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Molecular Characterization and Phylogenetic Relationships of Dalbergia Species of Eastern India Based on RAPD and ISSR Analysis

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Abstract:- The genetic relationships among six species of Dalbergia (D. sissoo, D. latifolia, D. volubilis, D. rubiginosa, D. paniculata and D. lanceolaria) with twelve accessions were assessed using RAPD and ISSR markers. Fourteen RAPD and thirteen ISSR primers were used for estimation of genomic variability among the species and accessions studied. High degree of polymorphism was observed with most of the primers used. All the species and accessions were related to each other with an average similarity of 0.49. Highest similarity (0.93) was observed between two accessions of Dalbergia volubilis (DV1 and DV2) and lowest (0.34) between Dalbergia rubiginosa (DR) and Dalbergia volubilis (DV3). The genetic closeness of D. latifolia, D. sissoo and D. rubiginosa was observed which is in partial agreement with the infra-generic classification of the genus Dalbergia proposed by Baker (1876), who placed all the three species under the sub-genus Sissoa. However, the genetic similarity observed between D. volubilis and D. latifolia, belonging to two separate sub-genera on the basis of molecular studies, could not be explained. In order to derive phylogenetic relationships among different species of the genus Dalbergia, more number of representative species and additional molecular markers need to be studied.

Keywords:- *Molecular phylogeny, RAPD, ISSR, Dalbergia.*

I. INTRODUCTION

The genus *Dalbergia* is represented by about 250 species and maximum number of species are known to occue in Central and South America, Africa, Madagascar and Asia (Klitgaard and Lavin, 2005). Several species of *Dalbergia* such as *D. latifolia, D. lanceolaria, D. sissoo* are source of quality timber used in furniture making and boat building (Hiremath & Nagasampige, 2004a). As many as 50 species of the genus bear aeschynomenoid type root nodules and fix nitrogen (Sprent, 2009). Indiscrimate felling of forest trees for timber and firewood extraction in the tropical dry and moist deciduous forests of India has been the main cause of loss of biological diversity of commercially important tree species including that of *Dalbergia* species.

The infra-generic classification and phylogeny of *Dalbergia* has been dealt in a number of taxonomic revisions, regional floras and inventories based morphological characters (Bentham, 1860; Carvalho, 1997; Chen *et al.*, 2010; Niyomdham, 2002; Prain, 1904; Sunarno and Ohashi, 1997; Thothathri, 1987). Bentham (1860) in his infra-generic classification of Dalbergia placed all the 64 known species of *Dalbergia* under six series (Triptolemea Americanae, Triptolemea, Sissoae Americanae, Sissoae Gerontogee, Dalbergariae and Selenolobia). The South East Asian species of *Dalbergia* were classified under two subgenera, five sections and 24 series (Prain, 1904). In an attempt to group morphologically allied species based on androecium and fruit characteristics, Thothathri (1987) put the forty six *Dalbergia* species then known to occur in the Indian subcontinent in four sections and seven series. Presently, the genus *Dalbergia* is recognized as having five sections defined by inflorescence and fruit characters such as: Sect. *Dalbergia, Triptolemea, Selenolobium, Pseudecastaphyllum* and *Ecastaphyllum* (Carvalho, 1997).

The intra-specific genetic variability of some species of *Dalbergia* has been assessed using RAPD and ISSR markers in several parts of the world (Hussain *et al.*, 2012; Kumar *et al.*, 2011; Amri *et al.*, 2009; Phong *et al.*, 2011; Wang *et al.*,2011; Ashraf *et al.*, 2010; Hien & Phong, 2012; Juchum *et al.*, 2007). Besides, the genetic variability and population genetics of many species have been assessed using markers other than RAPD and ISSR (Vatanparast et al., 2013; Pandey *et al.*, 2004; Ribeiro *et al.*, 2007; Andrianoelina *et al.*, 2009).

Dalbergia species exhibit a wide range of morphological variations and some of them have specific ecological and habitat preference. These attributes pose problems in placing the New World and the Old World species into natural groups (Bentham, 1860; Carvalho, 1989). With regard to the molecular systematic of the genus *Dalbergia*, scany information is available in published literature. Vatanparast et al. (2013) derived the molecular phylogeny of *Dalbergia* species and advocated the monophyletic origin of the genus. For Indian species of *Dalbergia*, very few studies have been undertaken till date (Hiremath & Nagasampige, 2004b; Mohana et al., 2001; Rout et al., 2003; Arif et al., 2009; Bakshi & Sharma, 2011; Bhagwat et al., 2015), making it imperative to conduct studies on genetic diversity and phylogeny of the genus occurring in Eastern Ghat region of India.

With a view to understand the genomic variability and molecular phylogeny of the genus *Dalbergia* occurring in Eastern India, molecular characterization of 12 accessions of six species of *Dalbergia* (*D. sissoo, D. latifolia, D. volubilis,*

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D. rubiginosa, *D. paniculata*, and *D. lanceolaria*) were made using RAPD and ISSR markers in the present study.

II. MATERIALS AND METHODS

A. Plant materials

Leaf samples of 12 individuals/ accessions belonging to six species of *Dalbergia* were collected from different forest areas of Odisha, Andhra Pradesh, West Bengal and also from the arboretum of Regional Plant Resource Centre (RPRC), Bhubaneswar. The accession number, locality of collection and abbreviation used for each taxon is shown in Table-1. The young tender leaves were used for genomic DNA extraction for molecular analysis.

Sl. No.	Samples collection sites	Species	Code used in text, tables and figures
1	Ghatikia, Bhubaneswar, Odisha	*Dalbergia sissoo*	DS1
2	Tirupati hills, Andhra Pradesh	*D. sissoo*	DS2
3	Barbara, Khurda Forest Division, Odisha	*D.latifolia*	DL1
4	Dhuanali, Khurda Forest Division, Odisha	*D. latifolia*	DL2
5	Paderu Hills, Vizag, Andhra Pradesh	*D. volubilis*	DV1
6	RPRC, Bhubaneswar, Odisha	*D. volubilis*	DV2
7	Barbara, Khurda Forest Division, Odisha	*D. volubilis*	DV3
8	Barunei hills, Khurda, Odisha	*D. volubilis*	DV4
9	RPRC, Bhubaneswar, Odisha	*D. volubilis*	DV5
10	Khandagiri, Bhubaneswar, Odisha	*D. rubiginosa*	DR1
11	Indian Botanic Garden, Howrah, West Bengal	*D. lanceolaria*	DLN
12	RPRC, Bhubaneswar, Odisha	*D. paniculata*	DP

Table 1. Details of plant samples used for study of genetic diversity and phylogeny

B. Genomic DNA extraction

Genomic DNA was extracted from the leaf tissues using the modified CTAB (cetyl-trimethyl-ammonium-bromide) proto- col (Doyle and Doyle, 1990). Two grams of leaf tissues from tender parts were ground with grinding buffer composed of 100 mM sodium acetate (pH 4.8), 500 mM NaCl, 50 mM EDTA (pH 8.0), 50 mM Tris (pH 8.0), 2% Polyvinyl pyrollidone (PVP) and 2% CTAB. Purification of DNA was done twice with extraction of phenol:chloroform: Isoamyl alcohol (25:24:1). RNAse @ 40 µl from 1 mg/ ml was applied in the supernatant to get rid of RNA. The quality and quantity of DNA were checked through 0.8% agarose electrophoresis with standard DNA before PCR amplification.

C. RAPD and ISSR analyses

Thirty RAPD and 30 ISSR primers (Operon Technologies, Alameda, USA) were used for PCR analysis based upon their performance and reproducibility. Among them, 27 primers showed distinct polymorphism. PCR mixture of 25 µl contained 25 ng of genomic DNA template, 0.6 µq of Taq DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 µM of decamer primers, 2.5 µl of 10 x PCR assay buffer (50 mM KCI, 10 mM Tris-Cl), 1.5 m $MgCl_2$) and 0.25 µl of pooled dNTPs. The PCR condition used for RAPD was: Initial denaturing step at 94°C for 5 minutes followed by 42 cycles of 94°C for 1 minute, 37°C for 1 minute and 72°C for 2 minute, the last cycle, primer extension at 72°C for 7 minutes. For ISSR amplification, the PCR condition was: Initial denaturing step at 94°C for 5 minutes followed by 42 cycles of 94°C for 1 minute, 45° - 55°C for 1 minute and 72°C for 2 minute, the last cycle, primer extension at 72°C for 7 minutes. The amplified products as developed by the primers were separated by agarose (1.5%) gel electrophoresis and documented in gel documentation system (Bio Rad XR, Biorad, USA). O'Gene Ruler™ 100 bp DNA Ladder plus (ladder range 3000 bp to 100 bp from Fermentas Life Sciences, USA) was used as molecular weight marker. Bands were scored for its presence/absence (1/0) for each primer genotypes combination. Software NTSYS-pc, version 2.1 (Rohlf, 2000) was used for estimation of genetic relatedness among the genotypes using Jaccard's similarity coefficient and clustering was done with UPGMA (unweighted pair group method using arithmetic averages).

III. RESULTS

A. RAPD analysis

Fourteen RAPD primers reproduced well and resulted in amplification of distinct bands. The DNA profiles obtained from RAPD analysis are represented in Fig. 1. A total of 92 amplified loci were generated which include 86 polymorphic, 6 monomorphic and 30 unique ones (Table-2). The resolving power of primers ranged from 2.0(N4) to 10.75(A11), whereas the primer index varied from 0.22 (N18) to 4.78 (A11). The RAPD banding pattern revealed that primer A11 produced highest number of amplified loci 11, followed by N7 (No. of bands=10) whereas N4 and N18 amplified least number of loci (1 and 2 respectively). Nine of the fourteen primers produced 100% polymorphic bands, whereas least polymorphism was observed with N18 (50%).

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The primer A3 and A10 also showed high level of polymorphism (87.5%). The average amplified and polymorphic band per primer was 6.57 and 6.14 respectively. The overall percentage of polymorphic band was around 93.48%. Out of total 6 monomorphic bands generated; N16 amplified maximum no. of monomorphic loci (2). The primers D18, A9 and N7 produced 6, 4 and 4 unique loci respectively.

All the accessions were related to each other with an average similarity of 40% as could be obtained from Jaccard's similarity co-efficient analysis. Highest similarity (90%) was observed between *Dalbergia volubilis* (DV2) and *Dalbergia volubilis* (DV1) and lowest of 17% between *Dalbergia volubilis* (DV2) and *Dalbergia latifolia* (DL2).

On the basis of data obtained from RAPD analysis, a cladogram was constructed for the 8 accessions and 4 species of *Dalbergia* which separated them into two distinct clusters of 6 and 2 having a common node at 24.5% similarity level (Fig. 2). The larger cluster of 6 was subdivided into a cluster of a lone accession of *Dalbergia sissoo* and rest of the accessions of *Dalbergia volubilis* and *Dalbergia latifolia*. While one of the accessions of *Dalbergia latifolia* formed a cluster with the four accessions of *Dalbergia volubilis*; all the genotype of *Dalbergia volubilis* exhibited close relationship among themselves with varying levels of similarity.

B. ISSR analysis

The details of ISSR analysis of 12 accessions of 4 species of *Dalbergia* is presented in Table-3. Out of the 30 ISSR primers screened, only 13 primers produced good amplified products. Total number of loci generated was 95; out of which 86 were polymorphic, 9 monomorphic and 10 unique ones. The size of amplicons ranged from 100bp to 2000bp. The resolving power of primers ranges from 3.83 (Oligo 3b) to 13.83 [T(GA)9] and the primer index from 0.83 to 4.99 for Primers (AG)10 and T(GA)9 respectively. The ISSR banding pattern is shown in Fig. 3

The primer T(GA)9 produced highest number of amplified bands (13), whereas Oligo 2a and Oligo 3b amplified the least number of loci (4 each). Six primers namely (CT) 8A, Oligo2a, Olio 3b, T(GA)9, (GAC)5and (AG)8C showed 100% polymorphism but the polymorphism observed in case of primers (AG)10 was only 66.66%. The average no. of amplified and polymorphic bands per primer was 7.31 and 6.62 respectively. (GA)9T and $(AG)_{10}$ were responsible for amplification of maximum no. of monomorphic loci (2 each) and most of the primers (GTGC)4, yielded three unique loci during amplification. The base sequences of these 13 primers indicate presence of repeated di-nucleotides (AG)n, (GA)n, (CT)n, tetra-nucleotides (GACA)n. The rate of polymorphism is highly dependent on di-nucleotides and higher % of GA content than other primer repeats.The rate of polymorphism is highly dependent on di-nucleotides and higher % of GA content than other primer repeats.

From the Jaccard's similarity table, it could be inferred that all the accessions were related to each other with an average similarity of 52%. Highest similarity (0.95) was observed between two accessions of *Dalbergia volubilis* (DV1 and DV2) and lowest between *Dalbergia sissoo* (DS1) and *Dalbergia paniculata* (DP) having similarity of 0.24. The single accession of *Dalbergia paniculata* got separated in the dendrogram in the first pace with very distinct genetic resemblance (30%) similarity. The rest 11 accessions was divided into two clusters, the smallest group contains the single accessions of *Dalbergia rubiginosa* and *Dalbergia lanceolaria* at 44% level of similarity Fig. 4.

Dalbergia lanceolaria and Dalbergia rubiginosa also separated from each other in the dendrogram showing a genetic similarity of 46.5%. The bigger cluster of Dalbergia volubilis – Dalbergia latifolia and Dalbergia sissoo was further subdivided into two distinct clades at 52.6% level of similarity; one contain two genotypes of Dalbergia sissoo and the other with accessions of Dalbergia volubilis and Dalbergia latifolia. The two accessions of Dalbergia sissoo had a genetic similarity of about 70%. Of the 7 accessions of Dalbergia volubilis and Dalbergia latifolia, all the five accessions of Dalbergia volubilis and 2 accessions of Dalbergia latifolia got separated from each at a similarity level of 53%. Both the genotypes of Dalbergia latifolia exhibited about 86% similarity between them. Further, all the 5 accessions of Dalbergia volubilis came together but shared varying genetic similarity in the range of 76% to 86% among the accessions.

C. RAPD and ISSR combined markers

By analysing both RAPD and ISSR data, it was found that 14 RAPD and 13 ISSR primers produced good and reproducible amplification products. All the species and accessions were related to each other with an average similarity of 0.49. Highest similarity 0.93 was observed between two accessions of *Dalbergia volubilis* (DV1 and DV2) and lowest 0.34 between *Dalbergia rubiginosa* (DR) and *Dalbergia volubilis* (DV3).

The dendrogram (Fig.- 5) generated from these data segregated the 8 accessions to distinct cluster of 3 and 5 sharing a common node at 42.5% similarity level. The small clade included the lone accession of Dalbergia rubiginosa (DR) and two accessions of Dalbergia latifolia (DL1 & DL2) and had 45.5% similarity among them. Further, the two accessions of Dalbergia latifolia shared a node at the 67% level of similarity. The bigger clade contains 4 accessions of Dalbergia volubilis and one accession of Dalbergia sissoo having a genetic similarity of about 43%. The accession of Dalbergia volubilis formed a clear cluster with varying levels of similarity among them. At the first instance, accessions DV 1 and DV2 got separated from the other two accessions DV3 and DV4 sharing a common node at a genetic 77% level of closeness. While D. volubilis (DV3 and, DV4) had a genetic relatedness of 79.8% between them, the other two accessions of DV1 and DV2 shared similarity of about 92.5% between them.

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IV. DISCUSSION

Vatanparast *et al.* (2013) used ITS nuclear sequence data and interpreted the molecular phylogeny of 64 species of *Dalbergia* and compared with infrageneric classifications suggested earlier on the basis of morphological data. In this study, they included almost the representative species of the various subgenera, sections and series to make the classification relevant. The results of the study revealed that sect. Triptolemea, with cymose inflorescences and thin samaroid pods and sect. Ecastaphyllum, with racemose or paniculate inflorescences and orbicular to suborbicular fruits, are potentially monophyletic in origin. However, the species of the sections *Dalbergia* and *Selenolobium* were found to be non-monophyletic. These results are in agreement with the findings of Ribeiro *et al.* (2007), who on the basis of ITS and trnL sequence data suggested that types of inflorescence and fruit may serve as sources of synapomorphies for classifications of *Dalbergia* as opined earlier by Carvalho (1997). Among the Asian species of *Dalbergia*, the members of sect. *Dalbergaria* (Prain, 1904) are condidered as monophyletic in origin. With reflexed standard petals and stamens in two bundles of five each, the species of this section are distributed throughout Southeast Asia including India.

Baker (1876) classified the 28 species of *Dalbergia* them known from British India under three sub-families namely, Sissoa, Dalbergaria and Selenolobium. Of the species investigated in the present study, *D. sissoo, D. latifolia and D. rubiginosa* came under the sub-genus Sissoa; and *D. lanceolaria, D. volubilis and D. paniculata* under the sub-genus Dalbergaria. Asian species of *Dalbergia* were placed in four sections viz. Sect. Sissoa, Sect. *Dalbergia*, Sect. Selenolobia and Sect. Ecastaphylla (Thothathri 1987)

The dendrogram constructed on the basis of RAPD data placed one accession of D. latifolia and one of D. rubiginosa in a single clade justifying their inclusion under the sub-genus Sissoa but the second accession of D. latifolia and D. sissoo were remotely placed. Similarly, two genotypes of D. sissoo and two of D. latifolia, which are members of the sub-genus Sissoa, came together in a common clade in the tree constructed using ISSR data their genetic proximity. However, closeness of D. volubilis and D. latifolia belonging to two separate subfamilies could not be explained from taxonomic point of view. As expected, accessions of D. latifolia and D. rubiginosa belonging to the same sub-genus Sissoa formed a cluster in the dendrogram constructed using RAPD and ISSR data in combination. However, Hiremath & Nagasampige (2004) on the basis of RAPD analysis of 10 Indian species of Dalbergia kept D. latifolia distinctly separate from other species. He also found close genetic similarity among D. lanceolaria, D. volubilis, D. rubiginosa, D. paniculata and D. sissoo. In the present study, D. sissoo was found to form cluster with D. volubilis, which is in agreement with the above findings of Hiremath & Nagasampige (2004).

Based on 4C DNA content and chromosome characteristics, Hiremath & Nagasampige (2004) detected genetic resemblance between *D. latifolia and D. sissoo*. They postulated that the species differentiation in these closely related tree species, *D. sissoo*, *D. latifolia* and *D. sissoides* have occurred through small increase in genome size. Close genetic resemblance could also be seen in the present study using RPD and ISSR markers.

As remarked by Carvalho (1989), sect. *Dalbergia* is an assemblage of heterogeneous species with pyramidal panicle sometimes arranged in bracteate compound panicles and samaroid fruits. Although the results of this study are congruent with some of the traditionally recognized sections of *Dalbergia*, sampling is too limited to derive a conclusion on phylogeny of this big genus. The pantropical distribution of *Dalbergia*, with higher species concentration in Amazonia, Indo-Asiaand Madagascar, nesessitates long term study of molecular phylogeny involving as many species as possible to throw light on the intra-generic classifications proposed by Bentham (1860), Prain (1904) and Carvalho (1989).

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Primer name	Sequence	Range of amplicons	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of Unique bands	% Polymorohic bands	Resolving power	Primer index
A8	5'GTGACGTAGG 3'	500-1500	5	5	0	2	100	3.5	1.69
A9	5' GGGTAACGC C 3'	800-1845	6	6	0	4	100	3	1.81
A18	5' AGGTGACCG T 3'	600-1845	6	6	0	1	100	4.25	2.41
A3	5' AGT CAG CCA C 3'	350-1000	8	7	1	3	87.5	9.5	1.69
A10	5' GTGATCGCA G 3'	100-1185	8	7	1	2	87.5	8.25	2.41
A11	5' CAATCGCCGT3'	200-1845	11	11	0	0	100	10.75	4.78
A12	5'TCGGCG ATA G 3'	450-1950	7	7	0	3	100	4.75	2.34
C2	5' GTGAGGGGTC3'	100-800	5	5	0	2	100	3	1.88
D18	5' GAG AGCCAA C 3'	100-1950	8	8	0	6	100	3.25	2.28
D20	5' ACCCGGTCA C 3'	200-1500	8	8	0	1	100	6.25	3.03
N4	5' GACCGA CCC 3'	1185	1	0	1	0	0	2	0
N7	5' CAG CCC AGA G 3'	180-1450	10	10	0	4	100	5.5	3.25
N16	5' AGGCGACCT G 3'	180-1000	7	5	2	1	71.42	11	3.25
N18	5'GGTGAGGECA3'	1185-1500	2	1	1	1	50	2.25	0.22

Table 2. Details of RAPD primers and band details in species and accessions of *Dalbergia*

Primer name	Sequence	Range of amplicons	Total no. of bands	No. of poly-morphic bands	No. of mono-morphic bands	No. of unique bands	% poly-morohic bands	Resolving power	Primer index
(CT)8A	5' CTCTCTCTCTCTCTCTA 3'	400-1950	6	5	1	0	83.33	5.17	1.65
(CT)8T	5' CTCTCTCTCTCTCTCTT 3'	600-2000	8	8	0	1	100	7	3
OLIGO 2a	5' AGAGAGAGAGAGAGAG 3'	100-400	4	3	1	0	75	6.33	1.03
OLIGO 3b	5' GACAGACAGACAGACA 3'	600-1780	4	4	0	1	100	3.83	1.54
(AGG)6	5' AGGAGGAGGAGGAGGAGG 3'	550-1500	6	6	0	0	100	6.83	2.29
T(GA)9	5' TGAGAGAGAGAGAGAGAGA 3'	345-1845	13	13	0	1	100	13.83	4.99
(GA)9T	5' GAGAGAAGAGAGAGAGAGAT 3'	200-900	7	5	2	1	71.42	10.67	1.28
(GTGC)4	5' GTGCGTGCGTGCGTGC 3'	150-1780	10	9	1	3	90	7.17	2.6
(GACA)4	5' GACAGACAGACAGACA 3'	400-1050	6	5	1	1	83.33	6	1.89
(GAC)5	5' GACGACGACGACGAC 3'	300-1500	10	10	0	0	100	11	3.81
(AG)10	5' AGAGAGAGAGAGAGAGAGAG 3'	100-800	6	4	2	2	66.66	6.67	0.83
(AG)8G	5' AGAGAGAGAGAGAGAGG 3'	300-650	5	4	1	0	80	6.83	1.07
(AG)8C	5' AGAGAGAGAGAGAGAGC 3'	200-1185	10	10	0	0	100	9.67	3.92

Table 3. Details of ISSR primers and bands amplified in different species and accessions of *Dalbergia*

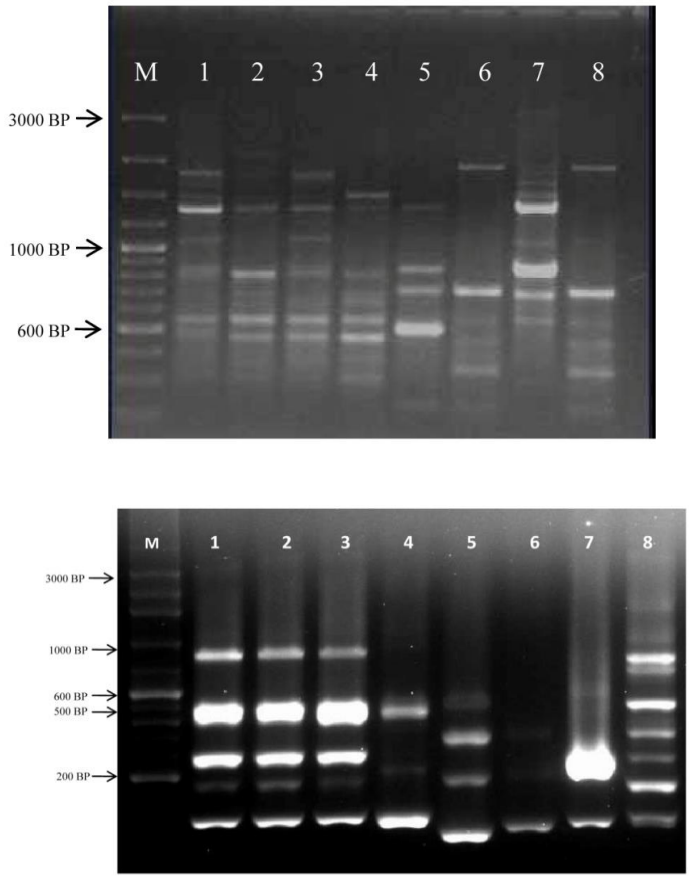

Fig 1:- RAPD banding pattern of different species and accessions of *Dalbergia*

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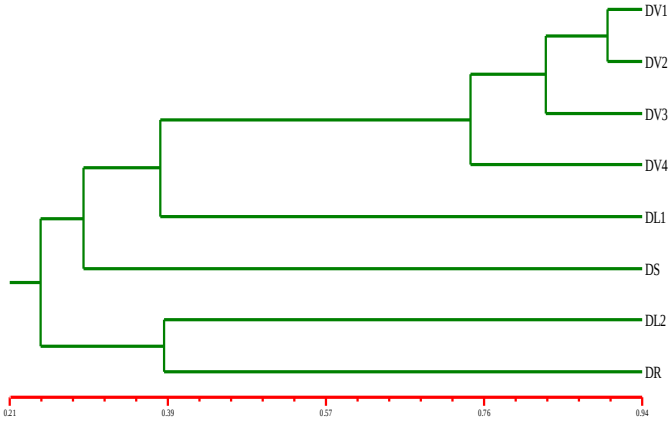

Fig 2:- Dendrogram showing genetic relationship among different species of *Dalbergia* as revealed from RAPD marker analysis

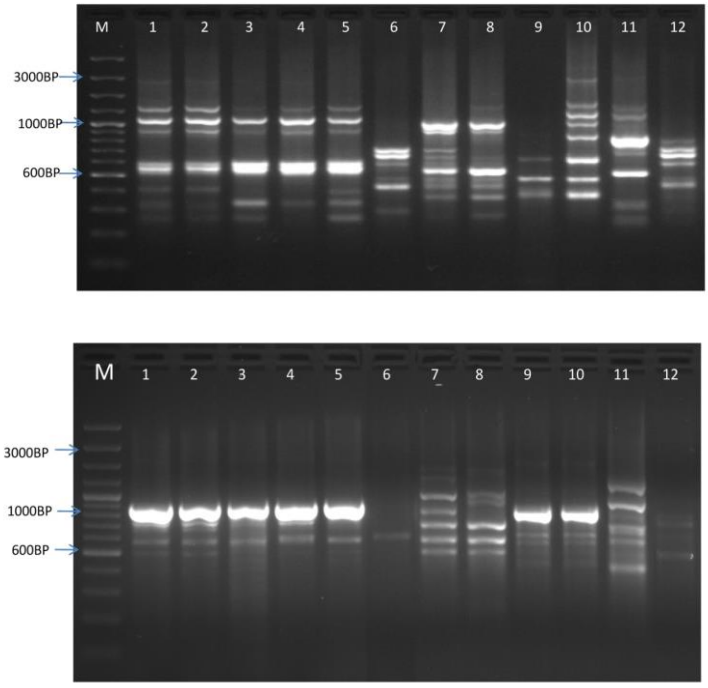

Fig 3:- ISSR banding pattern of different species and accessions of *Dalbergia*

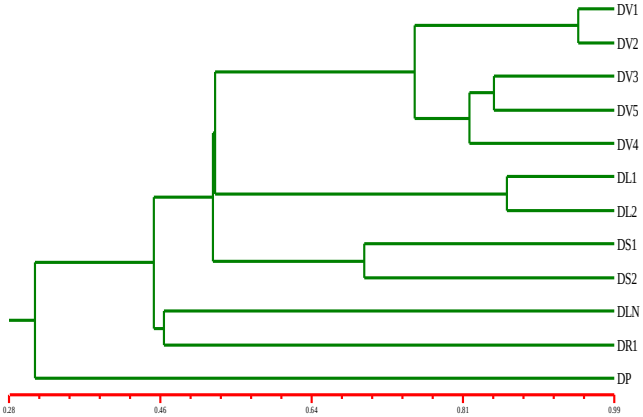

Fig 4:- Dendrogram representing relationship among the different species and accessions of *Dalbergia* as revealed from ISSR analysis

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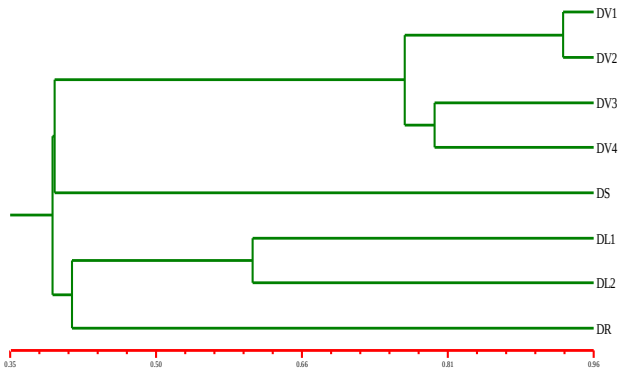

Fig 5:- Dendrogram of different species of *Dalbergia* using both RAPD and ISSR markers in combination.