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Research Article

OPTIMIZATION OF GENOMIC DNA EXTRACTION ROUTINE FROM ROSEWOOD (*DALBERGIA LATIFOLIA*) AND BEN TEAK (*LAGERSTROEMIA LANCEOLATA*)

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Abstract- *Dalbergia latifolia* and *Lagerstroemia lanceolata* are two economically important and expensive timber species of tropical countries (India, Indonesia and Madagascar, Burma) are exploited. An efficient and on site detection and legal confirmation of these timbers is possible using molecular methods, but molecular tools have not been employed due to lack of knowledge of DNA extraction and species specific identification. Purified genomic DNA, required for many applications in molecular genetics is frequently more difficult to obtain from trees than most other crop or annual plants. Here we describe the essential steps of a rapid DNA isolation protocol that can be used for diverse timber species, which contains huge amount of secondary metabolites such as alkaloids, flavonoids, phenols, gummy polysaccharides, terpenes and quinones. This modified DNA extraction protocol is applicable for fresh and mature dried leaf samples. This customized procedure has improved the efficiency of genomic DNA by enhancing the yield as well as purity of DNA that would facilitate efficient characterization and validation of these timber species. The isolated DNA has been proved to be acquiescent to PCR amplification though ISSR and SSR primers.

Key words- *Genomic DNA, timbers, phenolic, ISSR, SSR*

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Introduction

The future of the world's tropical forests has for some years been a major public concern in the industrialized tropical countries (India, Indonesia and Madagascar). The main issues have been deforestation, biological diversity conservation and the rights of indigenous people. *Dalbergia latifolia* and *Lagerstroemia lanceolata* are predominantly tropical timber species. They are premium quality timber species used to manufacture furniture, panelling and other ornamental products. The commercial values of these timbers are very high due to which illegal felling of trees is high. Molecular methods have proved to be the most useful in this regard. Species-specific molecular identification using molecular markers, through polymerase chain reaction techniques offers an important tool. In this regard, however, procurement of a high quality and quantity of total genomic DNA is essential. The problems with extraction of DNA from trees are generally attributed to impurities, such as terpenes, poly-phenolics and polysaccharides that are often abundant in the foliage of perennials and co-extract with DNA [1]. Such impurities also interfere in further DNA analysis. As a consequence, many tree species require more complex extraction methods than annual plants, utilising an initial organelle isolation step under acidic conditions [2] or special grinding procedures [3]. Many factors can cause shearing of DNA during extraction. Degradation of DNA due to endonucleases is one such problem encountered in the isolation and purification of high molecular weight DNA from plant, which directly or indirectly interfere with the enzymatic reactions [4, 5]. Several researchers have attempted to eliminate the use of hazardous chemicals, expensive kits, equipment, and labour-intensive steps for high throughput DNA extraction. However, these methods do have demerits such as limited shelf life, low purity, low recovery, and poor amplification [6]. Mostly the DNA extraction protocols recommend fresh leaf samples for genomic DNA isolation, but it seems impractical when the samples are collected from remote and rare locations.

These situations necessitate the development of the protocols for isolating DNA from dried leaf samples. Another major problem encountered with *D. latifolia* and *L. lanceolata* is most of the fully developed and mature leaves are accumulation of polyphenolics and tannins. These, when in oxidized, form covalently binds with DNA and sort it unaffected to restriction enzymes and give DNA a brown colour. The objective of this study was to develop a simple method to isolate DNA in a safe laboratory condition a method that eliminates the need to use of toxic phenol. The resulting optimized HEPES [7] modified protocol enables the isolation of high quality genomic DNA amenable to ISSR (Inter simple sequence repeats) and amplification of plant universal genes *matK* and *rbcL* or other microsatellite SSR (Simple sequence repeats) markers.

Materials and methods:

Sampling: To continue the DNA extraction processes, leaf samples of *Dalbergia latifolia* and *Lagerstroemia lanceolata* were collected from following regions in southern Karnataka.

Fig-1 Sample collection regions in IWST Bangalore, Hassan, Dharwad (Dandeli, Haliyal, Barchi) Karnataka

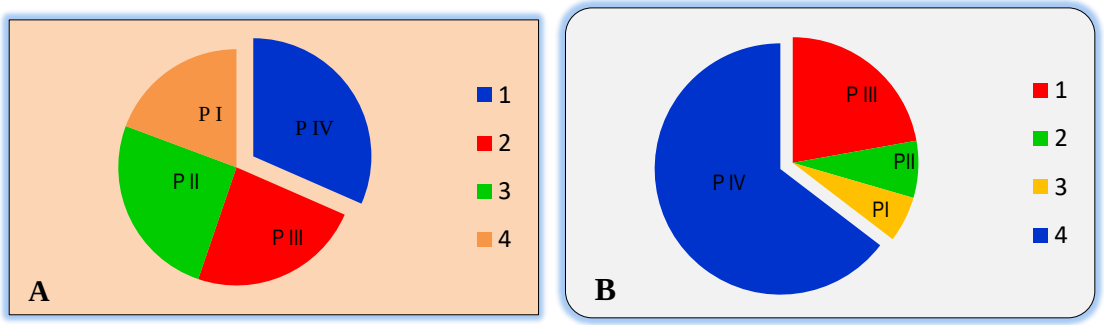

Fig-2 Pie chart showing the yielding of DNA extracted from different samples of two species (A) *D. latifolia* and (2) *L. lanceolata* by using the CTAB, modified CTAB and Plant DNA extraction kits ((GenElute Plant genomic DNA Miniprep Kit, Vivantis GF-1 Plant DNA extraction kit) and optimized HEPES buffer protocol

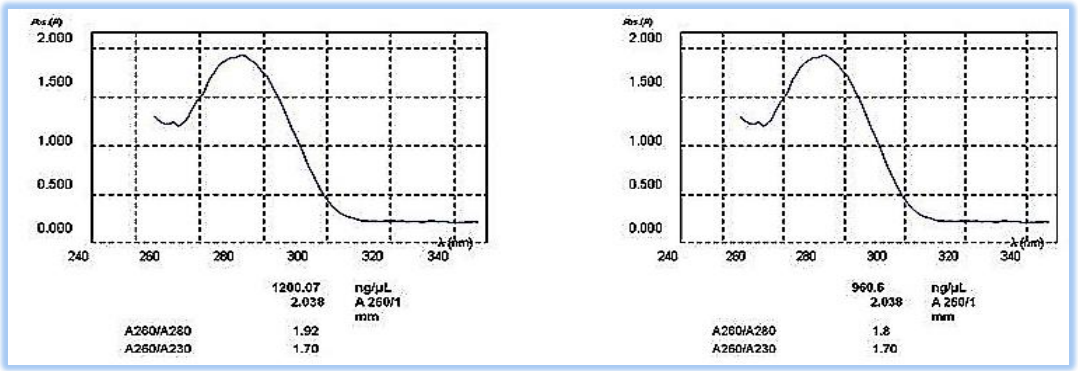

Fig-3 Graph profile of genomic DNA extracted by standardised protocol IV by using spectrophotometer factor$_{50}$ with 0.2 mm (Biospectrometer eppendorf).

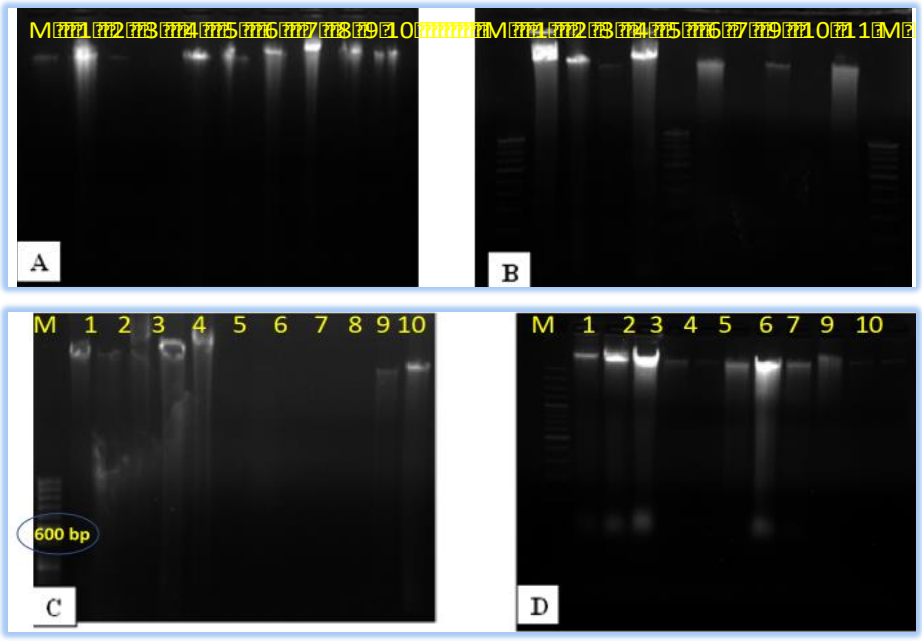

Fig-4 Ethidium Bromide stained isolated DNA from leaf samples of *D. latifolia* resolved on 0.8% agarose gel (A) Protocol I (CTAB protocol) (B) Protocol II (modified CTAB) (C) Protocol III (GenElute Plant genomic DNA Miniprep Kit, Vivantis GF-1 Plant DNA extraction kit) (D) (optimized HEPES buffer) Protocol IV.

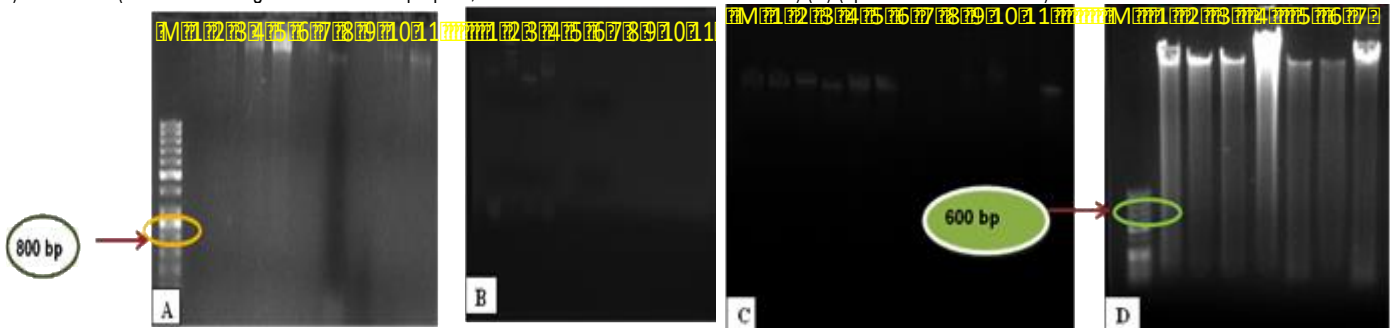

Fig-5 Ethidium bromide stained isolated DNA from leaf samples of *L. lanceolata*. resolved on 0.8% agarose gel. M-100 plus Ladder (Thermofisher pvt ltd) (A) Protocol I (CTAB protocol) (B) Protocol II (modified CTAB) (C) Protocol III (GenElute Plant genomic DNA Miniprep Kit, Vivantis GF-1 Plant DNA extraction kit) (D) (optimized HEPES buffer) Protocol IV.

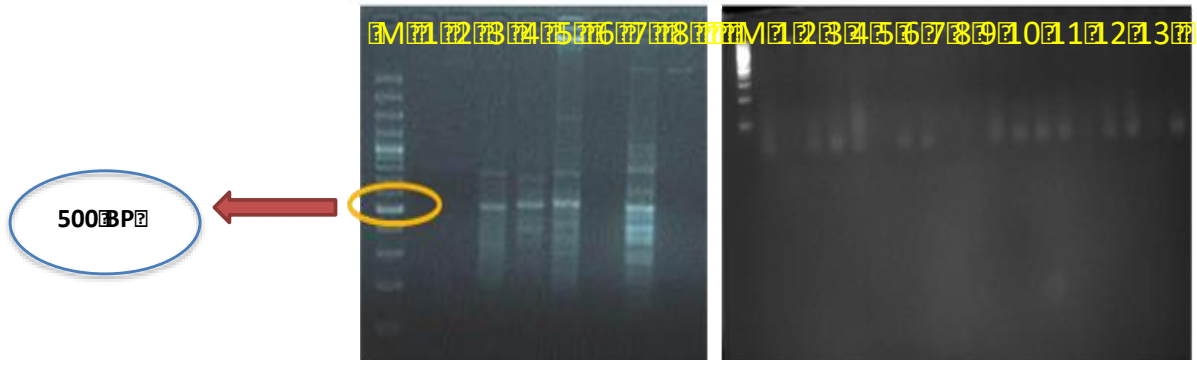

Fig-6 Amplification of genomic DNA isolated from CTAB and modified CTAB protocols of *D. latifolia* and *L. lanceolata* using 10 ISSR primers. M- 100 bp (Thermofisher pvt. ltd).

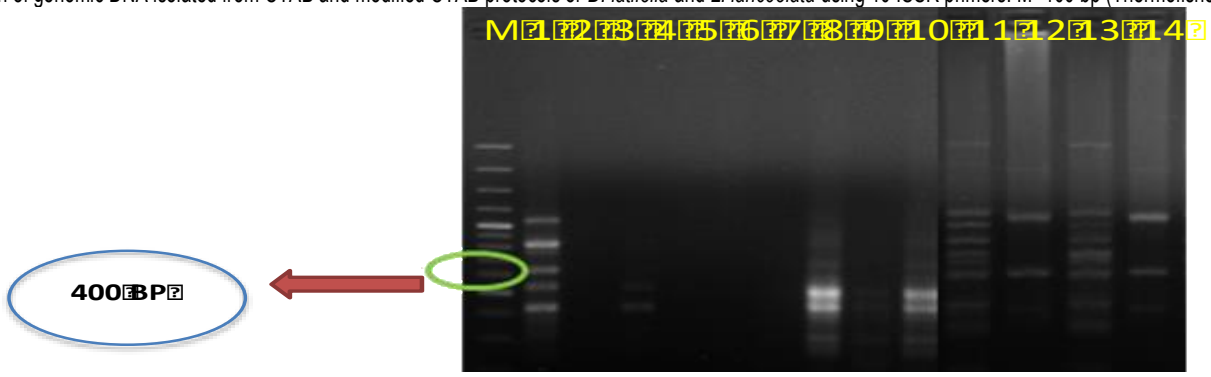

Fig-7 Amplification of DNA isolated by kits of *D. latifolia* and *L. lanceolata* through PCR by four ISSR primers.M-100 bp ()Thermofisher pvt. ltd).

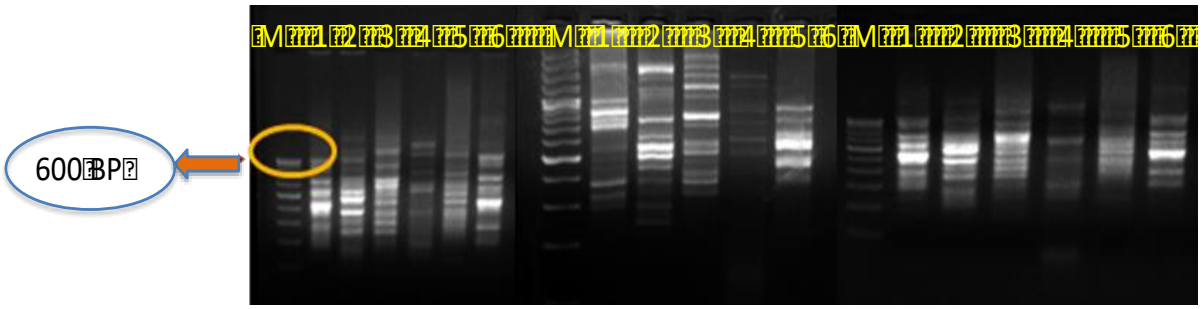

Fig-8 Amplification of isolated DNA from optimized protocol of *D. latifolia* (UBC810, UBC823, UBC841, UBC855, UBC856).M-100 bp (Thermofisher pvt. ltd).

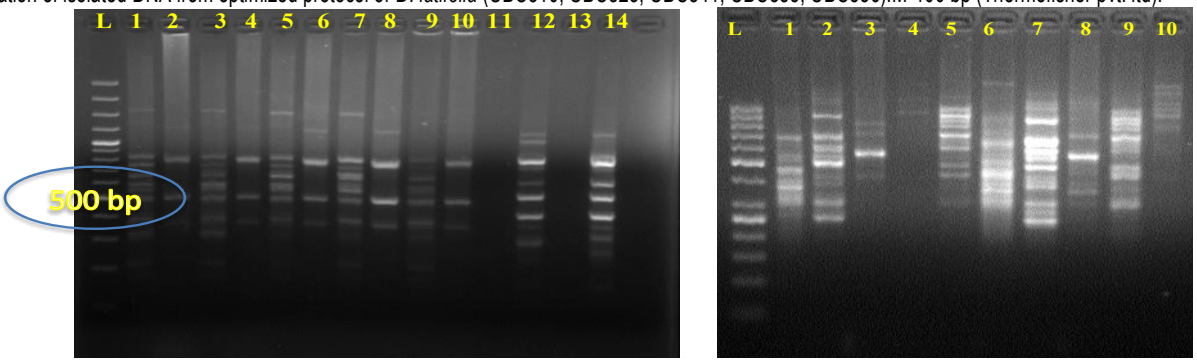

Fig-9 Amplification of isolated DNA from optimized protocol of *L. lanceolata* with 5 ISSR (UBC 815, UBC844, UBC845, UBC824, UBC867) primers with five samples. L-100 bp (Thermofisher pvt. Ltd).

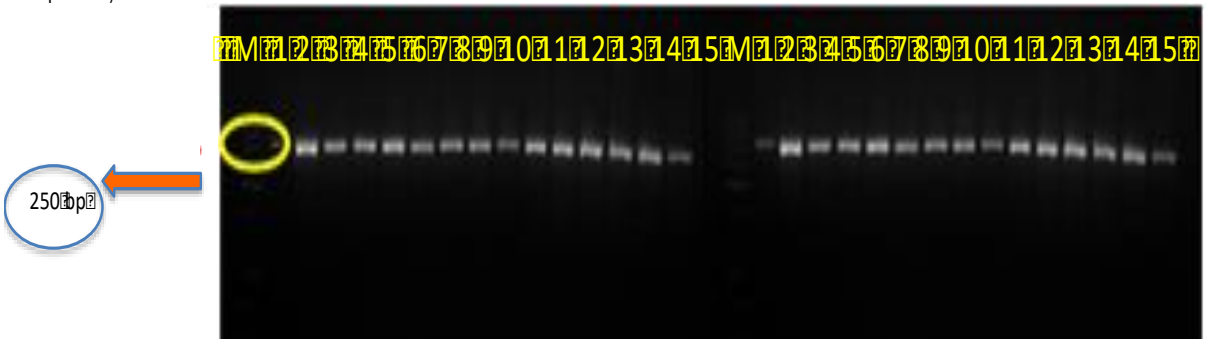

Fig-10 Amplification of *D. latifolia* and *L. lanceolata* through PCR using two universal SSR (*rbcL* and *matK*) microsatellite markers. M- 50 bp ladder (Thermofisher pvt. ltd.) (A) *rbcL* (B) *matK*.

Table-1 Details of *D. latifolia* and *L. lanceolata* identified across Karnataka

SN	Location	Taluk	Latitude (N)	Longitude (E)	Annual rainfall (mm)	Agroclimatic zone
1	Hassan	Hassan	13⁰ 00'29.9''	076⁰ 06'10.8''	1013	North West transition zone
2	IWST Blr	Bangalore	13⁰00'41. 3"	77⁰ 34'17. 6"	698	Eastern dry Zone
3	Dharwad	Dandeli	13⁰ 00'39.6"	077⁰ 34'21.7"	899	North west dry zone

Table-2 Details of selected ISSR primer sequences *L. lanceolata*

SN	Primer Number	Sequence (5'-3')	Amplified product range (bp)	Annealing temperature (⁰C)
1	UBC 810	(AC)8T	450-1200	50⁰C
2	UBC823	$(AG)_8$YT,	400-1250	50⁰C
3	UBC841	$(CA)_8$RG	303-1125	52⁰C
4	UBC855	$(AC)_8$YG)	310-1350	50⁰C
5	UBC856	$(CCT)_4$,	300-1050	52⁰C

Table-3 Details of selected ISSR primer sequences L. lanceolata.

Primer Number	Sequence (5'-3')	Amplified product range(bp)	Annealing temperature (⁰C)
UBC 815	$(CT)_8$G	400-1400	54 ⁰C
UBC844	$(CT)_8$RC	310-1135	55 ⁰C
UBC845	$(CT)_8$RG	312-1163	52 ⁰C
UBC824	$(TC)_8$G	305-1237	54 ⁰C
UBC867	$(CT)_8$CT,	500-1250	52 ⁰C

Table-4 Selected SSR (Microsatellite) Primers sequences

Loci	Primer information	Size (bp)	Amplified product (bp range)	Primer Tm (⁰C)
rbcL	*rbcL* 1 F ATGTCACCACAAACAGAAAC R724 R TCGCATGTACCTGCAGTAGC	679	215	60 ⁰C
matK	*F'matK* 472F CCRTCATCTGGAAATCTTGGTT R'1248RGCTRTRATAATGAGAAAGATTTCTGC	710	173	60 ⁰C

Table-5 DNA extraction protocols and yield of extracted DNA from D. *latifolia* and *L. lanceolata*

SN		Fresh leaves		Mature dried leaves	
D. latifolia	Protocol	DNA yield (ng/µL)	DNA purity (260/280)	DNA yield (ng/µL)	DNA purity (260/280)
1.	P I	320.5	1.5	150.6	1.4
2.	P II	100.6	1.5	90.0	1.4
3.	P III	90.5	1.4	83.2	1.5
4.	P IV	1200.07	1.9	960.6	1.7
L. lanceolata		-		-	
1.	P I	421.5	1.6	310.5	1.4
2.	P II	107.8	1.6	100.2	1.5
3.	P III	89.5	1.5	56.3	1.5
4.	P IV	960.6	1.8	580.2	1.7

Sample size: In case of *D. latifolia* sample size was (N= 29) and for *L. lanceolata* (N=30) mature leaf samples were collected from IWST Bangalore, Hassan, Dharwad, and Barchi Haliyal region.

Storage: Leaf samples were put into plastic cover and immediately placed into dried silica gel. After arrival to the laboratory the leaf samples that had been placed in the silica gel were cleaned with the help of tissue paper and distilled water to remove the dust and contaminations and kept for drying at least 3 to 4 days with the change of silica gel which absorb all moisture content of the samples and then kept the dried samples to -20°C cryogenic freezer to maintain the quality to further extraction process.

Genomic DNA extraction: Four methods were used *viz.* (i) the CTAB method [8,9], (ii) modified CTAB method [10,11], (iii) (GenElute Plant genomic DNA Miniprep Kit, Vivantis GF-1 Plant DNA extraction kit) DNA extraction kit and (iv) the modified HEPES buffer extraction method developed for highly mucilaginous leaves of respective species were carried out in the study. Protocol1 are described previuosly papers while the protocol2 are modified CTAB method which are also executed in number of research papers, protocol3 are kit manufacturer based routine process and the protocol4 developed (HEPES with modified CTAB protocol) has been described below. The CTAB based DNA extraction protocol was not efficient for *D. latifolia* and *L. lanceolata* as the DNA pellet obtained was brownish color and insoluble precipitation due to presence of high quantity of oxidative secondary metabolites and large amount of mucilaginous content. Therefore, the protocol was modified to reduce these oxidative agents (free radicals) and to obtain high quantity with good quality of soluble DNA from these timber species.

Protocol 4: *Optimized Modified HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) DNA extraction buffer method:*

1. Washing buffer: Solutions: 2% HEPES, 1.5 % PVP, and 2.5 % (v/v) β -mercaptoethanol.

2. CTAB extraction buffer: Solutions: 3% (w/v) CTAB, 0.25 % (w/v) PVP (M.Wt = 40000), 1.4 MM NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% (v/v) β-mercaptoethanol.

Protocol: This modified protocol was divided into two steps (i) Washing of samples with HEPES buffer and (ii) CTAB extraction buffer process.

(i) HEPES buffer washing treatment to samples: Approximately 500 mg. of leaf samples was taken into chilled autoclaved mortar and add 200 mg. PVP (Polyvinylpolypyrrolidone) and rapidly grind into fine powder using liquid nitrogen. The powder was transferred to 15 mL test tubes containing 4 mL washing buffer (HEPES) into each tube. The mixture was vigorously mixed by vortex. After the mixing process centrifuge the tubes at 8,000 rpm for 3 min. at 4⁰C and then discard the slurry supernatant and add 4 mL washing buffer and repeat the same step 4- 5 times until to obtained the clear supernatant solution.

(ii) Extraction of genomic DNA: After HEPES buffer washing treatment freshly prepared 5 mL extraction buffer pre-wormed at 65^{0}C was added. The mixture was gently mixed with the help of vortex and incubated the samples at 65^{0}C pre-heated water bath for 2.30 h with periodic inversion. After incubation equal amount of C: I 25:24:1 (v/v) was added with gentle overturn for 10 min. and centrifuged for 10 min. at 12,000 rpm at 4˚C. The supernatant layers were carefully transferred to the fresh test tubes with eppendorf pipettes and discarded the debris content and added equal volume of C: I 24:1 (v/v) in tubes. Inverted the tubes gently for 10 min to precipitate the protein and carbohydrate contaminations. Centrifuged the samples at 12,000 rpm for 10 min at 4˚C. The aqueous upper layer was carefully transferred to the fresh tubes then adds 30 µL RNase (Sigma aldrich) (10 mg/mL) in each tube. Incubated the tubes in a dry bath (Bio Bee pvt. ltd.) for 40 min at 37^{0}C with intermittent trembling. After RNase treatment further added 30 µL proteinase K (10 mg/mL) in respective tubes and again kept it in dry bath at 37^{0}C for 2 hours. Took out the tubes from dry bath and washed it again with C: I and centrifuged the samples at 12,000 rpm for 12 min at 4˚C. Transferred the 4 mL supernatant in new autoclaved 15 mL tubes and added 1 mL 5M NaCl, 1 mL 1M Sodium acetate and added equal volume of chilled isopropanol in each tube. Allowed it for incubation at -20 ºC for overnight. On next day centrifuged the tubes at 12,000 rpm for 15 min at 4˚C. Discarded the supernatant and retained the pellet. Transferred the pellet carefully into 1.5 mL reaction vials and washed it twice with 1 mL 70% ethanol following centrifugation for 5 min at 10,000 rpm to remove remaining salt content. The supernatant was discarded and the tubes were allowed to dry at room temperature until the ethanol smells evaporate. The pellet re-suspended in 50-80 µL (volume depends on pellet size) TE (10 mM Tris HCl, 1 mM EDTA at 8.0 pH) buffer and dissolved it properly and stored the DNA into -20^{0}C for further investigation.

Quantitative and Qualitative measurement of extracted DNA: Extracted genomic DNA quantity (from both optimized methods) was measured by Eppendorf UV spectrophotometer at the wavelength of 260/280, and 260/230.

F. Agarose gel electrophoresis: To check the DNA quality and purity for PCR compatible DNA were subjected to run the gel into 0.8% agarose gel electrophoresis unit [12]. After running the gel were visualized by UV gel documentation unit.

DNA amplification through ISSR and SSR primers: ISSR primers were used to validate the quality of extracted wood and leaves DNA in this study. DNA amplification was carried out in 13 µL reaction volume containing genomic DNA 1.5 µL (45 ng), 10 mM 2 µL primers, 1.5 µL 10x PCR buffer, 1.5 µL dNTPs, 1.5 µL MgCl2, 0.2 µL (3U/ µL) Taq polymerase (Bangalore genie) and 4.2 µL double distilled autoclaved RNase free water. Amplification cycle consist of an initial 3 min denaturation at 94^{0}C, 30 cycles for 30 seconds at 50-60^{0}C depends on the primer anealing temperature, 1 min 72˚C and final extension step for 10 min at 72^{0}C. The amplified product loaded with 4 µL 1x loading buffer (Bromophenol blue) were size fractionated by electrophoresis on a 1.5% for ISSR and 2% for SSR agarose gel with 0.2 % ethidium bromide and visualized on UV transilluminator to obtained the bands to validate the DNA quality and suitability for PCR reactions.

(i) List of selected ISSR Primers for *D. latifolia*: Initially, 10 primers were screened (UBC810, UBC823, UBC824, UBC826, UBC841, UBC845, UBC855, UBC856, UBC859, and UBC861) with all the species samples including wood and leaves [13]. Out of 10 we selected 5 primers which were giving amplification and polymorphism among the species and validated the HEPES buffer DNA extraction protocol[4] for mature and dried mature samples of *D. latifolia* [Table-1].

(ii) List of selected ISSR primers for *L. lanceolata*: Initially, 7 primers were screened (UBC 815, UBC844, UBC845, UBC824, UBC867, UBC861, and UBC869) with all the samples of *L. lanceolate* [14]. Out of 7 primers, 5 primers were amplified and showing polymorphism. These primers were validated the HEPES buffer DNA extraction protocol[4] for mature and mature dried leaf DNA of *D. latifolia* [Table-2].

(iii) List of selected SSR Primers: Two universal SSR markers recommended by COBOL (Consortium of Barcode of Life) were selected in this study to validate the DNA quality and PCR amplification [Table-3] [15].

Results and discussion

The CTAB method, modified CTAB protocol and the plant DNA extraction kit protocol were carried out to extract DNA from mature and mature dried leaf samples from selected species respectively. DNA obtained from these protocols were very brownish undissolved pellets and containing high viscosity. Three of these methods could not yield large quantity of DNA and the DNA was not amplifying in PCR due to presence of hidden proteins that act as a PCR inhibitor. In *L. lanceolata* a consistent amount of mucilaginous component was present which inhibits the appropriate DNA separation. Due to presence of high volume of polysaccharides, DNA could not be able to load and stuck to the wells during electrophoresis separation process. To overcome this problem, we modified the method and extract DNA through HEPES buffer, which reduces most of the slurry, slimy polysaccharide contaminations and removes the brown color precipitation. Extracted the genomic DNA from the optimized protocols when subjected to ISSR and SSR analysis, produced clear and highly reproducible bands. ISSR profiles of *D. latifolia* and *L. lanceolata* DNA which shows that the DNA extracted by this HEPES modified method were suitable for PCR amplification. The principle modification of this mode was washing of samples by using HEPES buffer, which reduced the polysaccharides contamination. The additional use of 3% CTAB, high conc. of PVP while sample grinding, reduces polysaccharides and PCR inhibitors. Precipitation of DNA with 2.5 M NaCl aided the removal of polysaccharides by increasing their solubilities in chilled isopropanol so they do not precipitate with DNA pellets. In conclusion the only modification that proved successful for DNA isolation from high polysaccharides containing species was HEPES buffer-based DNA extraction along with modification and purification dependent on the CTAB extraction buffer.

Discussion

In general, a larger quantity of DNA could have isolated by using the modified HEPES buffer protocol than that when using the CTAB, modified CTAB and Plant DNA extraction kits (GenElute Plant genomic DNA Miniprep Kit, Vivantis GF-1 Plant DNA extraction kit). The quantity of DNA of mature fresh leaves using HEPES buffer protocol was 58.2 % greater than the CTAB and modified CTAB protocol as well as using plant DNA extraction kits. In dried mature leaves, the quantity of DNA extracted using the optimized protocol was 60.3 % greater than isolated by using the CTAB and modified CTAB protocol as well as using plant DNA extraction kits. For fresh leaves there were significant differences in the quantity of extracted DNA between Dried and fresh ($P<0.05$) when using both the modified CTAB method and optimized HEPES protocol. In the same manner, the differences in the quantity of isolated DNA were also statistically significant between the mature and dried mature leaves ($P<0.05$). It shows that the quantity of DNA was greater in the fresh mature leaves and lesser in dried mature leaves [Fig-2,3] and [Table-4]. The purity of DNA extracted from the samples by the ratio of A260/A280 was between 1.4-1.5, which was lower than the normal purity range of DNA. The purity of DNA enhanced by using the modified HEPES buffer protocol, by the ratio of A260/A280 was between 1.8-1.92 which was the normal range of the purity of DNA, while the purity of mature fresh leaves was higher than the dried mature leaves. For both the fresh and dried leaves, there was no significant difference in the ratio of A260/A280 for the DNA isolated by using modified CTAB ($P<0.05$) [15]. However, there was a significant difference and the purity of the DNA isolated by using the HEPES buffer protocol was shown between the fresh and mature dried leaves of both the species ($P<0.05$).

Conclusion

DNA extraction from leaves of *D. latifolia* and *L. lanceolata* is difficult due to presence of high quantity of secondary metabolites, polyphenolics compounds and high concentration of polysaccharides.

Modification in this protocol includes HEPES buffer washing, various conc. CTAB, PVPP, β-mercaptoethanol, incubation time, RNAse treatment and Proteinase treatment.

Application of research: This method could be implemented as standard method for isolation of DNA from *D. latifolia* and *L. lanceolata* or similar timber species containing rich polysaccharides and defined here is hasty, uncertain and steady permitting the handling of large number of trials with easy routine.

Research Category: Wood Science and Technology

Abbreviations:
CTAB: Cetyltrimethyl Ammonium Bromide
Rbcl: Ribulose bisphosphate carboxylase
matK: Maturase K

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Author Contributions: All author equally contributed

Author statement: All authors read, reviewed, agree and approved the final manuscript

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

References

[1] Sahu S.K., Thangaraj M. and Kathiresan K. (2012) *ISRN Molecular Biology.Article* ID: 205049.
[2] Graham G. C., Meyers P. and Henry R. J. A. (1994) *Simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. BioTechniques*, 16, 48-50.
[3] Scott K. D., Playford J. (1996) *DNA extraction technique for PCR in rainforest plant species. Biotechnique*, 20, 974-978.
[4] Fang G., Hammar S. and Grumet R. (1992) *BioTechniques*,13, 1, 52-56.
[5] Weishing K., Nybom H., K. Wolff H. and Meyer W. (1995) *CRC Press, Boca Raton, Fla, USA*, 44–59, 1995.
[6] Dilworth E. and Frey J. E. (2000) *Plant Molecular Biology Reporter*, 18, 61–64.
[7] Lara D. Shephard and Todd G. B. (2011) *Journal of plant sciences*. 124, 2, 311-314.
[8] Doyle J.J. and Doyle J.L. (1987) *A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. Phytochemical Bulletin*, 19, 11-15.
[9] Murray M.G. and Thompson W.F. (1980) *Rapid Isolation of High Molecular Weight Plant DNA. Nucleic Acids Research*, 8, 4321-4325.
[10] Ginwal H.S. and Maurya S.S. (2010) *Indian Journal of Biotechnology*, 9, 69-73.
[11] Jiao L., Yin Y., Xiao F., Sun Q., Song K., Jang X. (2012) *IAWA*, 33, 4, 441-456.
[12] Sambrook J., Maccallum P. and Russell D. (2001) *3rd Edition, Cold Spring Harbor Press, Cold Spring Harbor*, 2344 p.
[13] Javaid A., Akram W., Shoaib A., Haider A. S., Ahmed. A. (2014) *Pak. J. Bot*. 46, 5, 1573-1576.
[14] Zukauskiene J., Paulauskas A., Varkuleviciene J., Marseliene , Gliaudelyte V. (2014) *American journal of plant sciences*. 5, 2741-2747.
[15] CBOL Plant Working Group. (2009) *PNAS*, 106, 31, 12794-12797.
[16] Dev S.A., Muralidharan E. M., Sujanapal P., Balasundaran M. (2014) *Annals of Forest Science*, 71, 517-522.
[17] Tung Nguyen C.T., Son R., Raha A.R., Lai O.M., and Clemente Michael W.V.L. (2009) *International Food Research Journal*, 16, 21-30.