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Research Article

Evaluation of Five DNA Extraction Methods for *Dillenia pentagyna* Roxb. and *Hardwickia binata* Roxb. Suitable for PCR Amplification

Raj Singh Yadav[1]*, Ankur Dahayat[1], Naseer Mohammad[1], Fatima Shirin[1], Harshita Agrahari[1]

[1]Genetics and Tree Improvement Division, ICFRE- Tropical Forest Research Institute, P.O. – RFRC, Jabalpur (Madhya Pradesh) – 482021, India

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***Corresponding author**
Raj Singh Yadav,
Genetics and Tree Improvement Division, ICFRE-Tropical Forest Research Institute, P.O. – RFRC, Jabalpur (Madhya Pradesh) – 482021, India
Email: rjy2410@gmail.com

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Abstract

High-quality genomic DNA extraction is crucial for the conservation of forest genetic resources, particularly for endangered species like *Dillenia pentagyna* Roxb. (Karmal) and *Hardwickia binata* Roxb. (Anjan), which hold significant value in traditional medicine and economics. The presence of contaminants such as polysaccharides, polyphenols, and secondary metabolites in forest trees complicates the isolation of sufficient, uncontaminated DNA. In this study, we evaluated five DNA extraction protocols, including those by Doyle and Doyle (1990), Michiels *et al*., (2003), Porebski *et al*., (1997), Khanuja *et al*., (1999), and Deshmukh *et al*., (2007). Quantification and quality analysis of the extracted DNA were conducted using a Nanodrop spectrophotometer and Agarose Gel Electrophoresis. Notably, minor modifications to the CTAB-based method outlined by Doyle and Doyle (1990) significantly enhanced DNA quality. The absorbance ratio at 260/280nm indicated mean purity ratios of 1.62 for *D. pentagyna* and 1.7 for *H. binata*, with DNA concentrations recorded at 341.6 µg/ml and 317.72 µg/ml, respectively. The Doyle and Doyle (1990) method consistently produced high-quality DNA, devoid of contaminants. Subsequent PCR amplification with SSR primers confirmed the suitability of the extracted DNA, exhibiting distinct and well-defined bands.

Introduction

The availability of high-quality genomic DNA is crucial for molecular studies of forest tree species employing molecular markers for evaluating plant genetic diversity and contribute to phylogenetic studies (Mohammad *et al*., 2018). Genomic studies and molecular characterization are vital for sustainable conservation amid climate change and habitat loss. High-quality genomic DNA is essential for effective molecular analyses especially for PCR-based DNA markers like RAPD, ISSR, SSR, and AFLP for finest amplification (Tewari *et al*., 2016; Dahayat *et al*., 2017). In forestry tree species, where many DNA-based experiments require pure genomic DNA, obtaining high DNA quality is crucial for successful amplification-based assays. Extracting intact DNA from forest tree species, which contain high levels of polysaccharides, secondary metabolites, or polyphenolics, poses challenges for PCR

amplification and other molecular studies (Dubey *et al*., 2007). Moreover, commercial kits are available for extracting genomic DNA from plants, but they are expensive and less suitable for long-term experiments (Xin and Chen, 2006; Dilworth and Frey, 2000). The aim of the DNA extraction procedure is to minimize the presence of polyphenols and polysaccharides in the sample (Magdum, 2013). Forest tree species play a crucial role in uplifting tribal communities economically by providing essential resources including traditional medicines, building and raw material for industries. Therefore, present study focused on two important forest tree species. *Hardwickia binata* Roxb. Commonly known as Indian black wood or Anjan belongs to the family Fabacea. The plant thrives in semi-arid and arid regions of Western, Southern, and Central India. It is prized for its medicinal and commercial value, yielding high-quality hardwood used for charcoal and firewood, including agriculture, wooden wheels, and construction for houses and bridges (Shingade and Kakde, 2021; Prabakaran *et al*., 2014). It contains flavonoids and used as a raw material for industries (Khare, 2008). In traditional medicine, it is used for a variety of ailments, including worms, indigestion, leprosy, and diarrhoea. (Rageeb *et al*., 2022; Deshmukh and Ghanawat, 2019). *Dillenia pentagyna* Roxb. has great medicinal importance and belongs to the family Dilleniaceae, commonly known as Nepali elephant apple or Karmal/Karkat (Suresh *et al*., 2015). It is native to tropical and subtropical southern Asia distributed in rain forest. It is rich in flavonoids and phenolic contents and traditionally used to treat multiple ailments like inflammation, cancer, and diabetes (Saxena *et al*., 2022; Patle *et al*., 2020; Sikarwar *et al*., 2016). The plant has diverse uses: green leaves for tusser silkworms and green manure, dried leaves as sandpaper, wood for construction and tools, and bark for cordage production (Gandhi *et al*., 2013). High-throughput DNA extraction protocols for each tree species is essential due to the variability in their secondary metabolites (Bellstedt, 2010). Therefore, the present study compares five DNA extraction methods to provide insights into the most effective and efficient methods for obtaining DNA from these investigated species. Additionally, the quality of extracted DNA samples was also assessed for PCR amplification.

Materials and Methods

In present investigation, five different DNA extraction methods *viz.* Doyle and Doyle (1990), Michiels *et al*., (2003), Porebski *et al*., (1997), Khanuja *et al*., (1999), and Deshmukh *et al*., (2007) were screened to evaluated their suitability for PCR amplification. Fresh leaf samples of both species (*D. pentagyna and H. binata*) were collected from central India in zip-lock polybags, brought to the laboratory, and stored at -20°C for further investigation (Table 1). Required chemicals in different extraction methods with their concentrations are summarized in Table 2. The procedures of the respective protocols are outlined here.

Table 1: Details of location of collected sample and the extraction method used for the present investigation

S. N.	Tree Species	Location	Sample	DNA extraction methods
1	Dillenia pentagyna Roxb.	Lanjhi, Balaghat, Madhya Pradesh	DP-1, DP-2, DP-3, DP-4, DP-5	Doyle and Doyle (1990) Michiels *et al*., (2003) Porebski *et al*., (1997) Khanuja *et al*., (1999) Deshmukh *et al*., (2007)
2	Hardwickia binata Roxb.	Narmadanagar, Khandwa, Madhya Pradesh	HB-1, HB-2, HB-3, HB-4, HB-5	

Table 2: Composition of buffers applicable in different protocol

M1. Doyle and Doyle, (1990) (pH=8.0)	**M2. Michiels *et al*., (2003) (pH=8.0)**	**M3. Porebski *et al*., (1997) (pH=8.0)**	**M4. Khanuja *et al*., (1999) (pH=8.0)**	**M5. Deshmukh *et al*., (2007) (pH=8.0)**
Extraction buffer: **100 mM Tris-HCl** **20 mM EDTA,** **2% CTAB** **1.4 M NaCl** **0.2% β-met (v/v)** **Wash buffer:** **70% EtOH** **10 mM ammonium acetate**	Extraction buffer: 100 mM Tris-HCl 20 mM EDTA, 2% CTAB 1.4 M NaCl 0.2% β-met (v/v) Wash buffer: 10 mM ammonium acetate 70% EtOH	Extraction buffer: 100 mM Tris-HCl 20 mM EDTA, 2% CTAB 1.4 M NaCl 0.3% β-met (v/v) 0.05 % PVP Wash buffer: 70% EtOH	Extraction buffer: 100 mM Tris-Cl 25 mM EDTA 1.5 M NaCl 2.5% CTAB 0.2% β-met (v/v) 1% PVP (w/v) High salt TE buffer: 1 M NaCl 10 mM Tris-Cl 1 mM EDTA	Extraction buffer: Sucrose 15% (w/v) 50 mM Tris-Cl 50 mM EDTA 500 mMNaCl Wash buffer: 100 mM HEPES, 0.1% PVP (w/v) 4% β-met (v/v) Resuspension buffer: 20 mM Tris-Cl 10 mM EDTA 10% SDS

M1: Preheated 5-7.5 ml of CTAB isolation buffer using a water bath (60°C) were taken in a 30 ml centrifuge tube. Fine powder prepared of leaf tissue was prepared using liquid nitrogen and transferred into centrifuge tube containing preheated buffer. After vertexing, the sample was incubated at 60°C for 30 minutes with occasional gentle swirling. Remove from water bath allow to cool at room temperature and treated with chloroform-isoamyl alcohol (24:1) by gentle inversion. Centrifuged the sample at 10,000rpm for 30 minutes and transferred the aqueous phase into a clean centrifuge tube, add 2/3 volumes of ice-cold isopropanol, gently mixed and stored at -20°C. Following day, centrifuged at 10,000rpm for 30 minutes, pellet was dried and washwd using wash buffer. Dissolved in TE buffer and stored at -20°C for further investigation.

M2: 1 gm leaf tissue was ground into a powder using liquid nitrogen and mixed with 15 ml of preheated extraction buffer at 60°C. Following a 60-minute incubation with occasional mixing, allowed it to cool at room temperature, chloroform: isoamyl alcohol (24:1) was added, vortexed the mixture, and centrifuged to collect the upper phase. This extraction step was repeated twice. The aqueous phase was then mixed with isopropanol and incubated at 25°C overnight. Centrifuged and the supernatant was removed, the pellet was briefly air-dried and resuspended in TE buffer.

M3: Leaf tissue was ground in liquid nitrogen and the powder was transferred into tubes containing 15ml of 60°C preheated extraction buffer. The sample was incubated at 65°C with shaking for 30 min. The suspension was emulsified with chloroform: isoamyl alcohol centrifuged for 20 min at 10,000 rpm. The upper aqueous was transferred to a fresh tube, added chloroform isoamyl alcohol and again centrifuged for 5 min at 10,000 rpm. Transferred the supernatant into fresh tube, ½ volume of 5 M NaCl, 2 volumes of chilled 95% ethanol were added and incubated the solution at 4°C overnight. The precipitated DNA was centrifuged for 10 min at 10,000 rpm. Poured off the supernatant, washed pellets with 70% ethanol, dried for 30 min at room temperature and pellet was dissolved in TE buffer.

M4: Leaf tissue was ground in liquid nitrogen and transferred to a 10 ml polypropylene tube containing 3 ml extraction buffer. The mixture was mixed properly and incubated at 60°C in a water bath for 2 hours. Added 3 ml of chloroform: isoamyl alcohol (24:1), mixed by inversion and centrifuged at 8000 rpm for 10 minutes. Transferred the aqueous layer to a clean 10 ml polypropylene tube and 1.5 ml of 5 M NaCl was added and mixed gently. Subsequently, 2/3 volumes of ice-cold isopropanol were added, and the mixture was allowed to stand at room temperature for 1 hour. Samples were centrifuged at 10,000 rpm for 10 minutes at 25–30°C after mixing with isopropanol. Discarded the supernatant, pellet was washed with 80% ethanol, dried in a vacuum for 15 minutes and the pellet was dissolved in high salt TE buffer.

M5: 1g of leaf was ground in liquid nitrogen and transferred the powder to tubes, 5 ml of wash buffer were added, vortexed, and then spin at 12,000 rpm for 3 minutes. The supernatant was dicarded, and washing steps was repeated five times. Five milliters of extraction buffer were added to the tube and centrifuged at 10,000rpm for 5 min. Discarded the supernatant, added 2.5 ml of resuspension buffer along with 500µl of 10% SDS and incubated at 70°C for 15 min. The sample was allowed to cool at room temperature, then 2 ml of 7.5 M ammonium acetate were added and the sample was placed on ice for 30 min. Spun at 12,000rpm for 15 min. The aqueous layer was transferred to another tube, an equal amount of ice-cold isopropanol was added and spun for 15 min at 12,000 rpm. Discarded the supernatant and washed the pellet twice with 70% ethanol. The pellet was air dried for 15 minutes and dissolved in TE buffer.

Purification

Add 5 µl of RNase A and incubate at 37°C for 30 minutes, then extract with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Transfer the aqueous layer to a fresh 1.5 ml eppendorf tube and add 2 volumes of cold ethanol. Centrifuge at 10,000 rpm for 10 minutes at 25–30°C, wash the pellet with 80% ethanol, and dry it in vacuum before dissolving it in 200 µl of sterile double distilled water. DNA concentrations can be measured by running aliquots on an 0.8% agarose gel or by taking the absorbance at 260 nm. Use approximately 40 ng for PCR amplification.

Quantity and Quality Checking

The quantity of the extracted DNA samples was determined using UV-spectroscopy (Cintra 404, Australia) at 260/280 nm to measure the concentration of DNA by assessing its absorbance (Table 3). Quality was evaluated through 1% agarose gel electrophoresis in 1x TBE buffer, containing 0.5 µg/ml EtBr at constant voltage (100V) for 30 minutes. After electrophoresis the gel was further visualized in Gel Documentation System (G: Box-F3, Syngene, USA).

Table 3: Comparison of five different DNA extraction methods for *Dillenia pentagyna* Roxb. and *Hardwickia binata* Roxb.

S. N.	Species	M1		M2		M3		M4		M5	
		P	Y	P	Y	P	Y	P	Y	P	Y
1	Dillenia pentagyna Roxb.	1.61	348.5	1.46	211.1	1.56	297.8	1.16	125.9	0.99	59.4
2		1.63	356.1	1.58	213.3	1.6	304.6	0.98	137.6	1.01	65.9
3		1.65	362.5	1.39	232.4	1.55	277.9	0.96	124.8	0.94	57.8
4		1.6	314.2	1.5	222.8	1.59	280.3	1.01	128.8	0.95	65.9
5		1.61	326.7	1.42	214.9	1.6	286.4	0.99	132.4	0.96	58.4
Average		1.62	341.6	1.47	218.9	1.58	289.4	1.02	129.9	0.97	61.48
1	Hardwickia binata Roxb.	1.75	322.6	1.32	222.7	1.16	101.5	1.05	117.2	0.89	99.8
2		1.69	328.1	1.3	216.9	1.19	99.8	1.08	114.4	0.88	102.4
3		1.68	305.4	1.25	207.8	1.15	117.5	0.99	109.6	0.9	97.7
4		1.72	312.6	1.29	213.6	1.21	103.8	1.05	113.3	0.96	109.5
5		1.66	319.9	1.24	229.8	1.19	120.9	0.98	103	0.92	108.6
Average		1.7	317.7	1.28	218.1	1.18	108.7	1.03	111.5	0.91	103.6

Where, M1-M5: Methods of DNA extraction, P: Purity of DNA (260/280), Y: Yield (µl/ml)

Table 4: SSR Primers for PCR amplification

Sl. No.	Primer Code	F/R	5'<-----Sequence----->3'	Temp.	Amplicon size
1	HB-29	Forward	CAGTGTACGGCGAAATCCTT	52°C	180-240
2		Reverse	CCAGACCGGCTTACTAATGG		
3	DP-13	Forward	ATCTCTTGGTTCTGGCATCG	51°C	190-220
4		Reverse	AATCCGCCGTTGTATTTCAG		

PCR Amplification and Gel Electrophoresis

The DNA samples were diluted according to their quantification results, and DNA with a concentration of 50 ng/µl was used for PCR amplification with the SSR marker (Table 4).

In a PCR reaction mixture of 12µl, the components included 50ng DNA, 1X Green PCR buffer (consisting of 10mM Tris-HCl and 50mM KCl, pH 7.5 at 25°C), 2.5 mM MgCl2, 0.2mM dNTPs, 1U of Go Taq Flexi DNA polymerase, 0.8µM of forward primer, and 0.8µM reverse primer. The PCR amplification process was carried out using a ProFlex Thermal Cycler (Thermo Scientific, USA). Amplification was performed with initial denaturation at 94°C for 5 minutes, followed by 35 reaction cycles. Each cycle comprised denaturation at 94°C for 30 seconds, annealing at primer-specific temperatures for 45 seconds, and extension at 72°C for 45 seconds, followed by a final extension at 72°C for 10 minutes. Subsequently, the amplified product underwent horizontal gel electrophoresis in a 3% (w/v) agarose gel with 0.5XTBE buffer containing ethidium bromide (EtBr) at a concentration of 0.5µg/ml. Electrophoresis was conducted at a constant voltage of 100V for 3 hours, and the results were documented using the gel documentation imaging system (SynGene).

Results and Discussion

Extracting high-throughput genomic DNA is essential for molecular research concerning the conservation and sustainable utilization of endangered forest tree species. Quality genomic DNA is a prerequisite for numerous DNA-based analyses, such as DNA fingerprinting, genome sequencing, diversity assessment, population structure evaluation, and other molecular investigations. (Mohammad *et al.*, 2017; Dahayat *et al.*, 2017). Different DNA isolation protocols are available, but none is

universally effective for all tree species. This is due to the variability of secondary metabolites like polyphenols and gums, which can impede genomic DNA isolation, leading to poor DNA quality and hindering long-term storage (Elhaj and Gamra, 2021; Ginwal and Mittal, 2010).

In present investigation, five distinct DNA extraction protocols were assessed for their efficacy in isolating high-quality genomic DNA from *Dillenia pentagyna* and *Hardwickia binata* (Table 2). The DNA extracted using the protocols outlined by Khanuja *et al*., (1999), Porebski *et al*., (1997), and Deshmukh *et al*., (2007) exhibited significantly poor quality. Whereas, the DNA isolated using the protocols described by Michiels *et al*., (2003) demonstrated low quality, as indicated by the presence of smearing (Fig. 1 & 2).

The CTAB-based DNA extraction protocol, originally outlined by Doyle and Doyle (1990), was notably enhanced through minor adjustments. These included upgrading to a 4% PVP concentration from 2%, prolonging the incubation time with the extraction buffer to 60 minutes, employing freshly prepared CTAB buffer, and repeating washing steps with the wash buffer. Additionally, the utilization of young leaves was implemented to improve quality and reduce impurities, following the recommendation by Sytsma *et al*., (1993). Minor adjustments to extraction protocols indeed enhance efficiency (Zidani *et al*., 2005). A high PVP concentration aids in removing polyphenols by forming complexes with them. Additionally, extended incubation and washing times effectively eliminate contaminants such as polysaccharides and polyphenols, ultimately improving DNA quality (Maliyakal, 1992). Maintaining DNA sample purity extracted from trees involves addressing various components such as polysaccharides, proteins, phenolic compounds, and RNA. Capeloto *et al*., (2005) recommend integrating RNase to remove RNA and enhance DNA purity. Meanwhile, Romano and Brasileiro (1999) acknowledge RNase as an optional step but underscore its role in improving sample purity. In our study, we observed that the utilization of RNase significantly enhances DNA cleanliness, irrespective of whether the samples are fresh or refrigerated for an extended period. After optimizing the protocol, twenty samples from both species were extracted using the Doyle and Doyle (1990) method and subsequently purified. After purification, agarose gel electrophoresis (1% agarose, 0.5 µg/ml EtBr) was performed to separate the large DNA fragments (Fig. 3 and 4).

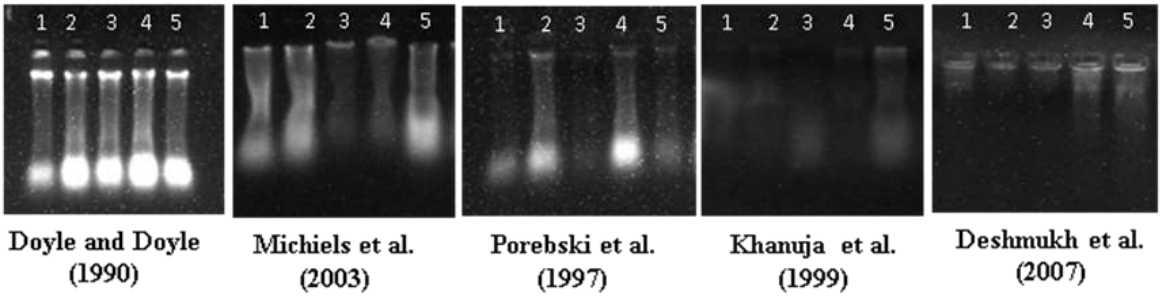

Fig. 1: Total genomic DNA extraction of *D. pentagyna Roxb*. in 1% agarose gel electrophoresis

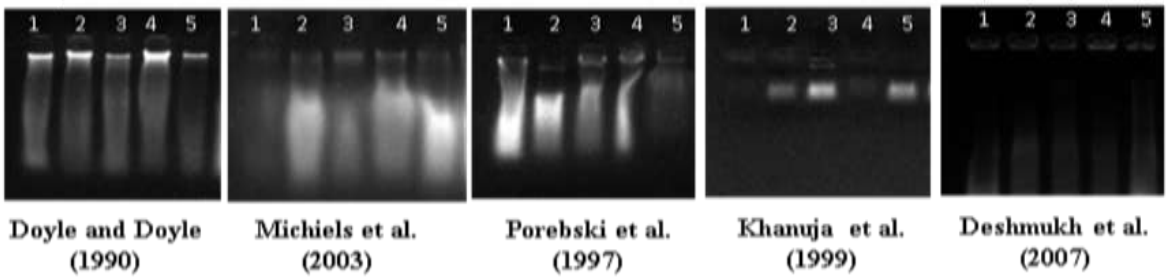

Fig. 2: Total genomic DNA extraction of *H. binata* Roxb. in 1% agarose gel electrophoresis

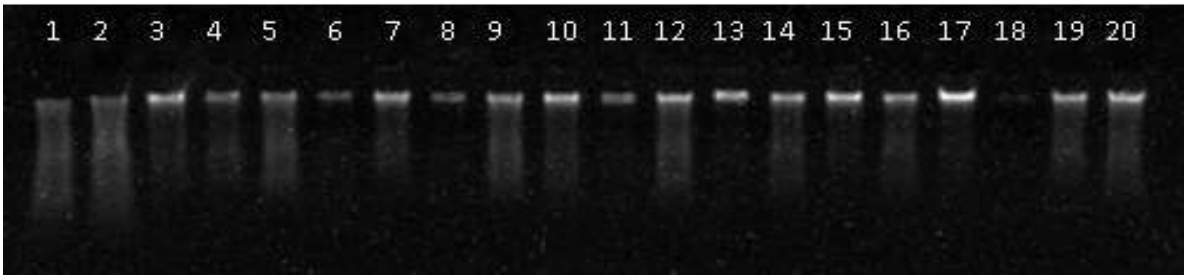

Fig. 3: Total genomic DNA extraction profile of *D. pentagyna Roxb*. after purification in 1% agarose gel electrophoresis

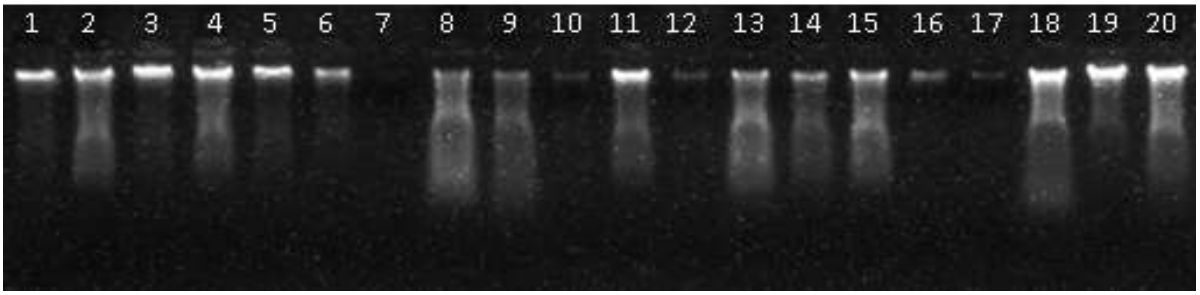

Fig. 4: Total genomic DNA extraction profile of *H. binata* Roxb. after purification in 1% agarose gel electrophoresis

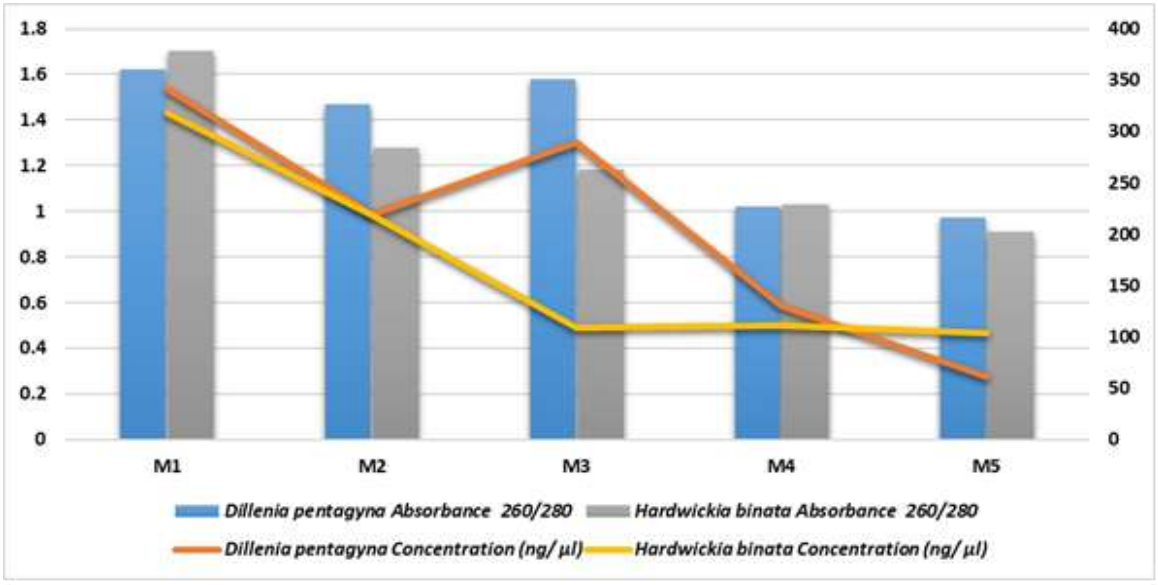

Fig. 5: Represents absorbance and yield of *D. pentagyna* and *H. binata* using different methods

Spectrophotometric observations were also recorded to assess the quality of the DNA. Table 3 provides a summary of the average DNA yield and the range of purity obtained from all sample extracts using five different extraction methods. Absorbance ratio 260/280 is found to be in the range of 0.97 to 1.62 in *D. pentagyna* and 0.91 to 1.70 in *H. binata*. The concentration of DNA showed differences in DNA yield ranging from 61.48 to 341.6 ng/µl in *D. pentagyna* and 103.6 to 317.72 ng/µl in *H. binata*. *D. pentagyna* yielded maximum DNA quantity with the CTAB method of Doyle and Doyle (1990) followed by Porebski *et al*., (1997) and Michiels *et al*., (2003). A very less quantity of DNA is visualized in Khanuja *et al*., (1999) and Deshmukh *et al*., (2007). Whereas in *H. binata*, Doyle and Doyle (1990) method show a maximum yield of DNA followed by Michiels *et al*., (2003), Porebski *et al*., (1997) (Fig 5). Although other methods yielded a lower amount of DNA compared to the Doyle and Doyle (1990) method, the purity of the extracted DNA was determined by the absorbance ratio of 260 and 280 nm. This ratio indicates that the isolated DNA was free from contaminants (Abdel-Latif and Osman 2017).

Secondary metabolites like polyphenols and polysaccharides can covalently bind to DNA during extraction, diminishing its suitability for PCR-based DNA markers such as SSR, ISSR, and RAPD (Mondal *et al*., 2014; Katterman and Shattuck, 1983). Therefore, assessing the efficacy of extracted DNA samples for PCR amplification is essential. In the present study, PCR amplification was conducted using the SSR marker, known for its high polymorphism, which necessitates high-quality genomic DNA. The amplification products observed in the PCR were notably clear and well-defined. For *Hardwickia binata*, PCR products ranged from 180 to 240 base pairs, and for *Dillenia pentagyna*, ranged from 190 to 220 base pairs (Fig. 6 and 7). This clear and distinct banding pattern of PCR amplification indicates high-quality genomic DNA. These results provide strong evidence for the efficacy of PCR amplification in generating reliable genetic data for further analysis.

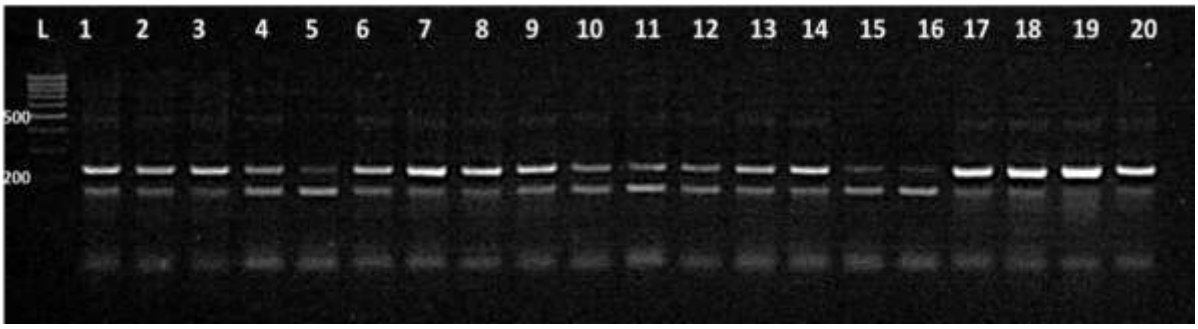

Fig. 6: PCR amplification profile of extracted DNA samples of D. pentagyna Roxb. using SSR primer, Where, L-Ladder (SM0383), 1-20 investigated tree accessions.

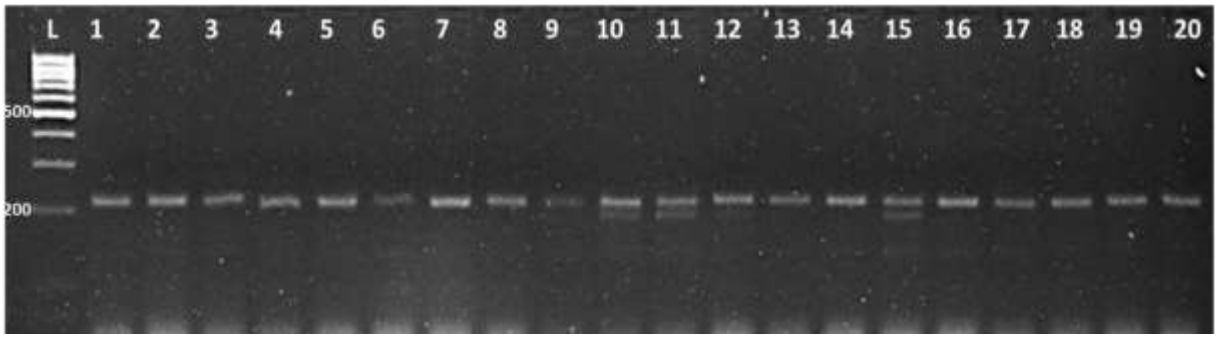

Fig. 7: PCR amplification profile of extracted DNA samples of *H. binata* Roxb. using SSR primer, Where, L-Ladder (SM0383), 1-20 investigated tree accessions.

Conclusion

In this study, we compared five DNA extraction methods to isolate high-quality DNA for PCR amplification. Minor adjustments to the CTAB-based method outlined by Doyle and Doyle (1990) significantly improved DNA quality for both Dillenia pentagyna Roxb. and Hardwickia binata Roxb. PCR amplification using SSR markers confirmed the suitability of extracted DNA for downstream molecular analyses. These findings highlight the importance of protocol optimization, particularly the Doyle and Doyle (1990) method, for reliable genomic data in endangered tree species conservation.

Authors' Contribution

All authors contributed equally at all stages of research, data analysis and manuscript preparation. Final form of manuscript was approved by all authors.

Conflicts of Interest

The authors declare that there is no conflict of interest related to this work.

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