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Optimisation of Genomic DNA Extraction and PCR Procedure for Sal (*Shorea robusta*)

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***Abstract*:** In the present study, leaves of Sal were collected from natural populations to isolate the genomic DNA. Unfortunately, genomic DNA extracted using protocols developed by Agbagwa et al (2012) and Doyle and Doyle (1987) was inadequate and failed to obtain genomic DNA of high quality and quantity. Woody tree species are rich in higher concentrations of polyphenols, polysaccharides, and secondary metabolites and interfere in the genomic DNA isolation process. To obtain the genomic DNA in good quality and quantity, the cetyl-trimethyl ammonium bromide (CTAB) method was modified. Modifications were carried out in the concentration of PVP and 2-mercaptoethanol and the process of utilising these reagents. The quantity of extracted DNA was evaluated through a NanoBio spectrophotometer (Analytical Technologies Ltd.), and the quality was checked by 0.8% agarose gel electrophoresis. The standardised protocol yielded high molecular weight DNA in the range of 830.12 ng μl^{-1} to 1597. 23 ng μl^{-1} with an average of 1278.28 ng μl^{-1}. The absorbance ratio at 260 to 280 nm ranged from 1.81 to 1.90, with an average of 1.84, which confirmed the purity of isolated DNA and indicated the presence of very low levels of protein, RNA, and polysaccharide contaminants. The extracted DNA was used to optimise the PCR conditions for microsatellite markers and obtain sharp polymorphic bands in an agarose gel.

***Keywords*:** Sal, Genomic DNA, CTAB, PCR, Microsatellite markers

Sal is one of the most important timber species and belongs to the family Dipterocarpaceae. As a perennial and long-lived tree species, shows higher genetic diversity within the population than among populations (Surabhi et al 2017). Pandey and Geburek (2011) reported a good amount of genetic variability present in natural populations of Sal, which may be due to continuous gene flow among the populations as evidenced by population genetic structure studies. Molecular biology techniques are helpful in understanding the genetic diversity and genetic makeup of a species. For the achievability and reproducibility of most molecular biology experiments, the isolation and availability of pure genomic DNA are pre-requisites. Quality is a key issue in genomic studies and most amplification-based assays because DNA amplification can be affected by co-purification inhibitors that reduce the efficiency of subsequent PCR (Anuradha et al 2013, Youssef et al 2015). To maximise the isolation of pure DNA in large quantities, researchers are continuously working on, modifying, and developing species-specific DNA extraction protocols. The principal goal of numerous DNA isolation techniques is the improvement of a pretty quick, cheaper, and steady protocol to extract excessively fine DNA with a higher yield (Singh and Singh 2015). Obtaining a high amount of DNA from small amounts of tissue is usually a difficult task (Pereira et al 2011). In the extraction process, objective is to minimise the quantity of polyphenols and polysaccharide content (Karthikeyan et al 2010, Sandip 2013). Commercially available DNA isolation kits provide higher throughput but are mostly specific to certain species. Additionally, their availability and excessive fees may be limiting factors. The most commonly used DNA extraction procedures are based on the cetyltrimethylammonium bromide (CTAB) method. Different procedures work best for different groups considering the great diversity of plant secondary metabolites that, in many cases, may interfere with a particular method of DNA isolation (Doyle and Doyle 1987). There are multiple procedures that have been standardised for the isolation of plant DNA, e.g., Doyle and Doyle (1987), Agbagwa et al (2012), Llongueras et al (2012). The purity and quantity of genomic DNA play an important role in downstream molecular studies. Therefore, it was needed to develop a DNA isolation protocol that could yield a good concentration of DNA with high purity. The primary objective of the present study was to develop a reproducible and efficient technique for the isolation of pure, high-quantity DNA from Sal leaf tissues and standardise their PCR conditions for SSR markers. To achieve the objective, several elements affecting DNA isolation from the leaf tissue have been investigated, together with buffer composition, replacement of reagents, and the addition of reagents that

improve the DNA quality. The reagent addition steps, as well as the reagent concentration, were modified to create a robust DNA isolation protocol that yielded high-quality and large amounts of genomic DNA from Sal. By utilising an optimised protocol, the extracted DNA yielded polymorphic and sharp bands when amplified using SSR markers.

MATERIAL AND METHODS

Plant leaf tissue sample collection and preparation: Fresh leaves were collected during the period May 2021 to August 2021 from natural populations of Sal distributed in different agro-climatic sub-zones of Jharkhand, India. The collected leaves were kept in an airtight polybag with silica beads to avoid excess moisture retention. The polybags were stored in an icebox, and, on return from the field, leaf samples were kept in a -40°C deep fridge to retain the freshness of the leaves. The DNA isolation experiment was carried out during the period October 2021 to March 2022 at the Forest Biotechnology Laboratory at latitude 23°2128 NL and longitude 85°1442. The collected samples were washed with distilled water to remove particulate contaminants and unwanted materials from the leaf surfaces. The extraction buffer was freshly prepared, containing 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 1.4 M NaCl, 2% CTAB, 3% PVP, and 4% 2-mercaptoethanol.

Grinding of plant materials: The leaf samples were taken from a -40^{0}C deep fridge and washed vigorously through distilled water to remove particles on the leaf surfaces. For 200 mg of leaf sample, midribs were removed for better grinding and chopped into pieces. The chopped samples were taken into a mortar and pestle with 1 mL of CTAB extraction buffer (preheated at 65^0 C for at least 30 minutes). During grinding, a little pinch of PVP powder may be added. After grinding, an equal volume of approximately 1 mL of the resultant paste was distributed into two separate 2-ml microcentrifuge tubes.

Extraction and purification protocol: Samples were incubated in a hot water bath at 65^{0}C for 1 hour and then kept at room temperature for 10–15 minutes. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added to each microcentrifuge tube for extraction. This was mixed gently but thoroughly by continuous rocking and inverting of the tubes for up to 10 minutes. Then the samples were centrifuged at room temperature (22^{0}C) at 14,000 rpm for 12 minutes to separate the phases. The top aqueous phase was transferred to new 2-ml microcentrifuge tubes by micropipette. The last step was repeated another time for better extraction. Then the top aqueous phase was transferred to new 1.5 ml microcentrifuge tubes along with 66% of the supernatant volume; chilled isopropanol was added to precipitate the DNA sample. This was gently mixed by continuous inversion for 2 minutes and kept at 4°C overnight. The next day, samples were centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The supernatant was discarded carefully to save the DNA pellets. The pellets were washed in 70% ethanol twice at 4000 rpm for 5 minutes and air-dried till the ethanol evaporated completely from the samples. This was facilitated by inverting tubes on tissue paper for a few minutes. After proper drying, the DNA pellets were rehydrated and dissolved in 100 µl $T_{10}E_1$.

Quantification and purity of genomic DNAL DNA yield and purity were determined through NanoBio UV spectrophotometer analysis. The yield was measured by checking the optical density (OD) with a UV spectrophotometer. The purity of DNA was determined with an absorbance ratio of A260 to A280 nm. The molar absorption coefficient is a property associated with a sample and is a measure of how strongly a sample absorbs light at a specific wavelength. The concentration is simply the molar mass L^{-1} (M) of the sample dissolved in solution, and the length is the length of the cuvette used for absorbance measurement, typically 1 cm. Absorbance is dimensionless and therefore should have no units. In the context of absorption spectroscopy, optical density is an older term synonymous with absorbance (Trumbo et al 2013).

Nano Bio UV spectrophotometer: The NanoBio UV spectrophotometer is a spectrophotometer capable of highly accurate analysis of 1 µl samples for DNA, RNA, protein, pigment, the entire UV-VIS (220–700 nm) spectrum and cell density testing, and has remarkable reproducibility. It is used to measure the concentration and purity of DNA, RNA, or protein samples for various downstream applications.

Agarose gel electrophoresis: The quality was checked by 0.8% agarose gel electrophoresis. Genomic DNA products were electrophoresed on a 0.8% agarose gel with 10x TBE buffer (100 mM TrisHCl and 20 mM EDTA, pH 8.0) and stained with ethidium bromide. The bands were digitally photographed and stored in the gel documentation system.

Efficacy comparison of extraction protocols: A comparison between the standard CTAB-based protocol and protocols suggested by Doyle and Doyle (1987) and Agbagwa et al (2012) was used. Using a spectrophotometer and 0.8% agarose gel electrophoresis, the yielded DNA from all protocols was compared for quality and quantity.

PCR amplification of extracted DNA through SSR markers: The PCR reaction conditions were optimised for the extracted genomic DNA for getting polymorphic bands. A 10µl of reaction mixture was prepared containing1X Taq DNA buffer, 0.6 U Taq DNA Polymerase, 0.2 mM dNTP Mix, 50ng template DNA, 0.25 µM of each forward and reverse primer

and nuclease free molecular grade water to maintain the volume. The details of chemicals used and their concentration are given in Table 1. The reaction mixture was mixed and a short spin was given. The thermal cycler [Eppendorf Mastercycler X50] was utilised for the amplification process (Table 2).

Results and Discussion

Agarose gel electrophoresis analysis of genomic DNA : Isolated genomic DNA through all the utilized protocols were assessed through 0.8 % agarose gel electrophoresis using 10x TBE buffer (100 mMTrisHCl and 20 mM EDTA pH. 8.0) and visualized by ethidium bromide staining. The DNA obtained through the Agbagwa et al (2012) protocol was not of good quality, as a smear of DNA was found with higher RNA contamination (Fig. 1). Doyle and Doyle (1987) method also yielded a very small amount of DNA with less purity (Fig. 2). The modified CTAB method yielded good quality and quantity of genomic DNA (Fig. 3).

UV Spectrophotometric analysis of DNA: The qualitative and quantitative analysis of genomic DNA samples was performed using UV spectrophotometry. DNA absorbs UV light very efficiently, making it possible to detect and quantify it's concentration. Nitrogenous bases in nucleotides have an absorption maximum of around 260 nm. The ratio of absorbance at 260 nm/280 nm is a measure of the purity of the DNA sample, should be between 1.80 and 1.90. The first-used protocol (Agbagwa et al 2012), yielded low quality and quantity of genomic DNA. The 260 nm/280 nm data was found to be greater than 2 in all samples, which indicated the presence of RNA as an impurity (Table 4). The second utilised protocol (Doyle and Doyle 1987) also yielded a low quantity and quality of DNA with RNA as an impurity (Table 5). The modified and standardised protocol yielded substantial quantity of genomic DNA ranged from 830.12 ngµl^{-1} to 1597.23 ngµl^{-1} with an average of 1278.28 ng µl^{-1} (Table 6). The obtained DNA was also found to be pure, as the absorbance ratio at 260 to 280 nm varied from 1.81 to 1.90, with a mean of 1.84, which indicated the absence of polysaccharides, polyphenols, and RNA.

Table 1. Chemicals and their concentration used in the PCR reaction

Chemicals	Concentration utilised
10X Taq DNA buffer	1X
Taq DNA Polymerase	0.6U/reaction
dNTP Mix	0.2 mM
Forward Primer	0.25 µM
Reverse Primer	0.25 µM
Template DNA	50ng

Table 2. Thermal profile utilised for PCR reaction

PCR steps	Temperature	Time	Cycle
Initial denaturation	95°C	5 min	33X
Denaturation	95°C	45 s	
Annealing	55°C to 60°C	30 s	
Extension	72°C	45 s	
Final extension	72°C	7 min	
Storage	4°C	∞	

Annealing temperature varied for different SSR primers

Table 4. Quantitative estimates of DNA concentration revealed by UV spectrophotometry isolated using Agbagwa et al (2012) method

Quantitative estimates of DNA concentration of Sal revealed by Nano-Bio Spectrophotometer			
Genotypes	Sample weight (mg)	DNA Concentration	
		ng/µl	A260/A280
JP-15-1	200 mg	80.21	1.70
JP-15-2	200 mg	145.56	2.19
JP-15-3	200 mg	200.23	2.10
JP-15-4	200 mg	204.45	2.25
JP-15-5	200 mg	217.62	2.28
JP-15-6	200 mg	187.65	2.20
JP-15-7	200 mg	233.90	2.22
JP-15-8	200 mg	170.80	2.14
JP-15-9	200 mg	10.43	1.63
JP-15-10	200 mg	90.78	2.15

Table 3. Details of SSR primers used in the PCR amplification

Locus	Forward/Reverse	Sequence (5' to 3')	Repeat Motif	No. of bases	T_a (°C)
SSR-53	Forward	TTGCATCTCCCTGGTAGAGA	(GAA)17	20	59.9
	Reverse	TCCACAGACTTCCTACCATCT		21	
SSR-54	Forward	GCTTCTTCTGCATGGCGATG	(TTG)21	20	59.9
	Reverse	CCTCTTTTGCATGGCATCAGT		21	
SSR-74	Forward	TCAGTTTTCCTTTGACAATGAGCA	(AG)18	24	55.6
	Reverse	TGAAGCTAGATGATACTGGCAGT		23	
SSR-80	Forward	CGTCCGGGCCAAAACATTTT	(AG)26	20	56.1
	Reverse	TGTTTGATGCGTATGTGTTGCA		22	

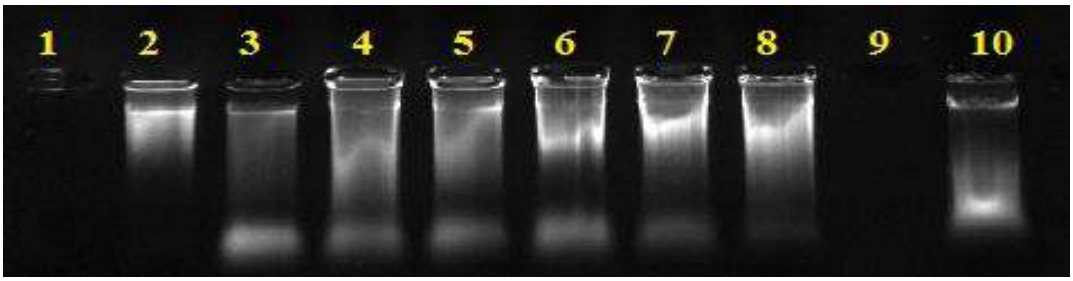
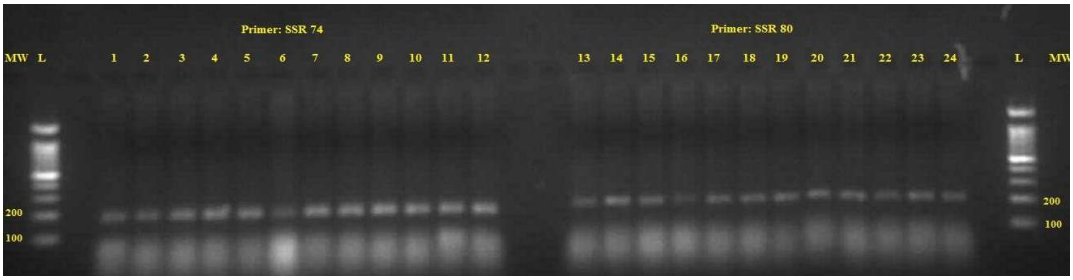


Fig. 1. Agarose (0.8%) gel electrophoresis of genomic DNA extracted using Agbagwa et al (2012) method

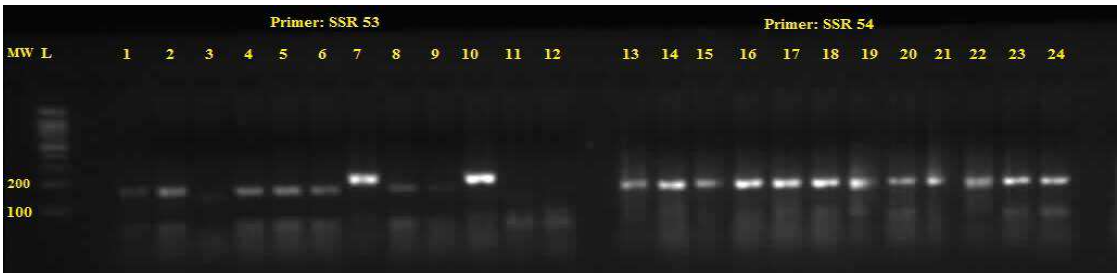
Fig. 2. Agarose (0.8%) gel electrophoresis of genomic DNA extracted using Doyle and Doyle (1990) method

Fig. 3. Agarose (0.8%) gel electrophoresis of gDNA extracted using our standardised modified CTAB method



Lane L: 50 bp DNA step ladder, Lane 1-12: Amplicons of Sal genomic DNA using primer SSR 74, Lane 13-24: Amplicons of Sal genomic DNA using primer SSR 80

Fig. 4. 2% agarose gel image of amplicons of Sal genomic DNA through PCR reaction using SSR primers (SSR 74 &SSR 80)



Lane L: 50 bp DNA step ladder, Lane 1-12: Amplicons of Sal genomic DNA using primer SSR 53, Lane 13-24: Amplicons of Sal genomic DNA using primer SSR 54

Fig. 5. 2% agarose gel image of amplicons of Sal genomic DNA through PCR reaction using SSR primers (SSR 53 & SSR 54)

Amplification of extracted genomic DNA using modified CTAB procedure by SSR markers: The genomic DNA extracted through an optimised protocol was subjected to amplification using SSR primers, viz., SSR-53, SSR-54, SSR-74, and SSR-80 (Table 3). The utilised primers amplified the targeted region of the extracted DNA and produced sharp and polymorphic bands in a 2% agarose gel.

Table 5. Quantitative estimates of DNA concentration revealed by UV spectrophotometry isolated using Doyle and Doyle (1990) method

Quantitative estimates of DNA concentration of Sal revealed by Nano-Bio Spectrophotometer			
Genotypes	Sample weight (mg)	DNA Concentration	
		ng/µl	A260/A280
JP-15-1	200 mg	120.23	1.99
JP-15-2	200 mg	94.45	2.00
JP-15-3	200 mg	50.62	1.68
JP-15-4	200 mg	187.65	2.10
JP-15-5	200 mg	263.90	2.02
JP-15-6	200 mg	71.80	2.08
JP-15-7	200 mg	150.43	2.04
JP-15-8	200 mg	172.67	2.12

Table 6. Quantitative estimates of DNA concentration revealed by UV spectrophotometry isolated using standardised modified CTAB method

Quantitative estimates of DNA concentration of Sal revealed by Nano-Bio Spectrophotometer			
Genotypes	Sample weight (mg)	DNA Concentration	
		ng/µl	A260/A280
JP-15-1	200 mg	1396.20	1.81
JP-15-2	200 mg	1528.34	1.86
JP-15-3	200 mg	1492.60	1.89
JP-15-4	200 mg	1597.23	1.82
JP-15-5	200 mg	1526.45	1.85
JP-15-6	200 mg	1228.68	1.81
JP-15-7	200 mg	1525.04	1.85
JP-15-8	200 mg	1267.91	1.86
JP-15-9	200 mg	1026.10	1.87
JP-15-10	200 mg	1324.13	1.82
JP-15-11	200 mg	1288.88	1.88
JP-15-12	200 mg	1450.24	1.81
JP-15-13	200 mg	1394.20	1.90
JP-15-14	200 mg	1487.81	1.85
JP-15-15	200 mg	1104.78	1.83
JP-15-16	200 mg	1304.24	1.86
JP-15-17	200 mg	982.76	1.84
JP-15-18	200 mg	974.15	1.84
JP-15-19	200 mg	835.77	1.85
JP-15-20	200 mg	830.12	1.88

CONCLUSION

The modified DNA isolation protocol successfully yielded high quality and quantity of genomic DNA with very low levels of protein, RNA, polyphenol, and polysaccharide contaminants compared to other utilised protocols. The genomic DNA isolated using the standardised and modified CTAB procedure underwent PCR amplification using primers such as SSR-53, SSR-54, SSR-74, and SSR-80 and resulted in polymorphic bands. This optimisation of PCR and genomic DNA isolation protocols for Sal can be utilised for its genetic improvement and molecular breeding programmes across the laboratory.

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