pH 8.0 (Ethylenediaminetetraacetic acid Di-sodium salt)	**4 ml**
4.	5 M NaCl	**28 ml**
5.	2-β- mercaptoethanol (2%)	**2 ml**
Adjusted all to pH 8.0 with HCL and made up to 100 ml by adding distilled water		
1gm of PVP dissolved in 10 ml of distilled water (10% PVP). (Used 100µl of PVP separately while grinding the sample)		

Other reagents and materials were chloroform: isoamyl alcohol (24:1), 10µl of RNase (20mg/ml), Ice chilled isopropanol (100%), ethanol (70 %) and TE buffer (10 mM Tris base and 1mM EDTA). Laboratory material and equipment includes, mortar-pestle, 1.5 ml and 2 ml microcentrifuge tubes, micropipettes, microtips, Eppendorf tube holding racks, Centrifuge, refrigerator etc.

Sample collection and preparation

Fresh juvenile and matured leaves were collected in zip bags from the field and kept in ice box. The leaves were stored in refrigerator at -40°C in the laboratory to maintain the freshness of the leaves. The collected samples were washed with distilled water to remove particulate contaminants and unwanted materials from the leaf surfaces. To facilitate the grinding of samples, the mid ribs and veins of leaves were removed and leaf lamina was chopped into pieces.

Extraction protocol:

In the standardized protocol, 300 mg leaf sample was taken, washed, mid ribs and veins were removed, leaf lamina were chopped and grinded in a fine paste with 1 ml DNA extraction buffer along with 100µl of 10% PVP in mortar-pestle. The paste was transferred to 2 ml microcentrifuge tubes and incubated at 65°C for 30 minutes in hot water bath. After incubation, microcentrifuge tubes were cooled down to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added to the microcentrifuge tubes, followed by gentle inversion of tubes for 15 minutes at room temperature. Then, tubes were centrifuged at 15,000 rpm for 10 minutes at 4ºC. Supernatant was transferred into a fresh 1.5 ml tube. Again chloroform: isoamyl alcohol (24:1) separation was done to obtained clear supernatant. Upper aqueous phase was taken into another fresh 1.5 ml microcentrifuge tube and 600µl of ice chilled isopropanol was added. Mixture was gently mixed by inverting the tubes and incubated at -20°C for 60 minutes (or at 4°C for overnight). Centrifugation was done at 10,000 rpm for 10 minutes at 4°C in order to obtain DNA pellet. Supernatant was discarded and DNA pellet was washed with 70% ethanol two times. The washing of DNA pellet was done with 500 µL 70% ethanol with centrifugation at 5000 rpm for 10 minutes at 4°C. The ethanol was discarded and pellet was air dried until ethanol was evaporated completely. The dried pellet was dissolved in TE buffer (10mM Tris base and 1mM EDTA) and stored at -20º C for further use.

Quality assessment and quantification of extracted DNA

The quantity and purity of extracted genomic DNA was estimated in Bio-photometer Plus (Eppendorf),

where 1µl DNA sample was taken in the stage and OD was measured at 260 nm. Purity of DNA was determined by calculating the ratio of absorbance at 260nm and 280nm wavelength. The extracted genomic DNA was electrophoresed in 0.8% agarose gel containing 10mg/ml EtBr submerged in 0.5X TBE buffer for 1 hr at 65V and image of the gel was captured using gel documentation system (Bio-Rad). Through the absorbance and transmittance value the DNA quantity estimated which can be subjected to make a dilution for PCR analysis.

RESULTS AND DISCUSSION

The genomic DNA of *Dalbergia latifolia* was isolated by using optimized DNA isolation protocol. The quality of isolated genomic DNA was confirmed by 0.8 % agarose gel electrophoresis, which confirmed the purity and quantity of DNA by depicting the clear band without smear (Figure 1). The genomic DNA was isolated from 89 individual plants by using the optimised protocol and assessed for its quantity and purity by using bio-photometer (Table 1). The protocol was also not utilized any lyophilization or liquid nitrogen or enzymatic digestion as reported by various authors (Manen *et al*., 2005, Chakraborti *et al*., 2006, Arbi *et al*., 2009, Agbagwa *et al*., 2012) for grinding/rupturing the cell wall and nuclear wall which reduces the cost of per sample DNA isolation and risk of health hazard.

Table 1: *Dalbergia latifolia* genomic DNA quantity and purity estimated through Bio-photometer

S.N.	Accessions utilized for DNA isolation	Concentration of DNA (ng/µl)	Purity of DNA (A_{260}/A_{280})
1	DLAD-1-1	2700.27	1.79
2	DLAD-2-3	2051.41	1.92
3	DLAD-3-2	2452.68	2.02
4	DLAD-4-1	2428.12	2.00
5	DLADA-1-3	2303.15	1.84
6	DLADA -2-1	1206.67	2.05
7	DLADA -3-1	1467.61	1.97
8	DLAGO- 1-1	1398.16	1.89
9	DLAGO -2-1	1725.66	1.98
10	DLAGO -3-2	1405.24	1.86
11	DLAGO -4-1	2890.98	2.00
12	DLAGO -5-1	1948.36	1.66
13	DLAGU-1-1	1166.68	1.57
14	DLAGU-4-2	1681.73	1.60
15	DLAGU-5-1	1951.55	1.95
16	DLAGU-6-2	1624.57	1.85
17	DLAGU-7-1	849.00	1.75
18	DLAGU-8-2	1001.40	1.63
19	DLAGU-9-2	536.45	1.74
20	DLAIFP-1-1	435.38	2.01
21	DLAIFP-2-2	534.71.	1.90
22	DLAIFP-3-1	363.55	1.99
23	DLAIFP-4-2	1216.80	1.86
24	DLAIFP-5-1	1166.24	1.48
25	DLAIFP-6-1	1123.75	1.86
26	DLAIFP-7-1	1053.17	1.92
27	DLAIFP-8-2	1358.75	1.66
28	DLAIFP-9-2	1144.97	1.86
29	DLAIFP-10-2	1356.69	1.63
30	DLAIFP-11-1	1289.16	1.84
31	DLABAU-1-2	2862.01	1.94
32	DLABAU-2-1	1763.43	1.86
33	DLABAU-3-2	1781.72	1.86
34	DLABAU-4-1	2441.56	1.94
35	DLABAU-5-1	2062.65	1.72
36	DLABAU-6-1	1464.37	1.70
37	DLARG-1-2	1626.44	1.85
38	DLARG-2-2	1624.70	1.63
39	DLARG-3-1	3762.23	1.76
40	DLARG-4-2	2289.90	1.91
41	DLARG-5-1	2415.30	1.70
42	DLARG-6-1	2884.45	1.95
43	DLARG-7-1	2202.15	1.73
44	DLARG-10-2	3599.13	1.99
45	DLARG-11-2	1666.46	1.91
46	DLARG-12-1	1581.84	1.90
47	DLARG-13-1	2726.46	1.62
48	DLARG-14-1	2190.69	1.76
49	DLARG-15-1	2530.34	1.86
50	DLARG-16-1	2397.88	1.67
51	DLACH-1-1	2700.51	1.66
52	DLACH-2-1	3602.17	1.66
53	DLACH-3-1	3534.33	1.53
54	DLACH-4-1	2313.23	1.64
55	DLACH-5-2	1844.14	1.87
56	DLACH-6-2	2527.79	1.70
57	DLACH-7-1	2875.94	2.04
58	DLACH-8-1	1891.42	1.90
59	DLACH-9-2	3850.11	2.00
60	DLACH-10-1	2362.08	1.86
61	DLACH-11-1	2267.52	1.95
62	DLACH-12-1	3504.48	1.93
63	DLACH-13-1	2905.79	2.00
64	DLACH-14-1	3018.28	1.87
65	DLACG-1-2	2055.53	1.56
66	DLACG-2-1	2306.25	1.85
67	DLACG-3-1	1879.52	1.81
68	DLACG-4-1	5501.86	1.55
69	DLACG-5-2	1724.98	1.83
70	DLACG-6-2	2883.77	1.85
71	DLACG-7-1	3060.43	1.63
72	DLACG-8-1	2752.22	1.74
73	DLACG-9-2	9671.25	1.37
74	DLACG-10-2	7963.34	1.54
75	DLACG-11-1	1972.14	1.61
76	DLACG-12-1	2744.33	1.80
77	DLACG-13-1	2665.95	1.74
78	DLACG-14-1	2455.38	1.60
79	DLACG-15-2	2171.65	1.62
80	DLACG-16-1	2126.80	1.80
81	DLACG-17-2	929.14	1.66
82	DLACG- 18-2	3904.84	1.91
83	DLAT-1-1	1077.15	1.82
84	DLAN-1-1	517.80	1.77
85	DLAK-1-1	3033.82	1.92
86	DLAL-2-2	1400.82	1.98
87	DLAL-3-2	1877.35	2.06
88	DLAL-4-2	683.71	2.05
89	DLAL-6-2	2446.54	1.90

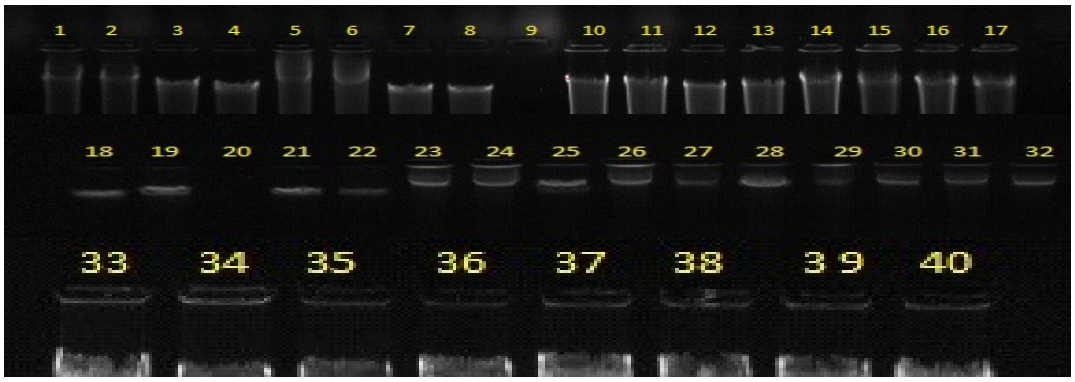

Figure 1: 0.8% agarose gel image of extracted genomic DNA of *Dalbergia latifolia*
Lane 1-40: Genomic DNA isolated by using optimized protocol.

The genomic DNA obtained by using optimizes protocol was whitish in colour and its purity was tested in 0.8% agarose gel electrophoresis (Fig. 1). The optimized protocol yielded significant amount of genomic DNA, varied from 500 ng/µl to 9000 ng/µl with A_{260}/A_{280} ratio ranged from 1.56 to 2.06 which showed the quantity and purity of extracted DNA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest and there is no financial interest to report. It is an original research work and not submitted to any other journal for consideration.

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