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Genetic Diversity Analysis by SSR Markers in *Melia dubia* Cav. -A Fast Growing Multipurpose Tree Species

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ABSTRACT

The present work was conducted in the year 2019 at the department of Forestry and Environmental Science, GKVK, University of Agricultural Sciences, Bengaluru Karnataka, India aimed to estimate the genetic variation among the populations of *Melia dubia*. 12 SSR primers producing polymorphic and monomorphic bands were analysed. The fresh and matured leaf sample was collected from the South India germplasm bank of *Melia dubia* established and maintained by Karnataka Forest Department (KFD) in collaboration with Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore at Hoskote near Bengaluru, is a part of rural Bengaluru district, Karnataka, India. Leaf surface was cleaned by wiping with alcohol and carried to the laboratory in the ice bucket. Samples were preserved at -86°C in a deep freezer. Genetic distances were calculated for all the accession lines studied by SSR PCR. The polymorphic information content (PIC) varied from 0.18–0.35, with an overall mean of 0.28. Nei's gene diversity (H) ranged from 0.22–0.45, with an overall mean of 0.34. Alleles maker^{-1} ranged between 1 and 4, with an average of 2.7 alleles marker^{-1}. Genetic distances were used to generate a dendrogram . An UPGMA dendrogram based on similarity coefficient indices revealed high genetic diversity and segregate into thirteen distinct clusters. This can be attributed to highly cross-pollinating nature of the species. and small distributional range in the area. Based on the genetic variability found, tree improvement strategies could be developed for conservation and further improvement of the species.

KEYWORDS: Heat ambience, sheep, water-deficit markers and hormone

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1. INTRODUCTION

Melia dubia Cav. synonym *Melia composita*, (Family-Meliaceae), is a large deciduous, fast growing tree species, commonly known as Malabar Neem. It is an indigenous multipurpose tree species, naturally distributed in Sikkim Himalayas, North Bengal, upper Assam, Khasi hills, Deccan and Western Ghats at altitudes of 1500–1800 m (Parthiban et al., 2009, Kumar et al., 2017, Ram et al., 2014, Thakur et al., 2018). This species is an excellent raw material for wood-based industries like paper and plywood due to its increased pulp recovery and strength (Neginhal, 2004, Suprapti et al., 2004). *M. dubia* has medicinal properties and the extract from different parts of the plant has antiviral activity (Vijayan et al., 2004, Goswami et al., 2020) and reported anticancer properties of *Melia dubia* plant extract (Anusuya et al., 2009). The leaf oil is reported to exhibit bacteriostatic, fungistatic (Nagalakshmi et al., 2003) and anti-feedant activity (Byeong et al., 2000). Its fruits are used in folk medicine as an antihelminth, astringent and in the treatment of colic. Paste made out of the green fruits is used in treatment of scabies and maggotinfested sores (Susheela et al., 2008). It is an ideal species for plywood and pulpwood industry apart from being extensively used in afforestation (Parthiban et al., 2009, Goswami et al., 2020, Prasad et al., 2009, Mohapatra et al., 2021). Considering its fast-growing ability and multipurpose uses, fruit pulp used as livestock feed (Parmar et al., 2018, Sukhadiya et al., 2020), it is also accepted as an ideal agroforestry species (Mohanty et al., 2017). Despite its large-scale cultivation and its importance, there is no information available on the extent of genetic diversity existing in this species.

The basic pre-requisite for genetic improvement and protection of genetic resource is the study of genetic variability. The species is reported to be predominantly self-fertilized (Johar et al., 2015). In order to identify the level of diversity, marker systems have almost become indispensable. The use of morphological markers for identification of elite genotypes has not been of much success in trees as visual inspection may not really be a reliable method. DNA markers, on the other hand, are highly reliable and most preferred choice for germplasm characterization (Kant et al., 2006). According to this technique is usually used to show the level of DNA variability among species and also among individuals within species which are closely related, as well as being able to detect the presence of variation of nucleotide arrangement within DNA (Saiki et al., 1988). The most crucial step for any molecular study is isolation of pure, intact and high-quality DNA (Tan and Yiap, 2009). However, due to excessive presence of secondary metabolites, isolating pure DNA from plants is very difficult as compared to animals and microorganisms. Various factors are responsible for degrading DNA during extraction. One such problem is the presence of endonucleases which directly or indirectly interferes with enzymatic reactions (Weising et al., 2005). Presence of polysaccharides makes DNA more viscous, inhibits Taq polymerase activity and restriction enzyme activity. Another major problem encountered with most of the fully developed and mature leaves is accumulation of polyphenolics and tannins. These, when in oxidized, form covalently bind with DNA ,make it resistant to restriction enzymes and give DNA a brown colour. Various methods have been developed for plants in order to encounter all these problems and isolate high-quality DNA (Dilworth and Frey, 2000). Documentation of genetic variation facilitate in carrying out programmes aimed at its improvement, conservation, sustainable management and utilisation. In this background present study is an effort to reconcile the molecular information of germ plasm lines of *Melia dubia*.

2. MATERIALS AND METHODS

2.1. Description of the study area

The study area is Hoskote, located at about 30 km away from Bengaluru is a part of rural Bengaluru district, Karnataka, India. It lies between 13°52'N latitude and 77°52'E longitude and characterized by undulating topography. The low-lying valleys and depressions are intensely cultivated. The climate is semi-arid (dry) with summer temperature reaching 31°C during May and the winter temperature is around 19°C during December–January. The relative humidity is around 67%. The study area receives an average rainfall of 650 mm. The experimental material composed of 42 germplasm lines of *Melia dubia* are primarily selected from low rainfall and dry conditions of South India and a plantation is established during late 2013. The experiment was conducted under rainfed conditions in Randomized complete block design (RCBD) with 4 replications planted at 4×5 m^2 spacing.

2.2. Sample collection

Young emerging leaves were collected in zip lock polythene covers after cleaning the surface with alcohol and brought to the lab in ice bucket and stored in a deep freezer at -80°C. The list of *Melia dubia* germplasm lines selected for characterizing for genetic diversity is given in Table 1.

2.3. DNA isolation

DNA was isolated following the protocol (Hua et al., 2006). Leaf sample (400 mg) was ground to a fine powder in a ceramic mortar and pestle using liquid nitrogen with 25 mg of PVPP. The powdered leaf tissues were immediately transferred to a 20 ml centrifuge tube containing 4 ml of pre-warmed extraction buffer-I (0.5 M Sucrose, 120 mM TrisHCl, 50 mM EDTA and 1.7 M NaCl) and 100 µl of β-mercaptoethanol was added to it and vortexed well. Freshly prepared extraction buffer-I was used. Tubes were

Table 1: List of lines used in the study

Sl. No.	Germplasm Accession codes	Sl. No.	Germplasm Accession codes
1.	25	22.	267
2.	MD111	23.	159
3.	MD058	24.	115
4.	94	25.	268
5.	53	26.	260
6.	64	27.	261
7.	257	28.	24
8.	241	29.	259
9.	128	30.	69
10.	20	31.	32
11.	271	32.	75
12.	MD112	33.	28
13.	MD118	34.	76
14.	MD117	35.	195
15.	MD115	36.	104
16.	MD120	37.	265
17.	MD121	38.	262
18.	MD122	39.	270
19.	263	40.	114
20.	MD126	41.	233
21.	MD123	42.	MD013

incubated at 65°C for 60 m and centrifuged at 10,000 rpm for 10 m and supernatant was transferred to a new tube.

Pre-warmed 4 ml extraction buffer-II (100 mM TrisHCl, 20 mM EDTA, 1.7 M NaCl, 2% CTAB) was added to the supernatant and tubes were kept incubated for 30 m at 65°C. After incubation an equal volume of chloroform: isoamylalcohol (24:1) was added and mixed vigorously by inverting 15–20 times. Samples were centrifuged at 12,000 rpm for 10 m. Aqueous phase was transferred to a new tube. 25 µl of RNase (20 mg ml^{-1}) was added to the tubes and incubated at 37 °C on dry bath for 1 h. 20 µl Proteinase K (20 mg ml^{-1}) treatment was also given simultaneously. Chloroform: Isoamylalcohol separations were done until clear solution was obtained. Clear supernatant was taken and 1/4th volume of 5 M NaCl and equal volume of chilled isopropanol was added and gently inverted for several times and kept at -20 °C overnight. Next day samples were centrifuged at 12,000 rpm for 10 m. DNA pellet was given 70% (v/v) ethanol wash 3 times, then air dried at room temperature for 2–3 h and dissolved in 50–100 µl of TE buffer (10 mM TrisHCl and 1mM EDTA pH 8.0) and stored at -20 °C for further use.

2.4. Quantitative and qualitative analysis of isolated DNA

The purified DNA was separated on 0.8% (w/v) agarose gel prepared using 1X TAE buffer and visualized on Gel Documentation system (Herolab, Germany). DNA was quantified using Bio-spectrophotometer (Eppendorf, Germany) at a wavelength of 260 and 280 nm. Purity was checked from ratio of absorbance 260:280 (Sambrook et al., 2001).

2.5. DNA Amplification

A total of 12 SSR primers from Life Technologies Corporation, USA (Table 2) were used for PCR amplification. PCR amplifications were performed using 96 well thermal cycler (Quanta biotech, Model S20143) using 25 µl reaction mixture, varying concentration of DNA (10–70 ng), dNTPs (100–300 µM), Taq polymerase (0.5–2.5 U) and $MgCl_2$ (0.0–3.5 mM) were tried in DNA thermocycler (Eppendorf mastercycler gradient, Germany). Different concentrations of agarose gel (1.5–3.0%) were also tried. The PCR condition was set at initial denaturation of 94 °C for 3 minutes, followed by 94 °C for 30 s, specific annealing temperature of primer for 30 s, elongation for 1 m at 72 °C and the cycle was repeated 39 times and a final extension step at 72 °C for 10 m and a hold temperature of 4 °C at the end. The PCR product was analyzed on 2% (w/v) agarose gel and visualized under gel documentation system (Herolab, Germany).

2.6. Band Scoring and Data Analysis

The frequency of SSR polymorphism between 42 genotypes of *Melia dubia* was calculated based on the presence of band '1' or absence of band '0'. The allele sizes were approximated by comparing the observed band with the DNA molecular weight ladder. Heterozygosity and polymorphic information content (PIC) were assessed from the polymorphic information. UPGMA-based analysis was carried out to estimate the molecular diversity using Nie's coefficient and the genetic distance between the teak clones using the software Darwin (version 5). Other diversity estimates such as proportion of heterozygous individuals in the Population (Heterozygosity), Gene diversity and Polymorphism information content were also estimated (Bostein et al., 1980).

3. RESULTS AND DISCUSSION

This study focused towards genetic diversity and relationships among *Melia dubia* germplasms lines is determined using with the use of 12 SSR primers with reproducible PCR bands. Since *Melia dubia* is an out-breeding tree species, it is most likely that the genomes may have significant heterozygosity at several loci. Hence, the use of co-dominant markers systems such as SSR is most crucial for assessing genetic diversity in *this species*..

Table 2: Details of SSR primers were used in present study

Sl. No.	Primer		Sequence	Annealing temp
1	Md5	Forward	GCGGTGGACTTGACTTTACC	55.0
		Reverse	ACCCGAAAGTGCATACGATG	
2	Md4	Forward	CCCGTTGCTGGAGAAGAAAA	58.9
		Reverse	GGTAAAGTCAAGTCCACCGC	
3	Md3	Forward	ACTTGAAGGGGAAAGGGACA	54.1
		Reverse	TACCTTCGCGAGCAAGATCT	
4	Md2	Forward	TGGCTTCAAAAGATACGCCTC	58.9
		Reverse	TCGGCTTACTGATGGGATGC	
5	Md1	Forward	TCAACTCCTTCGCTACTGGG	59.4
		Reverse	GCGTATCTTTTGAAGCCAGAATG	
6	MAC63	Forward	TCATTTGGAATCTGGGTTCT	55.2
		Reverse	TTATTTCGTACCCTTCGCTC	
7	C25	Forward	GTGACAGAGCGAGATTCCAT	53.2
		Reverse	CCCCTTCAGCTCCAAAATGT	
8	ccmp3	Forward	CAGACCAAAAGCTGACATAG	51.9
		Reverse	GTTTCATTCGGCTCCTTTAT	
9	ccmp4	Forward	AATGCTGAATCGAYGACCTA	49.5
		Reverse	CCAAAATATTBGGAGGACTCT	
10	ccmp6	Forward	CGATGCATATGTAGAAAGCC	52.2
		Reverse	CATTACGTGCGACTATCTCC	
11	MAC69	Forward	ATAAGCCAGATGACGGAACG	57.3
		Reverse	GCATACGGCTTTCTGGATGT	
12	sm07	Forward	GATAGCGGAGCCGGTGATT	57.2
		Reverse	GGATGGAAGGCTCAAGATTCG	

The gel profiles illustrated in Plate 1 was considered to fix appropriate annealing temperature. Molecular size of the amplified product was estimated using a 1 kb ladder. Allele scoring and sizing was done using the ladder as a standard. The representative gel showing polymorphisms of genomic SSR among germplasm lines are depicted in Plate 2.

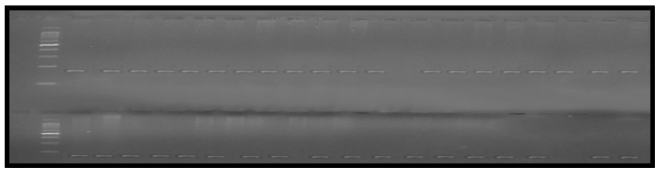

Plate 1: Gel profile depicting annealing temperature standardization of SSR primers

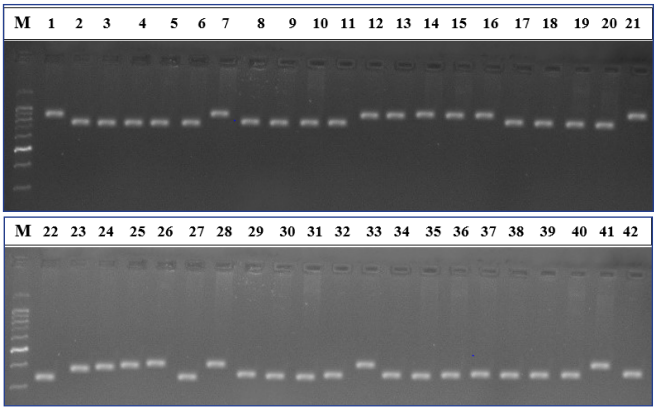

Plate 2: Gel electrophoresis pattern of amplified among 42 *M. dubia* genotypes using primer ccmp3; M – 1kb ladder, 1-267, 2-MD122, 3-MD115, 4-257, 5-128, 6-268, 7-114, 8-241, 9-MD117, 10-20, 11-159, 12-104, 13-75, 14-MD126, 15–195, 16-MD121, 17-25, 18-MD111, 19-233, 20-259, 21-MD120, 22-69, 23-MD123, 24-MD058, 25-115, 26-262, 27-263, 28-265, 29-64, 30-260, 31-261, 32-24, 33-32, 34-270, 35-28, 36–MD112, 37-76, 38-MD013, 39-MD118, 40-53, 41-271, 42-94

Gene diversity or expected heterozygosity is considered as an indication of genetic diversity among the population studied. The values of gene diversity ranged from 0.22–0.45 among the 42 lines studied. The mean Gene diversity from all the markers was 0.34 (Table 3). The markers revealed

Table 3: Details of genetic information obtained from SSR markers

Primers	Major allele frequency	Allele number	Gene diversity	Hetero-zygosity	PIC
Md5	0.64	2.00	0.44	0.44	0.34
Md4	0.83	4.00	0.22	0.44	0.18
Md2	0.75	3.00	0.31	0.68	0.31
Md1	0.80	3.00	0.31	0.46	0.26
C25	0.82	2.00	0.26	0.29	0.25
ccmp3	0.66	2.00	0.45	0.44	0.35
ccmp4	0.74	3.00	0.37	0.56	0.30
ccmp6	0.73	3.00	0.36	0.54	0.28
MAC69	0.73	3.00	0.39	0.58	0.31
sm07	0.81	2.00	0.30	0.29	0.25
Mean	0.75	2.70	0.34	0.47	0.28

an average of 2.7 alleles marker^{-1} at a given SSR locus and it ranged between 1–4. Major allele frequency was quite high with a mean of 0.75 ranging between 0.64–0.83. Polymorphism information content (PIC) of each locus was analyzed to determine the extent of diversity revealed by these markers. PIC value is a measure of similarity among the lines. The PIC values ranged from 0.18 in marker M02 to as high as 0.35 for the marker M06, with a mean of 0.28. The genetic similarity matrix generated from the 10 primer combinations was used to perform cluster analysis using the UPGMA clustering method following the Sequential Agglomerative Hierarchical Nested (SAHN) cluster analysis module of NTSYS (Rohlf, 2005). Dendrogram developed from the above information revealed high genetic diversity among the lines (Plate 3). The lines segregated into three main genetically distinct clusters. The germplasm lines were segregated into 13 clusters at 0.68 Jaccard's similarity coefficient. Cluster XIII was the largest that comprised of 12 germplasms. Cluster III had 5 germplasms. Cluster XII had 4 germplasms, Cluster V, VII and VIII had 3 germplasm each. The Clusters I, IV, VI, IX and XI had 2 genotypes each whereas the clusters II and X were solitary clusters. The clustering of genotypes for SSR profile is shown in Plate 3.

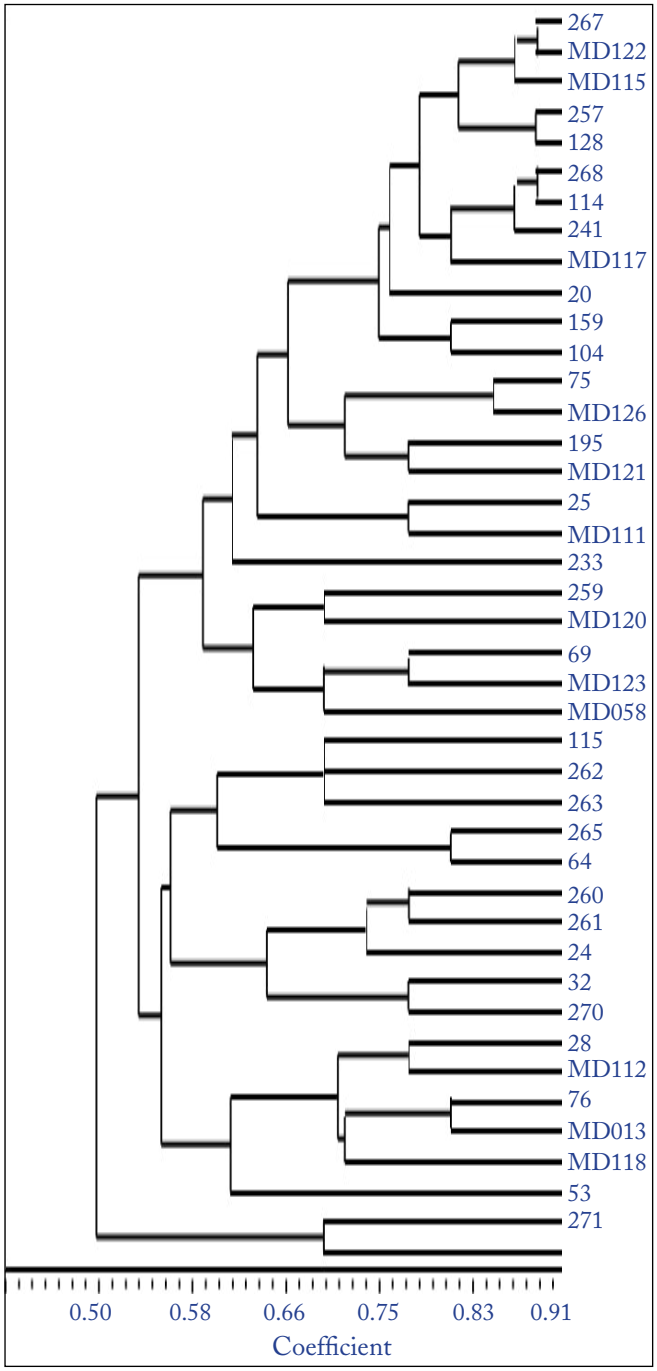

Plate 3: Dendrogram *Melia dubia* lines from SSR primers

In the present study, a total of 12 SSRs primers were initially screened for amplification, out of which, 10 positively amplified in *Melia dubia* and the other two were monomorphic. PIC ranged from 0.18–0.35 with an average of 0.28 primer pair^{-1}. Whenever SSRs are compared to other marker systems, it always revealed highest levels of polymorphism (Powell et al., 1996). The highest value of PIC was observed for the primer pair ccmp3. The average PIC 0.28 primer pair^{-1} observed in the present investigation was comparable to that reported earlier in *Dalbergia sissoo* (Shivani et al., 2019) using SSR markers. The utility of a given marker system was a balance between the level of polymorphism detected (information content) and the extent to which an assay could identify multiple polymorphisms.

Similar study in *Melia* composite was carried out and reported that the range of PIC value for SSRs ranged from 0.12–0.38 (Sharma et al., 2015). SSR markers present

a unique combination of several important advantages, such as co-dominant inheritance, abundance and uniform dispersion in plant genomes, high reproducibility, high polymorphism, enormous extent of allelic diversity and ease of assessing size variation by polymerase chain reaction with pairs of flanking primers (Weising et al., 2005). SSR markers have been extensively used for genetic diversity analyses in tree species such as *Eugenia dysenterica* (Zucchi et al., 2003) and *Populus* (Chen and Harrington, 2006, Tuskan et al., 2004). In Meliaceae many microsatellites have been developed to analyse level of polymorphisms in different species. Several factors dictate the level of polymorphism to be expected from different types of SSRs. In general, SSRs with many repeats have been shown to be more polymorphic than one with few repeats. Twelve microsatellites were developed and characterized for big leaf mahogany to enhance set of informative molecular markers available for application on population genetics, conservation and timber tracking in *Swietenia macrophylla* (Lemes et al., 2011). Eleven microsatellites were identified in *Swietenia humilis* for analyzing cross species amplification of these SSR loci in other species of Meliaceae (White and Powell, 1997).

4. CONCLUSION

Genetic diversity of *Melia dubia* lines with the use of microsatellite markers (SSR markers) were presented that forty-two lines segregated into thirteen different clusters of UPGMA dendrogram which indicates considerable high genetic diversity. This also indicated the possibility of gene transfer among the germplasm lines that are in close proximity and also suggest that, the lines of seedling origin which are collected from drier regions of Southern part of India under investigation, have reasonably high genetic diversity.

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