

An Effective Wood DNA Extraction Protocol for Three Economic Important Timber Species of India

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Abstract

Extraction of DNA from fresh tissues is routine in studies of tropical forest species, but DNA extraction from wood is considered as difficult due to its highly degraded nature and adequate quality of genomic DNA extraction is essential for molecular studies. Very few studies have validated the potential for isolating DNA from dried wood (Heartwood and Sapwood). Wood genomic DNA extraction is difficult from mature timber (Teak (*Tectona grandis* f; verbanaceae), Black Rosewood (*Dalbergia latifolia* f; Fabaceae) Ben Teak (*Lagerstroemia lanceolata* f; Lytheraceae) tissues due to presence of high quantity of secondary metabolites polyphenols, tannins and terpenoids and protein inhibitors. Mostly in laboratories DNA extraction kits are available but by using kits, DNA yield is very low and it is quite expensive too. We have standardized and validated the DNA extraction through manual protocol which is applicable for Bark, Sapwood and Heartwood samples of tree species which contains huge amount of inflexible tissues and fibers. The quality of the DNA was tested by spectrophotometer, gel electrophoresis and PCR (ISSR and SSR) amplification. An average DNA yield for heartwood ranges from 0.186 - 0.166 µg/µL and sapwood was ranges from 0.26 - 0.244 µg/µL. Modification of CTAB method was by addition of polyvinylpyrrolidone (PVP) appx 0.25%, variation in Rnase concentration, proteinase treatment with different concentration and incubation time. In order to evaluate the standardized wood genomic DNA extraction protocol, we compared it with the mature leaf and core samples (heartwood and sapwood) of the same timber species. The outcome was also quantified and proved by means of polymerase chain reaction analysis by using ISSR and SSR microsatellite markers conducted with isolated pure DNAs. This modified protocol made increased yield and purity of wood total genomic DNA and facilitate the important application of foren-

sic timber species effort.

Keywords

Wood Genomic DNA, Plant DNA Kits, Timber, ISSR and SSR Markers

1. Introduction

Preservation of endangered species is an indispensable part of accomplishment the objective of the Convention on Biological Diversity 2020 on cultivating the prominence of global biodiversity [1]. The first critical phase in protecting and managing threatened species is correct identification and delimitation of the target species [2]. Identification of plant species traditionally relies on morphological characters of especially leaf, flowers and fruits, which for trees can be time consuming to access and only present during parts of the year. Accurate identification in species-rich or taxonomically complex groups also typically requires genomic DNA that is not always available, especially in tropical forest samples [3] [4]. Teak (*Tectona grandis* f; verbanaceae), Black Rosewood (*Dalbergia latifolia* f; Fabaceae) Ben Teak (*Lagerstroemia lanceolata* f; Lytheraceae) are incredibly significant economic timber species in tropical countries chiefly in India, Indonesia, Myanmar and Burma. These three genres contain many valuable timber species threatened by illegal logging and deforestation, but knowledge on distribution and threats is often limited and accurate species identification difficult. Illegal logging and associated trade are the cause of many economic and ecological problems both in timber producer and timber consumer countries [5]. Although many legal instruments have been established to combat illegal logging and trade of illegally sourced timber, practical controls mechanisms to identify the tree species and geographic origin of wood and wood products are still lacking [6] [7]. The action of illegal logging crimes is hampered by a lack of available forensic timber identification tools and time of harvest for both screening of suspect material and definitive identification of illegally sourced wood, which were scam by forest department. Processed timber products such as decking, flooring and furniture are subjected to drying, engineering and treatment processes that degrade the DNA present in the wood, just as in ancient samples [8]. Extraction of whole genomic DNA from fresh tissues is routine in studies of tropical forest species [9] [10]. DNA extraction from wood (Sapwood and heartwood) is quite difficult due to presence of higher quantity of secondary metabolites phenolic and lignin compounds and the concentration of leaf DNA is higher than wood DNA [11] [12]. There are number of protocols for DNA extraction from various plant and animal species and tissues published, but these protocols are the best for leaf and soft tissues, while mature tree samples (core tissues) often narrows down the scope of DNA extraction [13]. Genotypes the tracing of timber origin, and species can be identified based on an investigation of wood if suitable DNA content and high amount of pure DNA is

available [14]. The aims for this study were to optimize the total genomic DNA extraction protocol by means of the standardized the modified CTAB wood (heartwood and sapwood) DNA extraction protocol [15] [16] and the comparison between the modified CTAB protocol, CTAB protocol and Plant DNA extraction kits (Nucleopore) protocols. This modified protocol made the intact wood DNA isolation that facilitates the important forensic timber species effort.

2. Materials and Methods

2.1. Sampling

Collection of leaves and core samples from naturally grown populations of southern regions of Karnataka such as Virnoli and Barchi range, Jangganmatti, (N15° 13'49.4" E074° 38'27.9"), Dharwad district (Haliyal region), and Hassan (Alur Taluk) **Figure 1(a)** were carried out.

Mature and dried leaves were collected by climbing of the tree with the help of forest guards **Figure 1(b)** and core samples were taken by penetrating increment borer in the trunk near breast height and free of limbs, knobs, or other growths of the tree **Figure 1(c)** and rotated manually from the selected samples and after collection of core **Figure 1(d)**. Sealed the holes with small dry twigs in the trunk and closed it with paraffin wax to reduce the fungal infections or other injuries to the selected trees.

2.2. Storage

Leaf and core samples were put into plastic cover and immediately placed into an ice storage box. After arrival to the laboratory the wood samples that had

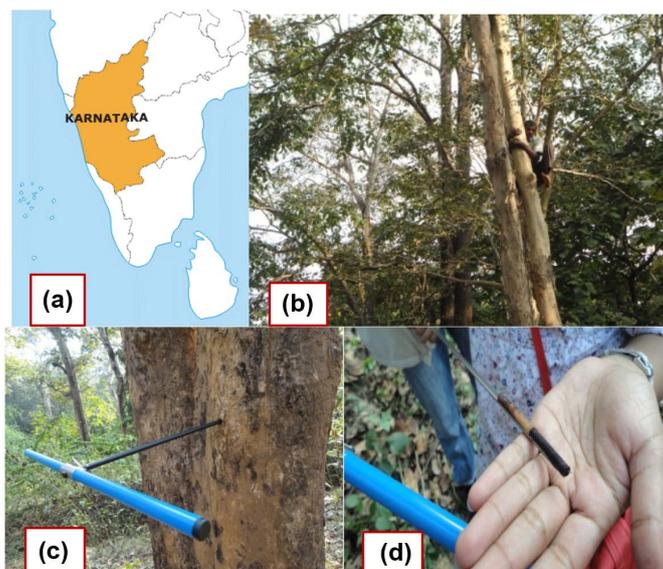


Figure 1. (a) Sample collection of leaves and core from virnoli Barchi range Dharwad district; (b) Collection of leaf samples; (c) Increment borer; (d) core samples of important (*D. latifolia*, *L.lanceolata* and *T. grandis*) timbers.

been placed in the ice storage box were transferred at once to a -20°C cryogenic freezer (Siemen pvt. ltd.) to maintain their freshness.

2.3. Sample Preparation

Before DNA extraction, core samples were cleaned and washed with distilled water and kept it in a fresh autoclaved glass bottles with distilled water for about 72 - 96 h and repeatedly changed the water at every 12 h to avoid the fungal or any other contaminations. Samples were cut into two Sections 1. Sapwood 2. Heartwood, cut the samples separately of an approx. 3 - 5 μM thickness was prepared from each samples using paper cutter or sliding microtome to produce small chips of wood samples. Kept the chipped slices for drying 5 - 10 min before imperilling to for CTAB based protocols for genomic DNA extraction which comprised of CTAB method [17], DNAsure plant mini kit (Nucleopore) [18], and CTAB method (protocol 3) developed at IWST laboratory. Protocol 1 are executed earlier while the protocol 2 are kit manufacturer based and all are a routine based protocol published in number of papers so here only the modified standardised developed *i.e.*, protocol 3 (**modified CTAB**) has been described below.

2.4. Protocol 3: CTAB Extraction Buffer

3.5% (w/v) CTAB, 0.25 % (w/v) PVPP, 1.4 mM NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2.5% (v/v) β -mercaptoethanol.

2.5. Method

Around 500 mg. samples (sapwood, heartwood and leaf separately) were taken into chilled autoclaved mortar pestle and added 250 mg. PVPP then rapidly ground it into a fine powder using liquid nitrogen. After grinding added pre-warmed at 65°C extraction buffer and vigorously shaken the tubes in vortex for few seconds and retained it at 65°C pre-heated water bath for $2^{1/2}$ h with periodic shuddering. Taken away the reaction tubes from water bath and retained it at room temperature to cool it for 30 min. Added 5 mL P:C:I (Phenol:chloroform:Isoamylalcohol 25:24:1) in each tube and moderately shaken it for 5 minutes. Centrifuged the tubes at 10,000 rpm for 10 min at 4°C temperature. Allocated the transparent color supernatant into fresh autoclaved tubes and cast off the debris content and added 6 ml. C:I (chloroform:Isoamylalcohol 24:1). Agitated the tubes gently without affecting the DNA in solution for 10 min to remove the protein and carbohydrate contaminations. Repeat the similar step twice due to presence of heavy amount of protein and lignin content to get clear supernatant. In the next step added 30 μL RNase (sigma Aldrich) (20 mg/mL) in each tube and gently mixed it with the help of micropipette. Allowed it into dry bath (Bio-Rad pvt. ltd.) at 37°C for 40 min. After RNase treatment, added 30 μL proteinase K (Sigma Aldrich) (20 mg/mL) [19] in respective tubes with gently mixing two three times and retained it in dry bath at 37°C to precipitate the

protein contaminations for 2 h. Carryout the samples from dry bath and washed it another time with C:I and spin the samples at 12,000 rpm for 12 min at 4°C. Transferred the 4 mL supernatant in new 15 mL reaction tubes and added 1 mL 5 M NaCl, 1 M Sodium acetate 1 mL and equal volume of chilled isopropanol in each samples. Allowed it to overnight incubation at -20°C for overnight and next day centrifuged it at 12,000 rpm for 15 min at 4°C. Discarded the supernatant and the retained transparent pellet transferred into anew autoclaved 1.5 mL reaction tubes. Washed the pellet twice with 1 mL 70% ethanol using short spin in mini centrifuge at 10,000 rpm for 5 min at to remove any remaining salts in tubes. Afterwards washing the pellet was allowed to dry. Re-suspended in 30 - 50 µL TE (Tris-EDTA) (pH 8.0) buffer. Dissolved it appropriately and kept the DNA containing tubes at 4°C for further analysis.

2.6. DNA Quantification

The yield of extracted DNA was quantified by nanodrop at 260/280 nm wavelength [20]. The purity of DNA was checked by running the samples on 0.8% agarose gel in 1X TAE buffer (8.0 pH) stain containing 4 µL of 0.3% Ethidium Bromide and visualized on UV transilluminator gel doc (Syngene version 7.0) to get the DNA bands.

2.7. DNA analysis through ISSR and Universal SSR (RbcL & TrnH) Markers

Ten ISSR primers were screened for analysis [20] [21] out of those four primers was amplified and showing high polymorphism in all three species. UBC834 (AG)₈YT, UBC874 (CCT)₄, UBC848(CA)₈RG, UBC857 (AC)₈YG) ISSR primers amplified. The universal SSR markers rbcL (5'-AACACCAGCTTTTRAATCCAA-3') and MatK (5'-GGGTTGCTAACTCAATGGTAGAG-3') primers [22] were used to validate the quality of extracted wood DNA. In this study, basically particular universal SSR primers which were used to carry out research in barcoding of timbers, which would be useful in illegal logging and tracing the timber origin. DNA amplification was carried out in 13 µL reaction volume containing genomic DNA 1.5 µL (30 ng/µL), 10 mM 2 µL primers, 1.5 µL PCR buffer, 1.5 µL dNTPs, 1.5 µL MgCl₂, 0.3 µL (3 U/µL) Taq polymerase (Bangalore genie) and 4.2 µLRNase freedouble distilled water (Sigma aldrich). Amplification cycle consist of an initial 3 min denaturation at 94°C, 30 cycles for 30 sec at 50°C, 1 min 72°C and final extension step for 10 min at 72°C. The amplified product loaded with 5 µL loading buffer were size fractionated by electrophoresis on a 1.5% for ISSR and 2.0% SSR agarose gel with 0.3% Ethidium bromide and visualized on UV transilluminator to determined the amplified clear bands to validated the DNA quality and suitability for PCR reactions.

3. Results

Isolation of DNA from CTAB method and using plant DNA extraction kits were

unable to extract DNA from wood tissues. Hence we developed a new modified protocol by modifying the CTAB protocol [23] by enhancing the incubation time, CTAB concentration, high concentration of proteinase and RNase treatment and most important keeping the sample in water to release the resilience of cells by process of osmosis. The ratio of extraction buffer and sample was 500 mg: 5 mL. The additional washing with C: I (24:1) helped to removal of polysaccharides and strong protein contamination.

Table 1 represented the quantity and quality of leaf, sapwood and heartwood tissues with all four protocols, which were used to standardize the protocol 3 to yield high genomic DNA with all selected timber species. For comparative purposes (**Figure 2**) DNA extraction of leaves, sapwood and heartwood tissues the graph of nanodrop were representing the quantity and qualitative analysis of all three selected species. It was summarized in which shows that the yield of DNA from sapwood from 0.26 - 0.244 $\mu\text{g}/\mu\text{L}$ in heartwood from 0.186 - 0.166 $\mu\text{g}/\mu\text{L}$ and in leaf samples from 1.30 - 1.511 $\mu\text{g}/\mu\text{L}$ according to spectrophotometer measurements with respect to purity from 1.7 - 1.8. In general, a higher quantity of DNA could be obtained using modified CTAB protocol (**Figure 3**). The quantity of DNA from Dried leaf samples and from wood samples was 56 % higher than the CTAB and other DNA kit protocol. In **Figure 4(a)** and **Figure 4(b)** were showing the DNA bands obtained by protocol 1 *i.e.*, CTAB method and protocol 2,3 *i.e.*, DNA isolation kits which is basically used for industrial purpose and specific for plant genomic DNA isolation. The bands were not clear and the pellet was dark brownish in color with undissolved PCR inhibitors (carbohydrate and proteins) contaminations. **Figure 4(c)** and **Figure 4(d)** was presenting the PCR amplification of DNA extracted through protocols 1,2 but in

Table 1. DNA extraction protocols and yield of extracted DNA from *T. grandis*, *D. latifolia* and *L. lanceolata*.

<i>T. grandis</i>		Mature dried leaves		Sapwood		Heartwood	
No.	Protocol	DNA Yield (ng/ μL)	DNA purity (260/280)	DNA Yield (ng/ μL)	DNA purity (260/280)	DNA Yield (ng/ μL)	DNA purity (260/280)
1	P I	200	1.5	120.3	1.5	95.3	1.3
2	P II	95.2	1.4	86.5	1.5	43.21	1.4
3	P III	1394.06	1.8	244.64	1.7	166.95	1.7
<i>D. latifolia</i>		-	-	-	-	-	-
1.	P I	163.5	1.6	195.9	1.4	76.8	1.4
2.	P II	98.6	1.6	51.6	1.5	40.2	1.3
3	P III	1494.42	1.8	262.67	1.7	186.50	1.7
<i>L. lanceolata</i>		-	-	-	-	-	-
1.	P I	173.7	1.3	90.2	1.5	65.1	1.3
2.	P II	90.3	1.6	51.6	1.4	40.2	1.3
3	P III	1511.38	1.8	224.024	1.9	193.80	1.8

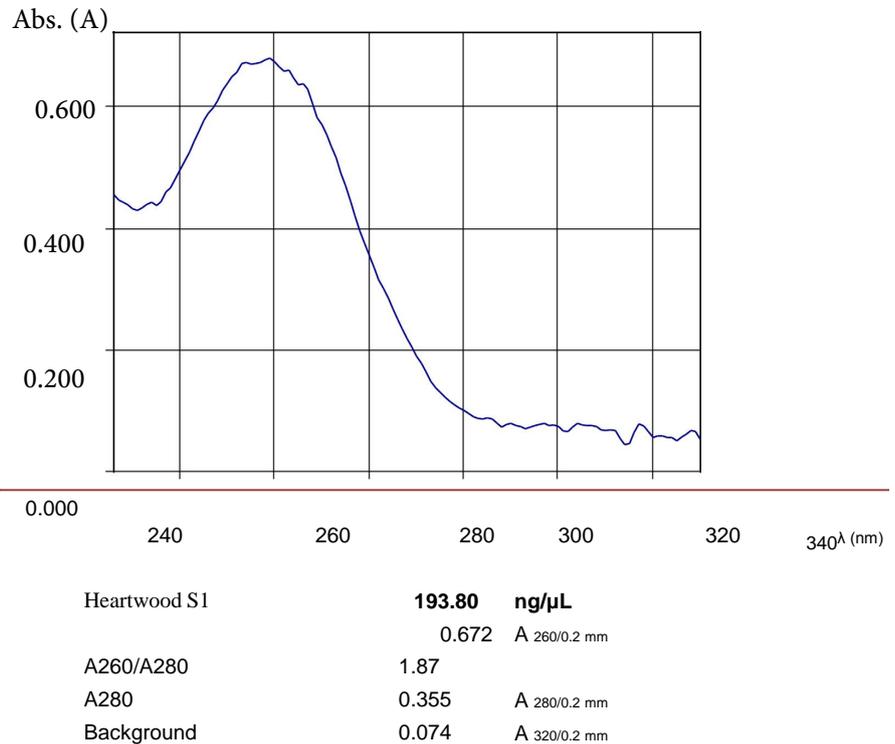


Figure 2. Nanodrop measurement profile of wood genomic DNA extractions from *D. latifolia* heartwood samples.

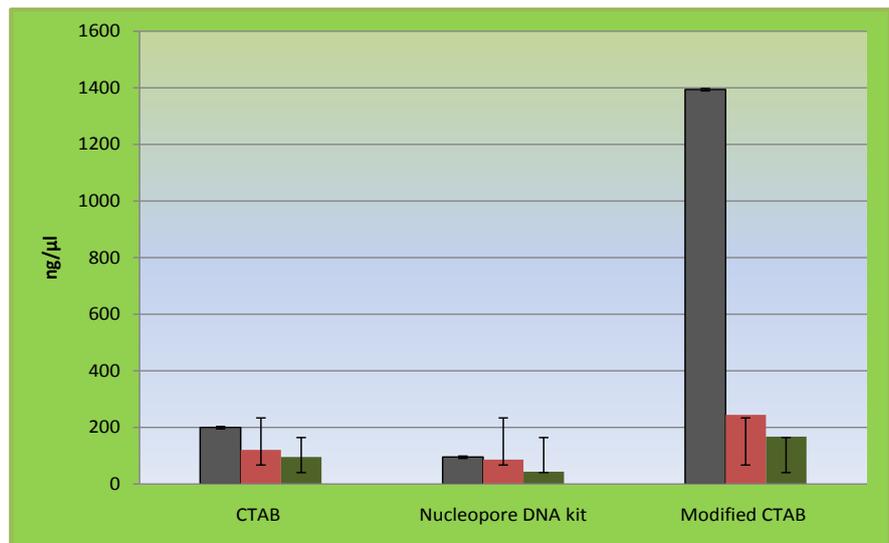


Figure 3. Comparison of the quantity of DNA (ng/mg) extracted from different plant tissues (Dried mature leaf, sapwood and heartwood) using the CTAB, Nucleopore DNA extraction kits, and modified CTAB DNA extraction protocol.

few samples it was some not clear bands. **Figure 4(e)** was presenting developed method *i.e.*, protocol 3 DNA bands and the same obtained DNA amplification through ISSR primers were in **Figure 4(f)**. DNA quality was always validated by its amplification through PCR with desirable primers. So to conclude the stan-

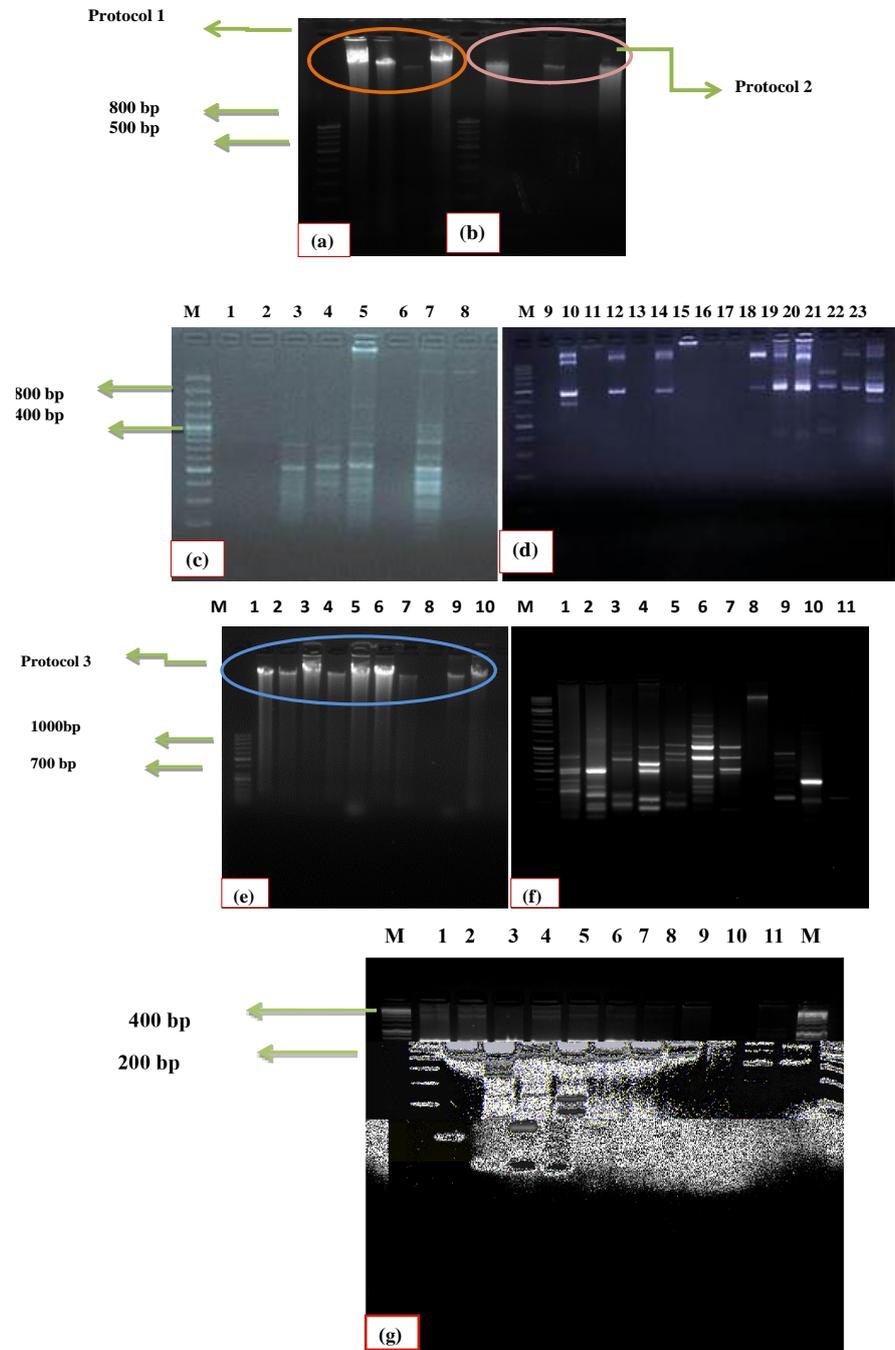


Figure 4. (a) Gel image of the genomic wood DNA of studied core samples isolated by Protocol 1; (b) Gel image of the genomic wood DNA of studied core samples isolated by Protocol 2,3; (c) ISSR bands obtained from DNA extracted from protocol 1 with UBC834M. Ladder 100 bp. (Bangalore genie); (d) ISSR bands obtained from DNA extracted from protocol 2,3 with UBC834M. Ladder 100 bp (Bangalore genie); (e) Gel image of the genomic wood DNA of studied core samples isolated by optimized protocol M. Ladder 100 bp (Bangalore genie); (f) ISSR bands obtained from DNA extracted from optimized protocol with UBC834M. Ladder 100 bp (Bangalore genie); (g) SSR primer amplification DNA fragment with RbcL. M. Ladder 50 bp (Bangalore genie) 1) Leaf 2) Sapwood 3) Heartwood (*T. grandis*), 4) Leaf 5) Sapwood 6) Heartwood (*D. latifolia*) 7) Leaf 8) Sapwood 9) Heartwood (*L. lanceolata*) 10) Negative control (DNA dilution 1:10).

standard protocol validation, the extracted DNA was amplified with universal SSR primers and the result was positively clear bands.

4. Discussion

DNA extraction from wood is difficult due to presence of hard tissues with high quantity of cellulose, hemicellulose and polyphenoliclignin compounds. These contents inhibits the DNA isolation and modification in the standardized protocol include svarious conc. CTAB, PVPP, β -mercaptoethanol, incubation time, RNase treatment and Proteinase treatment. By taking into consideration the important factors such as quantity, quality and suitability for PCR by using ISSR and SSR primers as well as the required time duration to extract DNA, among four methods the modified protocol of CTAB was found best protocol. In order to extract the DNA from wood, key footstep was to loosen the wood cell wall by placing it into water for three days without any fungal contamination. Due to hard nature of wood cell wall and there may be incomplete breakdown of the cell wall to release the cellular constituents, the penetration of water in the wood cells plays important role in sample preparation of DNA extraction. Result of the gel electrophoresis and spectrophotometer showed that the DNA purity was enhanced as well as polysaccharides and protein contaminations were removed by applying the modified protocol. The absence of RNA, polysaccharides and the amplification of desired primers (ISSR, SSR) with clear bands on 1.5% agarose gel were noticeable of a superior feature of DNA.

5. Conclusion

The present analysis clearly established the need for different tree species of appropriate DNA isolation methods for timber species. A single method may not be appropriate for extraction of DNA with good quantity and purity from all species but this method could be executed as standard method for isolation of wood DNA from *D. latifolia*, *L. lanceolata* and *T. grandisor* similar perennial timber species containing rich polysaccharides and defined here is hasty, uncertain and steady permitting the handling of large number of trials with easy routine. Previous studies [24] relying on relatively fresh samples but by using this developed protocol we could able to isolate pure DNA from mature dried wood samples of timbers or perennial tree species. Through **Figure 4** the final validation of qualitative DNA was proved that this protocol would be useful for each species of wood and mature dried or even disintegrating leaves of timber species. The isolated DNA as a result of using standardized protocol of various wood samples (fresh, dried, old and mature cores) was tested in PCR amplification for ISSR and ISSR profiling with selected primers to validate the protocol.

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