Naveen Shodh Sansar (An International Refereed / Peer Review Multidisciplinary Research Journal)
RNI No.- MPHIN/2013/60638, ISSN 2320-8767, E- ISSN 2394-3793, Scientific Journal Impact Factor (SJIF)- 8.054,
July to September 2026, E-Journal, Vol. I, Issue LV (55), ISO 9001:2015 - E2024049304 (QMS)

Genomic DNA extraction and quality assessment of *Pterocarpus marsupium* : Optimization of a modified CTAB-based protocol

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Abstract: *Pterocarpus marsupium* is a medicinally important woody tree whose conservation, authentication and future biotechnological utilization can benefit from reliable molecular resources.. Obtaining usable DNA from woody medicinal plants can be technically challenging because leaf tissues contain polysaccharides, polyphenols and other secondary metabolites that may interfere with DNA recovery, purity and downstream enzymatic reactions. In the present work, several published DNA isolation approaches, including protocols associated with Healey et al. (2014), Michiels et al. (2002), and Doyle and Doyle (1987), were evaluated. Minor modifications were introduced, and the Doyle and Doyle-based CTAB approach produced the most consistent DNA quality. The optimized procedure incorporated pre-chilling, liquid-nitrogen grinding, β-mercaptoethanol-containing extraction buffer, optional polyvinylpyrrolidone (PVP), controlled incubation at 65 °C, chloroform:isoamyl alcohol purification, RNase A treatment, salt-assisted ethanol precipitation, ethanol washing and resuspension in TE buffer. DNA quantity and quality were evaluated by NanoDrop UV/Vis spectrophotometry and 0.7% agarose gel electrophoresis. Twenty DNA samples were assessed using absorbance at 260 and 280 nm, A260/A280 ratio and calculated DNA quantity. The recorded table values showed quantities from 0.186 to 0.768 mg/ml and A260/A280 ratios from 1.545 to 1.886. The highest quantities occurred in samples T1, T2, T5, T10, T15, T16, T18 and T20, while lower quantities occurred in T3, T4, T6, T7, T8, T9, T12, T13, T14, T17 and T19. Clear, intact bands on agarose gel supported the integrity of the extracted DNA. The findings demonstrate that the modified CTAB procedure can provide DNA of useful quality from *P. marsupium* leaf tissue and establish a foundation for subsequent PCR-based marker analysis, genetic fidelity testing, genetic diversity studies and conservation-oriented molecular investigations.

Keywords: *Pterocarpus marsupium*; genomic DNA; CTAB; polyvinylpyrrolidone; β-mercaptoethanol; RNase A; A260/A280; agarose gel electrophoresis; molecular characterization; conservation genetics.

Introduction - *Pterocarpus marsupium.* Commonly referred as Vijaysar or the Indian kino tree, is a medicinally valuable woody species of the family Fabaceae. The species has substantial ecological, medicinal and economic importance and has a long history of use in traditional medicine. Different plant parts, particularly bark, heartwood and leaves, have been associated with diverse pharmacological properties and contain a broad range of phytochemicals. The importance of genomic DNA extraction extends beyond simply obtaining a DNA preparation. High-quality genomic DNA is the starting material for polymerase chain reaction (PCR), DNA sequencing, molecular marker analysis, genetic fidelity assessment, phylogenetic investigation and genetic diversity studies. The reliable DNA is important for future molecular characterization and conservation strategies. Molecular markers can be used to evaluate genetic variation, detect somaclonal variation arising during tissue culture, and establish relationships among accessions and support conservation of elite or threatened germplasm.

The molecular analysis of woody medicinal plants is frequently complicated by the biochemical composition of their tissues. *P. marsupium* leaf tissue contains secondary metabolites and phenolic compounds that can complicate DNA isolation. Polyphenols may oxidize during tissue disruption and subsequently interact with nucleic acids or other cellular components. Polysaccharides can co-precipitate with DNA and may interfere with downstream enzymatic reactions. Such contamination can reduce the reliability of spectrophotometric measurements, produce poor electrophoretic profiles and inhibit PCR.

Three approaches —Healey et al. (2014), Michiels et al. (2002), and Doyle and Doyle (1987)—were considered during optimization. Minor modifications were made to

accommodate the characteristics of *P. marsupium* leaves. The Doyle and Doyle-based approach ultimately gave the most consistent results. The resulting protocol was CTAB-based and incorporated measures directed at the major sources of contamination. PVP was used during tissue processing to reduce phenolic interference, β-mercaptoethanol was included in the extraction system, and phenol/chloroform-related purification and RNase treatment were used to improve purity. The combination was intended to generate DNA that could withstand subsequent molecular manipulation.

Molecular resources can complement propagation-based conservation by allowing researchers to compare genetic material, authenticate plant material, monitor diversity and identify elite genotypes. Establishing a reproducible extraction method is therefore a practical contribution to the larger conservation and biotechnology framework developed in the thesis.

Materials and Methods

Study material and sample collection: Plant material used was collected from three different sites of Barnawapara, Chhattisgarh, during June 2022 to May 2024, in the morning. Healthy bark, stem and leaf material was collected for the different objectives. For the genomic DNA component, leaf tissue was used. Their is use of secateurs, pruning knives, gloves and sterile sample bags during collection. Plant material was cleaned of foreign particles with distilled water, transferred in sterile bags and transported in an insulated box before laboratory processing. The DNA extraction work specifically required frozen leaf tissue, and the preparatory steps were designed to minimize thawing before grinding.

Protocol selection and optimization: Several DNA extraction protocols were evaluated during the study. There are several approaches associated with Healey et al. (2014), Michiels et al. (2002), and Doyle and Doyle (1987). Rather than treating these methods as interchangeable, the study compared their performance in the context of *P. marsupium* leaves. Minor modifications were introduced, and the Doyle and Doyle (1987)-based method produced the most consistent and high-quality results. The optimized procedure was consequently adopted for the twenty samples used in the DNA quality assessment.

Preparatory steps: Before tissue disruption, the mortar and pestle were pre-chilled to prevent thawing of frozen material. Ninety-five percent ethanol was maintained at -20 °C. W ater baths were pre-heated to 65 °C and 37 °C. The extraction buffer was prepared fresh and supplemented with 0.3% (v/v) β-mercaptoethanol. 10 ml extraction buffer per 1 g leaf tissue and notes that PVP could be added at this stage to improve removal of phenolic compounds. These measures were intended to preserve nucleic-acid quality while reducing the effects of oxidation and endogenous contaminants.

Grinding and tissue disruption: Approximately 1 g of frozen leaf tissue was ground to a fine powder using liquid nitrogen. The powdered material was transferred to a new 50 ml Falcon tube and mixed thoroughly with the pre-heated extraction buffer. The tubes were incubated at 65 °C for 30 minutes to 1 hour. During incubation, samples were gently inverted approximately every 10 minutes to support lysis and mixing. After incubation, the samples were centrifuged at 5000 × g for 5 minutes to pellet unlysed tissue and cellular debris. The supernatant was carefully transferred to a fresh Falcon tube for subsequent purification.

Protein removal and RNase treatment: An equal volume of chloroform:isoamyl alcohol (24:1) was added to the extracted solution and mixed by gentle inversion for five minutes. The mixture was centrifuged at 5000 × g for 10 minutes to achieve phase separation. The upper aqueous phase was transferred carefully to a new tube without disturbing the interphase. Five microlitres of RNase A at 10 mg/ml were then added, followed by incubation at 37 °C for 15 minutes with occasional gentle mixing. This step was included to remove RNA contaminants. After RNase treatment, an equal volume of chloroform:isoamyl alcohol was again added, followed by five minutes of gentle inversion and centrifugation at 5000 × g for 10 minutes. The aqueous phase was collected without carrying over the organic layer.

DNA precipitation and washing: For precipitation, half a volume of 5 M NaCl was added to the aqueous phase and mixed gently. Three volumes of cold 95% ethanol were subsequently added and the mixture was inverted gently to facilitate DNA precipitation. Tubes were incubated at -20 °C for one hour . Their should be cautions against extending this incubation period because prolonged exposure could encourage precipitation of CTAB and NaCl and interfere with efficient DNA recovery. Following precipitation, the tubes were centrifuged at 5000 × g for 10 minutes to pellet the DNA. The pellet was washed with 3 ml of 70% ethanol, followed by another centrifugation at 5000 × g for 10 minutes. The supernatant was discarded and the pellet was air-dried for approximately 15 minutes at room temperature. Finally, the DNA was resuspended in 200 µl of TE buffer and retained for further analysis and storage.

DNA quantity and quality assessment: DNA quantity and quality were assessed by NanoDrop UV/Vis spectrophotometry and agarose gel electrophoresis. The thesis describes the use of a 0.7% (w/v) agarose gel for evaluation of DNA integrity. Spectrophotometric assessment considered absorbance at 260 nm, absorbance at 280 nm and the A260/A280 ratio. A distinct absorbance peak at 260 nm and an A260/A280 ratio around 1.8 were treated as indicators of relatively clean DNA, with the methods section identifying 1.8–2.0 as the expected range associated with minimal protein contamination. Agarose electrophoresis was used to evaluate the physical integrity of the DNA and to check for major degradation or obvious contamination. The stock DNA was subsequently diluted to a working

concentration of 30–40 ng/µl for downstream analyses.

The A260/A280 ratio was interpreted as a purity indicator rather than as a direct measurement of DNA quantity. Absorbance at 260 nm is associated with nucleic acids, while absorbance near 280 nm is influenced strongly by proteins and aromatic compounds. Therefore, the ratio provides a rapid assessment of relative purity. The electrophoretic result adds a different type of information: a clear high-molecular-weight band indicates physical integrity, whereas extensive smearing can indicate fragmentation or degradation. The combined use of spectrophotometry and gel electrophoresis is consequently stronger than relying on either measurement alone.

Results

Overall performance of the modified protocol: The optimized CTAB-based procedure successfully produced genomic DNA from *P. marsupium* leaf tissues. The Doyle and Doyle (1987)-based method gave the most consistent and high-quality results among the approaches evaluated. The procedure was particularly effective after incorporation of tissue washing, liquid-nitrogen grinding, PVP-assisted reduction of phenolic interference, CTAB-based lysis, organic purification and RNase A treatment. Distinct DNA bands were observed on agarose gel electrophoresis, supporting the integrity of the extracted DNA.

The results demonstrate that extraction from *P. marsupium* leaves is feasible despite the presence of biochemical compounds that can complicate DNA isolation. There is identification of polysaccharides, polyphenols and secondary metabolites as potential interfering substances. Their removal or suppression was therefore a central feature of the optimized procedure. PVP was considered important because it reduces the impact of phenolic compounds, while the organic purification and RNase treatment were intended to improve the purity of the aqueous DNA preparation.

DNA quantity and purity of twenty samples: Twenty DNA samples, designated T1 to T20, were evaluated. The absorbance at 260 nm, absorbance at 280 nm, A260/A280 ratio and DNA quantity. Table 1. DNA quantification and purity measurements

Sample	A260	A280	A260/A280	Quantity (mg/ml)
T1	0.077	0.044	1.749	0.768
T2	0.076	0.044	1.745	0.761
T3	0.027	0.015	1.789	0.272
T4	0.019	0.011	1.794	0.192
T5	0.064	0.036	1.747	0.636
T6	0.020	0.011	1.886	0.198
T7	0.027	0.015	1.819	0.271
T8	0.027	0.015	1.818	0.269
T9	0.019	0.010	1.788	0.186
T10	0.063	0.037	1.699	0.632
T11	0.056	0.036	1.545	0.561
T12	0.026	0.014	1.799	0.259
T13	0.020	0.011	1.773	0.195
T14	0.027	0.015	1.813	0.272
T15	0.076	0.043	1.757	0.759
T16	0.063	0.038	1.678	0.631
T17	0.020	0.010	1.875	0.195
T18	0.065	0.036	1.817	0.654
T19	0.019	0.012	1.619	0.191
T20	0.063	0.036	1.751	0.634

The highest recorded DNA quantity was 0.768 mg/ml in T1, followed by 0.761 mg/ml in T2, 0.759 mg/ml in T15, 0.654 mg/ml in T18, 0.636 mg/ml in T5, 0.634 mg/ml in T20, 0.632 mg/ml in T10 and 0.631 mg/ml in T16. These samples correspond to the group showing higher DNA concentrations. In contrast, T9 showed the lowest tabulated quantity at 0.186 mg/ml, followed by T4 at 0.192 mg/ml, T13 and T17 at 0.195 mg/ml, T6 at 0.198 mg/ml, T19 at 0.191 mg/ml, T12 at 0.259 mg/ml, T8 at 0.269 mg/ml, T7 at 0.271 mg/ml and T3/T14 at 0.272 mg/ml.

The A260/A280 values were generally close to the range expected for relatively pure nucleic acid. The lowest recorded ratio was 1.545 in T11, while the highest was 1.886 in T6. Most samples fell between approximately 1.68 and 1.82. T3, T4, T5, T7, T8, T9, T10, T12, T13, T14, T15, T16, T18 and T20 were within or near the range expected for comparatively clean DNA, while T11 was notably lower. The overall pattern indicates that quantity varied more strongly among samples than the purity ratio.

The groups T1, T2, T5, T10, T15, T16, T18 and T20 as higher-yield samples, with concentrations stated as 0.631–0.768 mg/ml. It groups T3, T4, T6, T7, T8, T9, T12, T13, T14, T17 and T19 as lower-yield samples, with concentrations stated as 0.186–0.272 mg/ml. This classification is consistent with the individual measurements in Table 1 and illustrates that extraction efficiency was not uniform across all twenty samples.

Electrophoretic integrity: Agarose gel electrophoresis produced distinct DNA bands, as evidence of DNA integrity and absence of substantial degradation. The electrophoretic assessment was important because a spectrophotometric ratio alone cannot demonstrate whether DNA molecules remain physically intact. The clear bands therefore complemented the NanoDrop measurements by showing that the extracted DNA was not extensively fragmented.

The observed integrity is especially relevant for PCR-based applications. Fragmented DNA can reduce amplification reliability, while co-purified polysaccharides, phenolics and proteins can inhibit polymerase activity. The combined observation of acceptable optical-density ratios and distinct gel bands suggests that the optimized protocol successfully addressed several major extraction challenges encountered in *P. marsupium* leaves.

Discussion

Genomic DNA extraction is challenging in *P. marsupium*

The present findings are understandable in the biochemical context of *P. marsupium*. This species as rich in phenolic

compounds and other secondary metabolites. Such compounds are valuable from a pharmacological perspective but can become problematic during nucleic-acid isolation. A DNA extraction method must therefore separate the desired nucleic acid from a chemically complex plant matrix. The problem is particularly relevant to woody medicinal plants, where mature tissues can contain abundant phenolics, polysaccharides and structural components.

The CTAB approach is well suited to this type of material because it is designed to assist nucleic-acid extraction in plant tissues containing substantial polysaccharide and secondary-metabolite loads. In the present study, the CTAB-based protocol was further supported by measures aimed at reducing oxidation and contamination. The use of PVP during grinding was intended to bind or reduce the impact of phenolic compounds, while β-mercaptoethanol contributed to the reducing environment during extraction. The combination was therefore logically aligned with the specific biochemical obstacles..

The study also used two purification stages involving chloroform:isoamyl alcohol, together with RNase A treatment. Organic extraction helps separate nucleic acids from proteins and other hydrophobic contaminants, whereas RNase A reduces RNA carryover. The second organic purification following RNase treatment provides an additional opportunity to recover a cleaner aqueous phase. The final salt and ethanol precipitation then concentrates DNA and permits removal of soluble impurities through ethanol washing.

Importance of tissue disruption and temperature control: Efficient disruption of plant tissue is essential because DNA is enclosed within cells and nuclei. In the present protocol, approximately 1 g of frozen leaf tissue was ground using liquid nitrogen. Maintaining the tissue in a frozen state during grinding facilitates efficient mechanical disruption while reducing the opportunity for enzymatic degradation and phenolic oxidation, pre-chilling of the mortar and pestle. The subsequent incubation at 65 °C in extraction buffer allows chemical lysis and promotes release of nucleic acids.

The protocol specified intermittent gentle inversion during incubation rather than vigorous mixing. This is relevant because genomic DNA is a long, high-molecular-weight molecule and excessive mechanical shear can reduce its integrity. The later centrifugation step removed insoluble debris, allowing the DNA-containing supernatant to proceed to purification. Thus, the sequence of frozen grinding, controlled lysis, debris removal and gentle handling contributed to the observed intact electrophoretic bands.

Role of PVP and β-mercaptoethanol: PVP was an important modification because phenolic compounds can interfere with DNA isolation. PVP was incorporated to reduce phenolic interference and that its use during tissue grinding played a crucial role in minimizing phenolic oxidation and preventing DNA degradation. This is consistent with the practical logic of using a polymer capable of interacting with polyphenolic compounds before they become strongly oxidized and associated with nucleic acids. â-mercaptoethanol was included at 0.3% (v/v) in the freshly prepared extraction buffer. Its inclusion provided an additional reducing condition during tissue processing. PVP and â-mercaptoethanol, together with extended incubation, as modifications contributing to the effectiveness of the optimized procedure. The importance of these modifications is particularly apparent because *P. marsupium* is pharmacologically rich in phenolic constituents. The very compounds that make the plant interesting for phytochemical research can make molecular extraction more demanding.

Interpretation of A260/A280 ratios: The A260/A280 ratio provides a rapid indication of nucleic-acid purity. In the present dataset, ratios ranged from 1.545 to 1.886. Values near 1.8 were common, the extracted DNA was generally of acceptable purity. The comparatively low value of 1.545 in T11 deserves attention because a lower ratio can indicate greater contribution from protein or other absorbing contaminants. Nevertheless, the thesis considers the overall set acceptable, and the gel results provide additional evidence that the DNA was usable.

It is important to distinguish purity from quantity. A high DNA concentration does not automatically mean that DNA is pure, and a lower concentration does not necessarily mean that a sample is unusable. For molecular applications, both concentration and purity influence performance. The present dataset demonstrates this distinction clearly. Some samples had high quantities with ratios around 1.7–1.8, whereas other samples had lower quantities but ratios close to 1.8. Consequently, selecting DNA for downstream PCR should consider both parameters and, where necessary, perform an additional cleanup or dilution step.

Sample-to-sample variation: Variation among the twenty samples is an important result rather than an experimental failure. Their is variability to possible differences in leaf maturity, endogenous metabolite levels, tissue handling and grinding efficiency. These factors can influence the amount of recoverable DNA and the extent of contamination. Differences in leaf age may alter secondary metabolite composition, while variation in tissue handling can affect the time between collection, freezing and extraction. Grinding efficiency can also change the degree of cellular disruption and therefore influence yield.

The relatively consistent purity ratios across many samples suggest that the protocol controlled contamination reasonably well even when DNA yield varied. In other words, the main variation was quantitative rather than a complete loss of DNA quality. This is a useful characteristic for a protocol intended for future molecular work because concentration can often be normalized before PCR, whereas severe contamination or degradation can be more

difficult to correct.

Relevance to molecular markers and clonal fidelity: One of the most important implications of the extracted DNA is its potential use in molecular marker analysis. The experiment discusses molecular markers as tools for assessing genetic diversity, genetic relationships and somaclonal variation. This is particularly relevant to the mass multiplication component of the research. When plants are regenerated through tissue culture, molecular assessment can help determine whether regenerated plants remain genetically similar to the source material.

ISSR markers have previously been used in *P. marsupium* to evaluate genetic variation and clonal fidelity. It also refers to molecular fingerprinting and genetic relationship studies among accessions. Reliable DNA extraction is therefore directly connected to the future application of these marker systems. If DNA is sufficiently intact and free of major inhibitors, it can be standardized to a working concentration and used in PCR-based analyses.

Relevance to conservation genetics: *P. marsupium* has conservation significance because of poor natural regeneration, limited success of conventional vegetative propagation and pressure associated with medicinal and commercial use. Conservation strategies should ideally preserve both the number of plants and the genetic diversity represented within populations. Molecular tools can contribute to this process by distinguishing accessions, estimating relationships, identifying genetically distinct material and helping prioritize germplasm for conservation. The ability to obtain DNA from leaf tissue is especially practical because leaves can provide a non-destructive or relatively low-impact source of molecular material compared with destructive sampling of wood or bark. Once an efficient extraction protocol is standardized, multiple accessions can be processed using a common workflow. This creates opportunities for population-level studies in which material from different geographic locations can be compared using appropriate marker systems. Such work would extend the present experiment from DNA preparation to conservation genetics.

Relevance to plant biotechnology: The experiment combines plant tissue culture with pharmacological and molecular approaches. From a biotechnology perspective, the extracted DNA can support several future applications. These include confirmation of clonal fidelity in micropropagated plants, molecular characterization of elite genotypes, genetic diversity analysis and marker-assisted conservation. DNA-based authentication may also help distinguish genuine plant material from morphologically similar material in contexts where medicinal plant identity is important.

The molecular component also provides a bridge between propagation and phytochemical research. If future studies identify accessions with superior levels of desirable phytochemicals, DNA-based approaches could be used to characterize the genetic background of those accessions. Similarly, tissue-culture-derived plants with desirable growth or metabolite characteristics could be compared molecularly with donor plants. Such integrated work could improve the reliability of selecting and maintaining elite germplasm.

Significance: First, the study did not rely on a single untested extraction procedure; multiple approaches were considered and the most consistent method was selected. Second, the protocol addressed several known sources of plant DNA contamination through PVP, β-mercaptoethanol, organic purification and RNase treatment. Third, the extracted DNA was evaluated by two complementary techniques—spectrophotometry and agarose gel electrophoresis. Fourth, twenty samples were examined, providing evidence that the protocol could produce DNA across multiple individual preparations rather than from only one sample.

Another strength is the direct connection between the molecular objective and the conservation and micropropagation goals of the thesis. The study did not treat DNA extraction as an isolated biochemical exercise. Instead, it was positioned as a foundation for future molecular analysis of a medicinal tree for which sustainable propagation and conservation are important. This integrated perspective increases the practical significance of the optimized protocol.

Conclusion: Modified CTAB-based method for extraction of genomic DNA from *Pterocarpus marsupium* leaf tissue. Evaluation of several approaches led to selection of a Doyle and Doyle (1987)-based procedure that incorporated modifications designed to reduce phenolic interference and improve DNA purity. The combination of liquid-nitrogen grinding, β-mercaptoethanol-containing extraction buffer, PVP-assisted processing, controlled CTAB lysis, chloroform:isoamyl alcohol purification, RNase A treatment, salt-assisted ethanol precipitation and ethanol washing produced DNA preparations that were generally acceptable in purity and showed distinct bands on agarose gel electrophoresis.

Across the twenty tabulated samples, DNA quantity varied from 0.186 to 0.768 mg/ml and A260/A280 values varied from 1.545 to 1.886. Several samples showed comparatively high DNA quantities, while most samples exhibited ratios close to the expected range for relatively clean DNA. The variation in yield can reasonably be associated, as discussed in the thesis, with differences in leaf maturity, endogenous metabolites, tissue handling and grinding efficiency. Importantly, the observed electrophoretic integrity indicates that the extraction procedure was able to recover intact genomic DNA from this chemically complex woody medicinal plant.

The establishment of a reproducible molecular starting point for *P. marsupium* research. The extracted DNA can support future PCR-based marker analysis, genetic fidelity testing of micropropagated plants, genetic diversity

assessment, accession authentication and conservation genetics. The molecular protocol complements the successful in vitro propagation and pharmacological profiling components and strengthens the overall biotechnology framework for sustainable utilization and conservation of *P. marsupium*.

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