Optimization of Total Genomic DNA Extraction from Fresh and Mature Leaves of Eighteen Tropical Forest Tree Species without treatment of Proteinase K

Tanzeem Fatima* and Sreedevi C.N.
Tree Improvement and Genetics Division Institute of Wood Science and Technology, Bangalore, 560003, INDIA
*tanzeem.fatima@gmail.com

Abstract
Extraction of genomic DNA is necessary for many applications in molecular genetics and it is difficult in forest tree species due to presence of high amount of secondary metabolites polyphenols, poly-terpenes in mature forest trees than the annual crops, shrubs and flowering plants. DNA isolation from the routine protocol is quite expensive due to use of highly priced chemicals such as proteinase K used as a protein degradable enzyme.

In this study, we optimized the modified DNA extraction protocol developed for eighteen forest tree species (mature dried and fresh leaf tissues), Santalum album, Melia dubia, Bambusa balcooa, Tectona grandis, Dalbargia latifolia, Pterocarpus santalinus, Aquilaria malaccensis, Nothapodytes nimmoniana, Pongamia pinnata, Terminalia catappa, Swietinia macrophylla, Bambusa vulgaris, Bambusa Guadua, Diospyrous ebenum, Bamboosa brandisii, Madhuca indica, Garcinia indica and Garcinia gummigatta without proteinase K enzyme. DNA purified with this modified method resulted in the DNA extraction at much reduced cost. DNA obtained by this method was found an average of 19.47 µg /sample of DNA for selected species with a purity level of 1.87. The extracted DNA from all the selected species was successfully amplified using microsatellite markers (rbcL and Actin), confirming the absence of hidden and strong proteins as PCR inhibitors. This alternative method for isolating DNA from different tree leaf samples would facilitate cost effective molecular work and therefore is more economical when compared to other protocols.

Keywords: Enzymes, Forest tree species, Genomic DNA, Proteinase K, PCR inhibitors

Introduction

Forest trees require more effort than other annual crops, shrubs and plants to isolate high quality of DNA for molecular genetic studies. Previous reported standard methodologies suitable for other plants may not be necessary acquiescent to trees DNA extraction[24]. Mostly forest tree species are containing large number of secondary metabolites which is a challenging task as most of these compounds precipitate with DNA and inhibit further down-streaming utilization. Most genetic diversity study, genetic linkage mapping, marker-assisted selection, population and genetic studies need high quality of DNA from forest trees[4].

DNA isolation is the routine protocol and it is quite expensive due to highly priced chemicals such as proteinase K which is used as a protein degradable enzyme.

In this study we explained the optimization of cost effective DNA extraction method developed for various forest timber species (Mature dried or fresh leaves) *Santalum album (*Sandalwood*), Melia dubia* (Malabar neem), *Bambusa balcooa* (Plain bamboo), *Tectona grandis* (Teak), *Dalbargia latifolia* (Rosewood), *Pterocarpus santalinus* (Red Sanders), *Aquilaria malaccensis* (Agarwood), *Nothapodytes nimmoniana* (Mappia), *Pongamia pinnata* (Indian beech tree), *Terminalia catappa* (Leadwood), *Swietinia macrophylla* (Mahogany), *Bambusa vulgaris* (Clumping bamboo), *Guadua angustifolia* (Clumping bamboo), *Diospyros ebenum* (Ebony), *Bambusa brandisii* (Velvet leaf bamboo), *Madhuca indica* (Mahua), *Garcinia indica* (Kokum) and *Garcinia gummi-gatta* (Malabar tamarind). Several methods have been standardized for isolation of total genomic DNA from trees and plants.[2,4,7-9,11,12,14,19,22,24,26]

The previous reported standard protocols to isolate DNA are quite costly due to the use of highly prized chemicals, so that was not economically effective. The important modification in the optimized protocol included no use of proteinase K enzyme, less quantity of RNase, incubation time and inversion time of the C:I wash (15-20 min). The absence of proteinase K, DNA extraction cost would reduce and would be economically effective.

In the selected forest trees, the standardized CTAB method produced moderate yields and consistent quality of DNA. The yield and quality of DNA were assessed by UV spectrophotometry and gel electrophoresis method. Result showed that the modified protocol could be used successfully as template in PCR amplification reactions with microsatellite molecular markers rbcL and actin.

Therefore, confirming the absence of hidden and strong proteins as PCR inhibitors, this alternative method for isolating DNA using different tree leaf samples facilitates molecular work with mature samples in a much-reduced cost.

Material and Methods

(A) Sample collection: Fresh and mature dried leaves of *S. album, M. dubia, B. balcooa, T. grandis, D. latifolia, P. santalinus, A. malaccensis, N. nimmoniana, P. pinnata, T. catappa, S. macrophylla, B. vulgaris, G. aungustifolia, D. ebenum, B. brandisii, M. indica, G. indica* and *G. gummigatta* were collected from all the selected tree species. A minimum of two replicates was taken for each species.

(B) Genomic DNA isolation:
Solutions
(i) Suspension buffer: 0.5 M Sucrose, NaCl, Tris-HCl, EDTA and 2% β-mercaptoethanol [22 and 20].
(ii) Extraction buffer: 3.5% CTAB, 5M NaCl, 1M Tris-HCl, 0.5M EDTA and 0.5% β-mercaptoethanol.[8]

Method
The optimized protocol for DNA isolation was divided into three major steps:

a. Suspension buffer treatment: This step included pre-heated suspension buffer treatment and incubation at 65 °C for 15- 30 min.

Step I: Grind 500 mg of leaf samples and 50 mg. PVPP into fine powder using liquid nitrogen. Transfer the powder to 15 mL test tubes containing 5 mL pre-warmed suspension buffer and add 100 µL β-mercaptoethanol in each tube. Vortex the tubes vigorously and incubate it at 65 °C pre-heated water bath for 20 min followed with periodic inversion. Centrifuge the tubes at 10,000 rpm for 10 min at 4 °C temperature in high-speed refrigerated centrifuge (KUBOTA).

b. Extraction buffer treatment: This included pre-heated extraction buffer and β-mercaptoethanol followed by incubation at 65 °C for 1 h. This step also involves C:I washing and RNAse treatment.

Step II: Transfer the supernatant in fresh autoclaved tubes and add pre-warmed extraction buffer and add 50 µL β-mercaptoethanol in each tube. Keep the tubes at 65 °C pre-heated water bath for 1 h with periodic inversion. After incubation, add equal volume of C: I (24:1) (v/v) and gently invert each tube for 20 min and centrifuge it for 10 min at 12,000 rpm at 4 °C. Repeat this step 2-3 times. Transfer the aqueous upper layer to the fresh tubes and add 10 µL RNase (Sigma Aldrich) (10 mg/mL) in each tube followed by incubation in dry bath at 37 °C. After incubation, add equal volume of C:I, 24:1 (v/v) with very gentle inversion for 15 min and centrifuge the tubes at 12,000 rpm for 12 min at 4 °C. Transfer the final supernatant to fresh autoclaved 15 mL tubes and add equal volume of chilled isopropanol followed by overnight incubation at -20 °C.

c. Elution of DNA: This step is final stage of DNA isolation. It includes DNA pelleting and washing it by 70% ethanol followed by dissolving it in TE buffer.

Step III: Centrifuge the tubes at 12,000 rpm for 15 min at 4 °C. Discard the supernatant and transfer the pellet into 1.5 mL reaction vials and wash it twice with 1 mL 70% ethanol followed by spinning for 5 min at 10,000 rpm. The pellet was allowed to dry at room temperature until the ethanol smells evaporate. Re-suspend the pellet in 70-100 µL TE buffer (8.0 pH) and store the DNA at -20 °C.

DNA Quantification: The yield of extracted DNA was quantified by nano drop at 260/280 nm wavelengths in Eppendorf spectrophotometer[23]. The purity of DNA was checked by running the samples on 0.8% agarose gel in 1X TAE buffer stain containing 4 µL of 0.3% ethidium bromide solution in gel documentation unit (Syngene G: box).

DNA quality validation through PCR amplification by microsatellite markers (rbcL and Actin): Universal microsatellite markers rbcL and actin[8, 9](60-60.3°C) were used to validate the quality of yielded DNA in this study. DNA amplification was carried out in 13 µL reaction volume containing genomic DNA 1.5 µL (45 ng/µL), 2 µL (10 picomole/ µL) primers, 1.5 µL PCR buffer, 1.5 µL (10 mM) dNTPs, 1.5 µL $MgCl_2$, 0.2 µL (3U/µL) Taq polymerase (Bangalore genie) and 4.2 µL double distilled water to retain the volume.

Amplification cycle consists of an initial 3 min denaturation at 94 °C, 30 cycles for 30 seconds at 50 °C, primer annealing at 55–65 °C 1 min 72 °C and final extension step for 10 min at 72 °C[10]. The amplified product 2 µL loaded with 4 µL loading buffer (Bromophenol blue) was size fractionated by electrophoresis on 2% agarose gel with 0.2% ethidium bromide and visualized on UV transilluminator (Gel documentation Syngene G: box) to obtain the bands and to validate the DNA quality suitability for PCR reactions.

Results

The optimized DNA isolation method is simple and very low cost due to absence of proteinase K and low volume of RNAse enzymes compared to other protocols, which are significantly expensive. The result obtained in fig. 1 shows thick layer of impurities of carbohydrates and proteins after 1st and 2nd wash by C:I (Fig. 1A and B). The results revealed that the modified method could yield highly pure DNA, which was transparent non-sticky pellet of DNA (Fig. 1C).

The concentration of DNA yielded 7.94 ± 33.54 µg/sample with the average of 19.47 µg and the purity at 260/280 1.79±1.87 (Table 1). The result of 0.8% agarose gel electrophoresis (Fig 2A and 2B) showed that the standardized protocol for selected species enhanced the DNA quality and quantity by removing proteins, carbohydrates and RNA impurities Lane 1. *Santalum album*-31 *Pterocarpus santalinus*. DNA from fresh and dried leaf samples of selected eighteen forest trees was successfully amplified by RbcL and Actin SSR primers and visulaised in 2% agrose gel. Lane: (1) *Santalum album* - (30) *Bambusa bambos* (Fig. 3A and 3B).

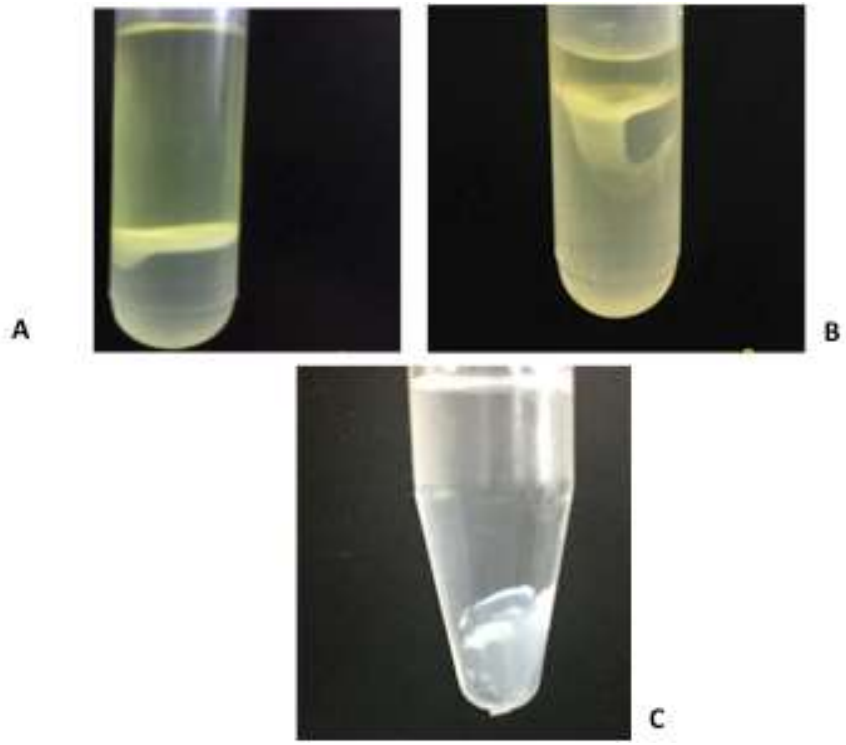

Fig. 1: Represented reaction tubes illustrating the addition of C:I followed by centrifugation showing a thick band of contamination between supernatant and the C: I separated by this modified method. (A) First C: I treatment (B) Second C:I treatment with inversion (C) The tube contains samples extracted using our standard protocol Pellet of genomic DNA from samples lyophilized and treated with PVP showing a clean and transparent

Table 1
DNA extraction without proteinase modified CTAB method-ratio and yield of selected tree species.
***- fresh leaves, **- dried leaves**

S.N.	Tree species	Quantity µg/ µL	Purity 260/280
1.	*Santalum album**	33.54	1.82
	*S. album***	28.27	1.79
2.	*Melia dubia**	24.23	1.80
	*M. dubia***	24.55	1.82
3.	*Bambusa. balcooa **	23.38	1.75
	*B. balcooa **	16.97	1.79
4.	*Dalbergia latifolia **	17.78	1.79
	*D. latifolia **	17.53	1.79
5.	*Tectona grandis **	16.86	2.06
	*T. grandis **	18.26	1.89
6.	*Diospyros ebenum **	7.94	2.03
7.	*Dendrocalamus brandissii **	13.44	1.93
8.	*Aquilaria malaccensis **	17.51	1.92
9.	*Pongamia pinnata **	19.61	1.80
10.	*Bambusa vulgaris **	16.11	1.98
11.	*Nothapodytes nimmoniana **	13.27	1.95
12.	*Pterocarpus santalinus**	13.39	1.93
13.	*Madhuca indica**	19.63	1.79
14.	*Guadua aungustifolia**	17.71	1.89
15.	*Terminalia catappa**	18.21	1.89
16.	*Swietinia macrophylla**	19.64	2.02
17.	*Garcinia Indica**	26.30	1.92
18.	*Garcinia gummigatta**	26.78	1.84
Average		19.47	1.87

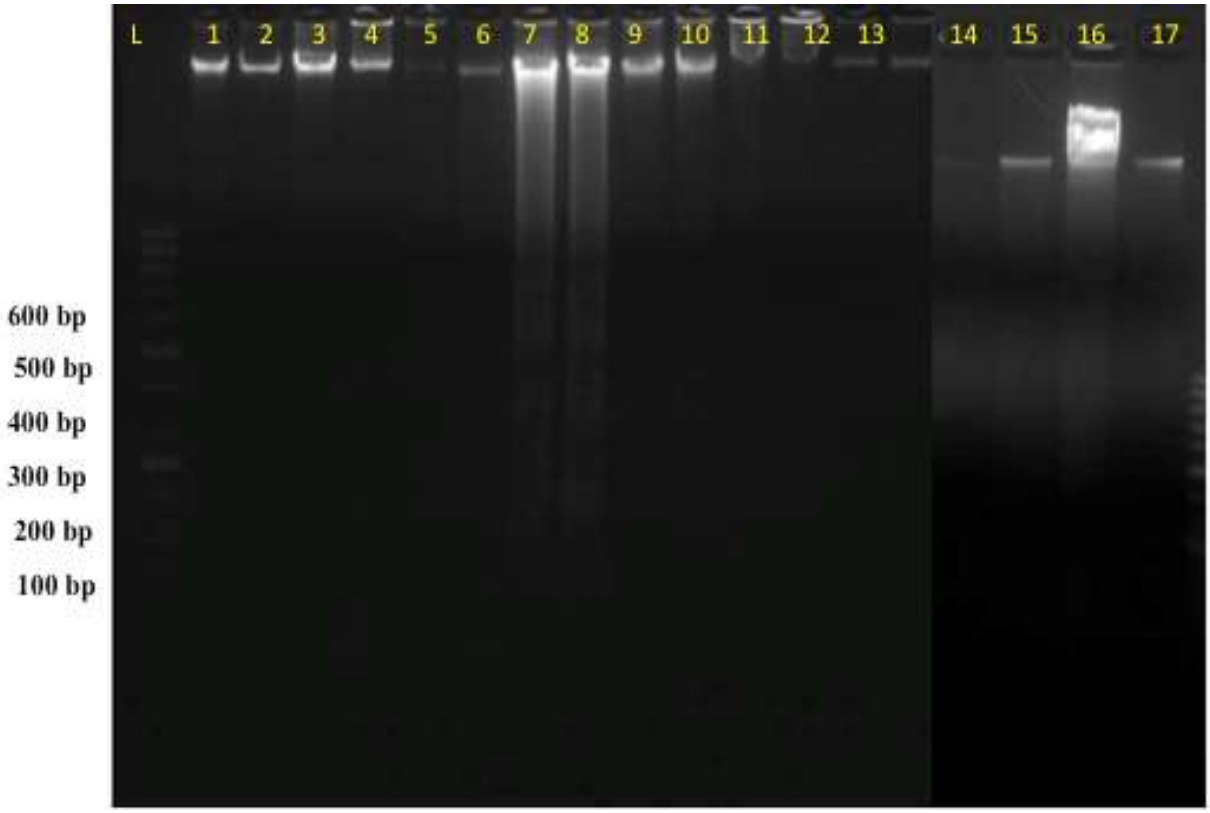

Fig. 2A: Genomic DNA isolated from selected forest tree species: L- Ladder 100 bp plus ladder. 4 ng of genomic DNA (Lane:1-17) from each species was separated on 0.8% Agarose gel in 1X TAE buffer with 2 µL (0.5 µg/mL) ethidium bromide. 1. *S. album* (FL) 2. *S. album* (DL) 3. *M. dubia* (FL) 4. *M. dubia* (FL) 5. *M. dubia* (DL) 6. *M. dubia* (DL) 7. *B. bambus* (FL) 8. *B. bambus* (DL) 9. *D. latifolia* (FL) 10. *D. latifolia* (FL) 11. *D. latifolia* (DL) 12. *D. latifolia* (DL) 13. *T. grandis* (FL) 14. *T. grandis* (DL) 15. *S. album* old Sapwood 16. *S. album S*apwood 17. *S. album* old Sapwood *FL- fresh leaves, **DL- dry

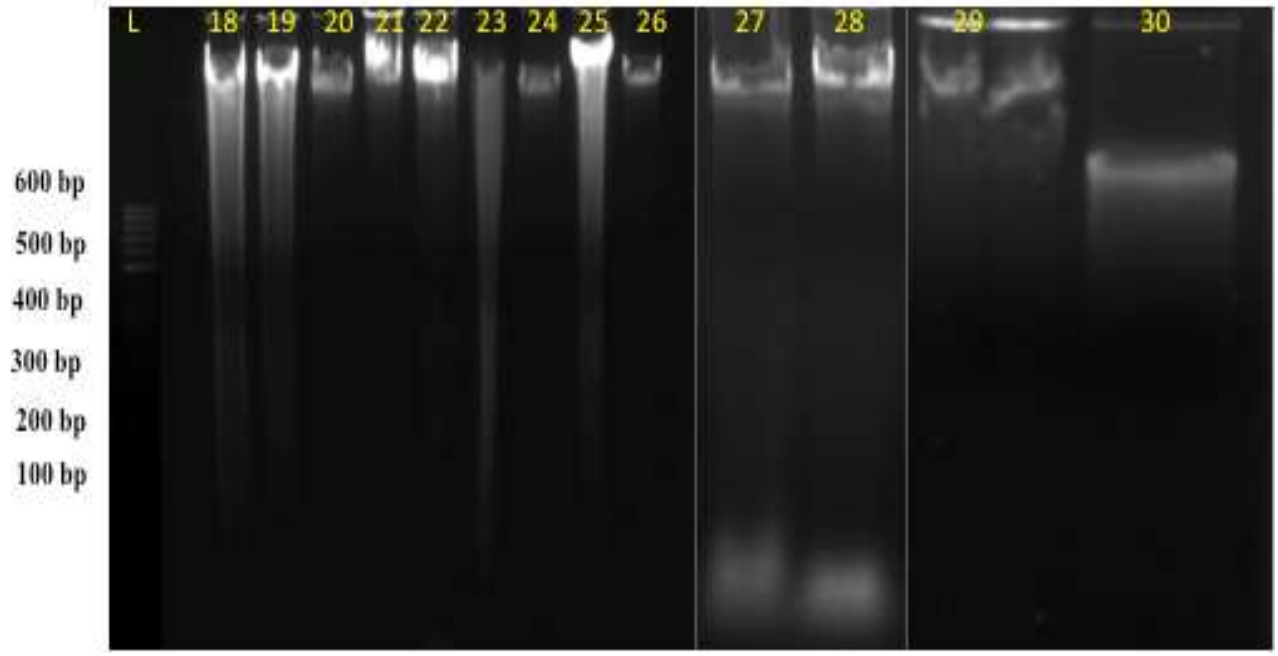

Fig. 2B: Genomic DNA isolated from selected forest tree species: A. L- Ladder 100 bp plus ladder. 4 ng of genomic DNA (Lane:18-31) from each species was separated on 0.8% Agarose gel in 1X TAE buffer with 2 µL (0.5 µg/mL) ethidium bromide. 18. *D. ebenum* 19. *D. brandissii* 20. *A. malaccensis* 21. *P. pinnata* 22. *B. vulgaris* 23. *N. nimmoniana* 24. *P. santalinus* 25. *M. indica* 26. *G. aungustifolia.* 27. *T. catappa* 28. *Garcinia indica* 29. *Garcinia gummigatta* 30. *M indica* 31. *Pterocarpus santalinus*

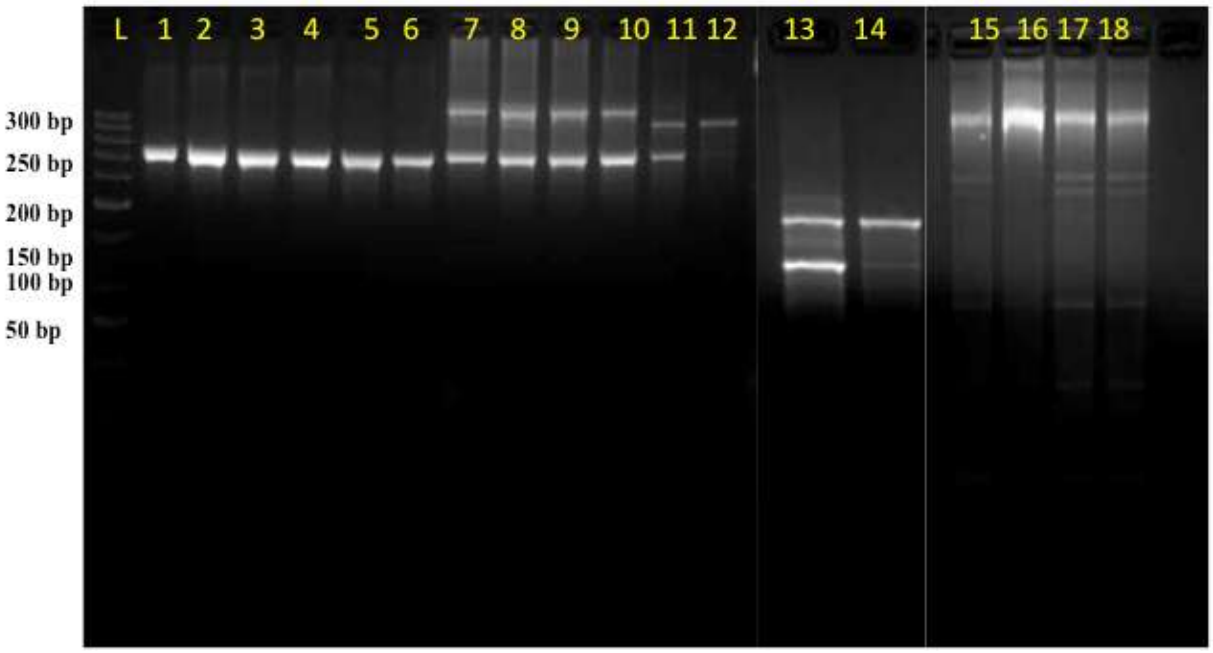

Fig. 3A: Gel of PCR amplification from microsatellite loci presented with DNA template extracted by the current optomized protocol (Lane:1-18) of genomic DNA extracted from selected tree species by using the microsatellite primers (rbcL and Actin). L-Ladder 50 bp Gene ladder 1. *S. album* (FL) rbcL 2. *S. album* (FL) rbcL 3. *S. album* dried leaves rbcL 4. *S. album* (DL) rbcL 5. *M. dubia* (FL) rbcL 6. *M. dubia* (DL) rbcL 7. *S. album* (FL) Actin 8. *S. album* (FL) Actin 9. *S. album* (DL) Actin 10. *S. album* (DL) Actin 11. *M. dubia* (FL) Actin 12. *M. dubia* (DL) Actin 13. *B. bambus* (DL) rbcL 14. *B. bambus* (DL) actin 15. *T. grandis* (DL) rbcL 16. *T. grandis* (DL) Actin 17. *S. album* sapwood rbcL 18. *S. album* Actin * FL-fresh leaves, **DL-dried leaves

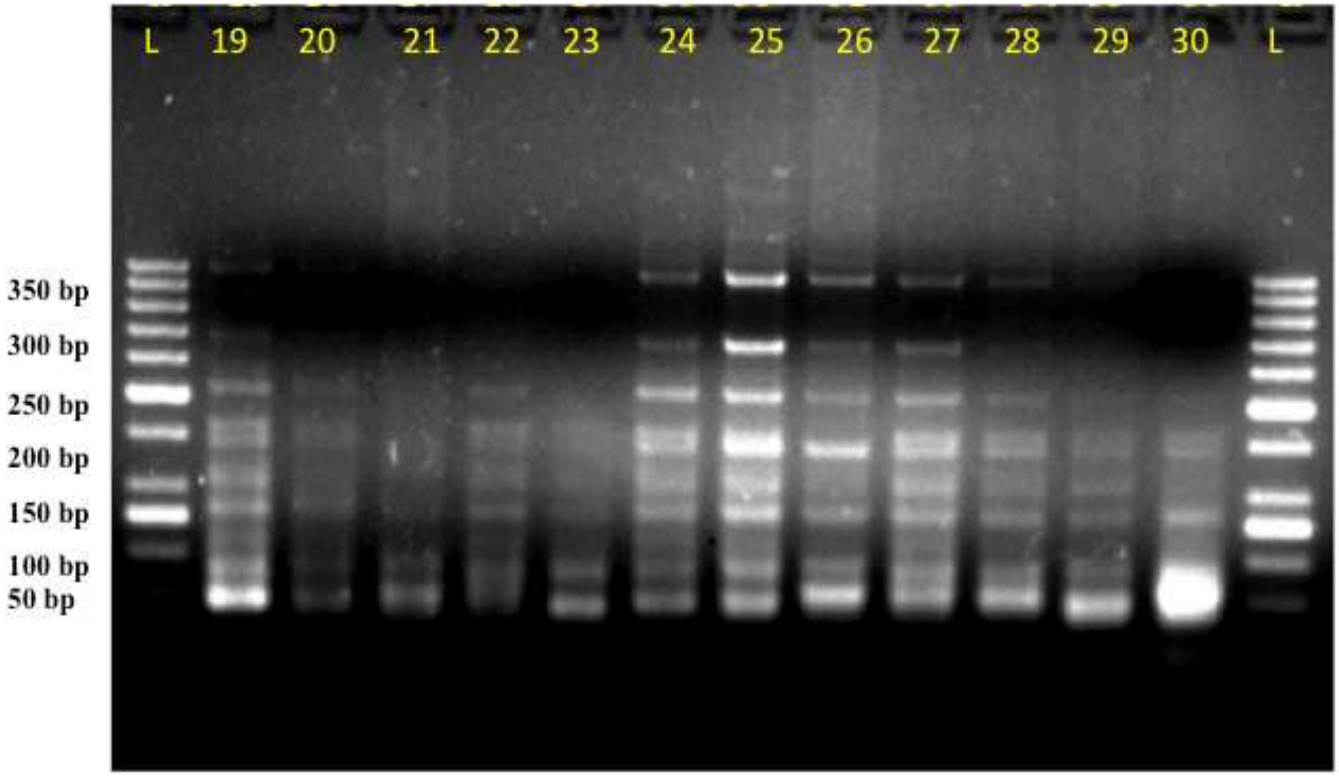

Fig. 3B: Gel of PCR amplification from microsatellite loci presented with DNA template extracted by the current optomized protocol (Lane:19-30) of genomic DNA extracted from selected tree species by using the microsatellite primers (rbcL and Actin). L-Ladder 50 bp Gene ladder 19. *D. latifolia* dried leaves rbcL 20. *D. latifolia* (FL) rbcL 21. *T. grandis* (FL) rbcL 22. *S. album* dried leaves rbcl 23. *S. album* sapwood sample rbcL 24. *B. bambos* (FL) rbcL 25. *D. latifolia* (DL) Actin 26. *D. latifola* (FL) Actin 27. *T. grandis* (FL) Actin 28. *S. album* (DL) Actin 29. *S. album* sapwood Actin 30. *B. bambos* (FL) Actin

Discussion

This study aims to extract DNA from many forest tree samples with the DNA quality to be high enough to allow for PCR based analysis such as ISSR, RAPD and SSR based genetic diversity, barcoding and association mapping study. A major limitation of applying molecular techniques in forest tree species is the lack of easy and low-cost quality DNA extraction and analysis. The significant modifications incorporated in the protocol were as follows: extended time duration during C:I wash (minimum 15 min), 5% PVPP and no use of proteinase K enzyme. By expending this protocol, it has been revealed that the proteins and RNA contaminations could have been precipitated during C: I treatment with a long trembling process and keeping the tubes at 37 ^{O}C for 1 h.

Borges et al[5]extracted DNA from leaves of *Melanoxylon brauna* and yielded 36.89 ng/µL DNA (purity ratio of 1.80) by using proteinase K. The range of DNA yield isolated by using standardized protocol from fresh and dried samples of *Aquillaria malaccensis* was 68.70- 723 ng/µL[2]. Mekonnen et al [17]optimized DNA extraction without liquid nitrogen and without proteinase K and the DNA purity at A260/280 ratio of 1.8. Similarly, in our study DNA yield was 7.94 ± 33.54 µg/sample with the average of 19.47 and the purity at 260/280 1.79 ±1.87 (Table 1). Latif and Osman[16] compared three DNA extraction methods with proteinase K enzyme. The overall DNA yield was in a range 100 to 200 ng/100 mg material whereas, in our study the yield of DNA was high (>200 ng).

Xia et al[27] isolated DNA by using modified SDS-based method from raw soybean with the purity ratio was 1.862-1.954. Bhandari et al[3] isolated genomic DNA from dried leaves of Taxus sps with the purity range 1.70 to 2.25. Ki et al[15] isolated genomic DNA from various plant tissues without using proteinase K and the yield of DNA were 14.00±4.30 to 6.60±2.20 µg/sample. Sambrook et al[23]extracted high quality DNA from different plant and tree species and 47.0±20.0 to 1.6±0.7 µg of high quality DNA was obtained. Aydin et al[1] isolated genomic DNA by using optimized method without enzyme (Proteinase K and RNAse) from the cottonseed varieties and the DNA yield ranged from 5.72 to 23.76 µg with the mean value of 11.26 µg/sample.

Mahuku[18] optimized protocol for bean leaves and the DNA extraction DNA concentration ranged from 20-50 µg/250 µg samples. The DNA concentration of *Pinus radiata* (buds, cambium and leaves) ranged from 8.58±15.29 µg by using 200 µL protein precipitation solution[26]. Extraction methods significantly induced DNA quantity from the tree species investigated. In the current study, the result obtained (Fig. 3A and 3B) with different samples from different species by PCR, were visibly exhibiting the successfully amplified bands by SSR (microsatellites) primers. Similarly, Sahu et al[22] and Davi et al[6] optimized DNA extraction protocol for plants containing high secondary metabolites ad polysaccharides, the DNA concentration ranged from 8.80-9.90 µg/µL and clear banding patterns were observed with successfully amplified barcode genes (rbcL and MatK).

Silva and Ribeiro[25] extracted DNA from *Bambusa vulgaris* and *Guadua angustifolia* and yielded 487.50 ng/µL of DNA with the purity level of 1.91 followed by evaluation of the amplification efficiency of the matK and rbcL regions of the DNA. The DNA extracted by this method yielded reproducible and clear amplified bands resulting in its suitability for PCR applications. This modified protocol would be useful in other plant species DNA extraction process at very reduced cost.

Conclusion

The optimized method developed to improve genomic DNA extraction from mature and dried leaf tissues of forest tree species is economically cost effective over other protocols as it does not require highly prized chemicals. Using this protocol, high quantity and pure DNA was extracted from 18 different forest tree species that was effectively amplified by microsatellite markers despite presence of high amount of phenolic compounds in the leaf samples.

The modification explains without proteinase enzyme protocol, provides the less expensive and high qualities DNA from mature dried or fresh samples in productive way for further studies of genetic diversity, DNA fingerprinting and association mapping and markers studies.

Acknowledgement

Authors are thankful to the Director, IWST, Group Co-Ordinator Research, Head- Genetics and Tree Improvement Division, Institute of Wood Science and Technology.

References

1. Aydin A., Ince A.G., Gocer E.U. and Karaca M., Single Cottonseed DNA extraction without the use of enzymes and liquid nitrogen, *Fresenius Environmental Bulletin*, **27(10),** 6722-6726 **(2018)**

2. Azhari M.H.N. and Azhar M., Crucial optimization steps in getting premier quality of *Aqularia malaccensis* genomic DNA for molecular activities, Conference: Nuclear Technology Convention, Bangi, Malaysia, 1-9 **(2013)**

3**.** Bhandari A.N., Lamichhane S. and Shakya P., An Efficient Method for Isolation of Genomic DNA from Silica Gel Dried Leaves of *Taxus sps*, *International Journal of Biosciences,* **5(5),** 147-152 **(2014)**

4. Bashalkhanov S. and Rajora O.P., Protocol: A high-throughput DNA extraction system suitable for conifers, *Plant Methods,* **4(20)**, 1-6 **(2008)**

5. Borges D.B., Amorim M.B., Waldschmidt A.M., Neto E.M., Vivas C.V. and Pereira D.G., Optimization of DNA extraction from fresh leaf tissues of Melanoxylon brauna (Fabaceae), *Genetics and Molecular Research,* **11(2),** 1586-1591 **(2012)**

6. Devi D.K., Punyarani K., Singh N.D. and Devi H.S., An efficient protocol for total DNA extraction from the members of order Zingiberales- suitable for diverse based downstream applications, *Springer Plus,* **2(669)**, 1-9 **(2013)**

7. Doyle J.J. and Doyle J.L., A rapid DNA isolation procedure for small quantities of fresh leaf tissue, *Phytochemistry Bulletin*, **19** 11-15 **(1987)**

8. Fatima T., Srivastava A., Hanur V.S. and Rao M.S., An Effective Wood DNA Extraction Protocol for Three Economic Important Timber Species of India, *American Journal of Plant Sciences,* **9**, 139-149 **(2018a)**

9. Fatima T., Srivastava A., Somashekar P.V. and Hanur V.S., Optimization of genomic DNA extraction routine from Rosewood (*Dalbergia latifolia*) and Ben Teak (*Lagerstroemia lanceolata*), *International Journal of Genetics,* **10(6),** 446-451 **(2018b)**

10. Fatima T., Srivastava A., Somashekar P.V., Vageeshbabu H.S., Rao M.S. and Bisht S.S., Assessment of morphological and genetic variability through genic microsatellite markers for essential oil in Sandalwood (*Santalum album* L.), *3 Biotech,* **9(252)**, 1-16 **(2019c)**

11. Ginwal H.S. and Maurya S.S., Evaluation and optimization of DNA extraction method for *Dalbergia sissoo* leaf, *Indian Journal of Biotechnology,* **9**, 69-73 **(2010)**

12. Healey A., Furtado A., Cooper T. and Henry R.J., Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species, *Plant Methods,* **10(21)**, 1-8 **(2014)**

13. Inglis P.W., Pappas M.D.C.R., Resende L.V. and Grattapaglia D., Fast and inexpensive protocols for consistent extraction of high quality DNA and RNA from challenging plant and fungal samples for high throughput SNP genotyping and sequencing applications, *Plos One,* **13(10),** e0206085 **(2018)**

14. Kumar A., Gayakwad A., Panse U., Khasdeo K., Narayan C., Ansari S.A. and Lazarus A.D., Optimization of DNA extraction methods for some important forest tree species, *International Journal of Advanced Biotechnology and Research,* **4(3),** 364-371 **(2013)**

15. Ki J.S., Chang K.B., Roh H.J., Lee B.Y., Yoon J.Y. and Jang G.Y., Direct DNA Isolation from Solid Biological Sources without Pretreatments with Proteinase-K and/or Homogenization through Automated DNA Extraction, *Journal of Bioscience and Bioengineering*, **103(3)**, 242–246 **(2007)**

16. Latif A.A. and Osman G., Comparison of three genomic DNA extraction methods to obtain high DNA quality for maize, *Plant Methods,* **13(1)**, 1-9 **(2017)**

17. Mekonnen T., Sethelly K., Sharma M., Tesfaye K. and Kaul T., Genomic DNA isolation method from fresh wheat leaf samples without liquid nitrogen, *African Journal of Biotechnology,* **16(20),** 1192-1196 **(2017)**

18. Mahuku G.S., A simple extraction method suitable for PCR based analysis of Plant, Fungal and Bacterial DNA, *Plant Molecular Biology Reporter,* **22,** 71-81 **(2004)**

19. Porebski S., Bailey L.G. and Baum B.R., Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components, *Plant Molecular Biology Reporter,* **1**, 8-15 **(1997)**

20. Rawat S., Joshi G., Annapurna D., Arun Kumar A.N. and Nataraja K.N., Standardization of DNA Extraction Method from Mature Dried Leaves and ISSR-PCR Conditions for *Melia dubia* Cav-A Fast Growing Multipurpose Tree Species, *American Journal of Plant Sciences,* **7,** 437-445 **(2016)**

21. Sane A., Kareem V.K.A., Rajasekharan P.E. and Kumar R.T., Optimization of DNA isolation ad PCR protocol for ISSR analysis of *Nothapodytes nimmoniana*: A threatened Anti-cancerous medicinal plant, *Asian Journal of Biotechnology,* **4(2),** 100-107 **(2012)**

22. Sahu S.K., Thangaraj M. and Kathiresan K., DNA Extraction Protocol for Plants with High Levels of Secondary Metabolites and Polysaccharides without Using Liquid Nitrogen and Phenol, *International Scholarly Research Notices,* **205049(6)**, 1-6 **(2012)**

23. Sambrook J., Fritseh E.F. and Maniatis T., Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY **(1989)**

24. Shepherd M., Cross M.J., Rhonda L.S., Scott L.J. and Jones M.E., High-throughput DNA extraction from forest trees, *Plant Molecular Biology Reports*, **20(4),** 425 **(2002)**

25. Silva R.M. and Ribeiro N.P., Establishment of a genomic DNA extraction protocol for bamboo species, *Revista de Ciências Agrárias*, **41(3),** 825-831 **(2018)**

26. Tibbits J.F.G., Mcmanus L.J., Spokevicius A.V. and Bossinger G.A., rapid method for tissue collection and high throughput isolation of genomic DNA from mature trees, *Plant Molecular Biology Reports,* **24**, 81–91 **(2006)**

27. Xia Y., Chen F., Du Y., Liu C., Bu G., Xin Y. and Liu B.A., modified SDS based DNA extraction method from raw soybean, *Bioscience Reports,* **39(2),** BSR20182271 **(2019)**.

(Received 30th April 2020, accepted 09th July 2020)