Genetic Relationships among Some Species of *Dalbergia* Using PCR Based DNA Markers

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Summary Random amplified polymorphic DNA (RAPD) markers were used to study the genetic relationships among 10 species belonging to 2 sections of the genus *Dalbergia*. Of the 20 primers initially screened for polymorphism, only 10 amplified genomic DNA across all the 10 species. A total of 224 RAPD bands were observed with an average of 22.4 bands per primer, of which 99% were polymorphic. Pair wise genetic distances were calculated between species according to Jaccard, then used to construct UPGMA dendrogram. Cluster analysis based on the genetic similarities placed 10 species of *Dalbergia* in 2 clusters, with *D. latifolia*, *D. sissooides*, *D. horrida* and *D. melanoxylon* in Cluster A, and *D. lanceolaria*, *D. paniculata*, *D. volubilis*, *D. rubiginosa*, *D. malabarica* and *D. sissoo* in cluster B. These 2 clusters corresponds to sections: Sissoa and Dalbergia respectively. The results show that *D. paniculata* is very closely related to *D. lanceolaria* and suggested that *D. paniculata* can be considered as a subspecies of *D. lanceolaria*. The results of the present investigation distinguished the *D. sissooides* from *D. latifolia*, which was considered as a variety of *D. latifolia*. RAPD data supports the independent species status of the 2 species. The relationship between other species of *Dalbergia* in each cluster is discussed. This is the first report on genetic relationships among some species of *Dalbergia* using PCR based DNA markers.

Key words *Dalbergia*, Genetic relationships, RAPD, Indian rosewood, PCR based markers.

The genus *Dalbergia* Linn.f. belongs to the family Fabaceae of the tribe Dalbergiae. It comprises over 100 species of trees, shrubs and woody climbers widely distributed throughout the tropics of the world. In India about 35 species are reported and about 10 species are known from western ghats of Karnataka (Thothathri 1987). Thothathri (1987) placed Asian species of *Dalbergia* in 4 sections viz. Sect. Sissoa, Sect. Dalbergia, Sect. Selenobia and Sect. Ecastaphylla. The *Dalbergia* species included in the present study belongs to Sect. Sissoa and Sect. Dalbergia. The sect. Sissoa includes *D. latifolia*, *D. sissoo*, *D. sissooides*, *D. malabarica*, *D. rubiginosa*, *D. horrida* and *D. melanoxylon*, whereas Sect. Dalbergia includes *D. lanceolaria*, *D. paniculata* and *D. volubilis*. *Dalbergia* is an exclusively diploid genus with $2n=20$ chromosomes.

The genus is of economic importance, as many of its species yield valuable timbers of commerce. The Indian rosewood is derived from *D. latifolia*, whose timber is greatly valued for fine furniture and cabinet works. The timber of *D. sissoo* used for boat building, carts, carriages and rail-sleepers. The *D. sissooides* wood, commonly known as Malabar blackwood is used for cabinet works. *D. melanoxylon* better known in commerce as African or Chinese black wood or Sudan ebony is another species whose timber is used for musical instruments and handles of surgical instruments. The *D. lanceolaria* wood is used for agricultural implements (Thothathri 1987). In spite of their economic importance, no information is available on the genetic relationships between *Dalbergia* species. However, some species of *Dalbergia* have been included in reports on cladistic analysis of Dalbergiod legumes (Lavin *et al.* 2001). A clear understanding of the genetic relationships among various species is essential for successful and efficient utilization of the genetic vari-

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ability present in the related wild species (Kimber 1984). Such information is lacking in the genus *Dalbergia*.

The selection of appropriate technique for elucidating genetic relationship depends on several practical considerations. These include the speed, simplicity, cost and reproducibility of the protocol, as well as the ability of the technique to distinguish between large numbers of closely related species. Random amplified polymorphic DNA (RAPD) markers (Williams *et al.* 1990) have been successfully used for establishing the genetic and species relationships among plant species. RAPD markers have been extensively employed in establishing phylogenetic relationships in *Cicer* (Iruela *et al.* 2002), in *Fagopyrum* (Sharma and Jana 2002), within *Digitalis* (Nebauer *et al.* 2000), among 9 annual *Cicer* species (Ahmad 1999), *Hordeum* (Bustos *et al.* 1999), *Capsicum* (Rodriguez *et al.* 1999), *Chenopodium* (Ruas *et al.* 1999), *Persea* (Fiedler *et al.* 1998), *Eleusine* (Hilu 1995), to study genetic relatedness among developing seeds and intra fruit seed abortion in *Dalbergia sissoo* and taxa. (Mohana *et al.* 2001) However, no attempt has been made to study the phylogeny of *Dalbergia* using PCR based DNA markers. The aim of the present work is to determine the genetic relationships between 10 species of the genus *Dalbergia* using RAPD markers.

Materials and methods

Plant material

The following 10 *Dalbergia* species belonging to 2 sections of this genus: *D. latifolia* Roxb., *D. sissoo* Roxb., *D. sissooides* Grah., *D. malabarica* Prain, *D. rubiginosa* Roxb., *D. horrida* var. *concanensis* Thoth. and *D. melanoxylon* Guill. and Perr. (Sect. *Sissoa*); *D. lanceolaria* Linn.f., *D. paniculata* Roxb. and *D. volubilis* Roxb. (Sect. *Dalbergia*) were sampled throughout the natural distribution in Western Ghats of Karnataka, India (Table 1). Seeds were collected from large number of the maternal trees of each species. Seedlings were raised and young leaves were used for RAPD analysis. Three accessions of each species were included in the present study. From each accession, a number of leaf samples were randomly selected for DNA extraction.

DNA isolation and PCR amplification

Genomic DNA was extracted from young leaves following the modified CTAB method described by Doyle and Doyle (1990). Amplification reactions (25 μ l final volume) contained 40 ng of DNA, 250 μ M dNTP's, 0.3 μ M Primer (Operon Technologies, Alameda, California, USA), 1X Taq polymerase buffer, 1 unit of Taq polymerase (Bangalore Genei, India) and 1.5 mM MgCl₂. Each reaction mixture was overlaid with 30 μ l of mineral oil. DNA amplification was performed on a PTC-100 thermal cycler (MJ Research, USA) programmed to 1 cycle of 4 min at 94°C (initial strand sep-

Table 1. Seed lot No., Botanical section and collection sites of 10 *Dalbergia* species used for RAPD analysis

Sl. No.	Species	Seed lot No.	Section*	Collection site in Karnataka, India
1	<i>D. latifolia</i>	DL-478	S	Shimoga
2	<i>D. sissooides</i>	Ds-475	S	Coorg
3	<i>D. paniculata</i>	DP-412	D	Dharwad
4	<i>D. lanceolaria</i>	DL-407	D	Belgaum
5	<i>D. volubilis</i>	DV-436	D	Khanapur
6	<i>D. rubiginosa</i>	DR-428	S	Sirsi
7	<i>D. malabarica</i>	Dm-426	S	Yellapur
8	<i>D. horrida</i> var. <i>concanensis</i>	DH-424	S	Belgaum
9	<i>D. melanoxylon</i>	DM-405	S	Dharwad
10	<i>D. sissoo</i>	DS-406	S	Dharwad

* Section S: *Sissoa*, D: *Dalbergia*.

aration); followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 36°C (annealing) and 2 min at 72°C (extension) and a final 4 min at 72°C. A negative control was added in each run to test for contamination. After amplification, PCR products were resolved by electrophoresis in a 1.4% agarose gel with 1X TBE buffer, stained with ethidium bromide and photographed under UV light (Sambrook *et al.* 1989). EcoRI-HindIII double digest (Bangalore Genei, India) was used as a molecular weight marker to determine the size of the DNA fragments. The reproducibility of the amplification products was tested for each experiment at different times using similar reaction conditions.

Data analysis

Each amplified product was considered to be a unit character and the populations were scored for the presence (1) or absence (0) of a band. Faint bands were not considered. The molecular size of the amplification products were calculated from a standard curve based on the known size of DNA fragments of the EcoRI-Hind III double digest (Bangalore Genei, India). Estimates of genetic similarity were calculated between all pairs of species according to Jaccard's Similarity Index (1908).

The resulting similarity index was used to evaluate the genetic relationship among species of *Dalbergia* with cluster analysis using an unweighed pair group method with arithmetic averages (UPGMA) (Sneath and Sokal 1973). Principal Coordinate Analysis (PCA) was also performed to geographically display relationships between the species. All computations were carried out using the phylogenetic software NTSYS-pc version 2.0 (Rohlf 1997).

Results and discussion

This is the first report on genetic relationships in *Dalbergia* by PCR based DNA fingerprinting. RAPD markers represent an efficient and inexpensive tool to generate molecular data and thus, have been used successfully in various taxonomic and phylogenetic studies (Iruela *et al.* 2002, Sharma and Jana 2002, Nebauer *et al.* 2000).

A total of 20-decamer primers screened initially to amplify the genomic DNA of *Dalbergia*, only 10 primers generated robust and reproducible bands. These primers differed greatly in their efficiency for revealing polymorphism (Table 2). Ten primers were finally selected and used to analyze all 10 species of *Dalbergia* for polymorphism. All the reactions were repeated twice and only highly reproducible bands were considered. In all, 224 reproducible fragments were amplified, 99% of which were polymorphic, which were consistently generated from ten primers across *Dalbergia* species. Each primer generated 15 to 31 polymorphic amplification products with an average of

Table 2. The DNA sequence of random decamer oligonucleotide primers used for *Dalbergia* species
DNA amplification and % polymorphism

Primers	Nucleotide Sequence 5' —————>3'	No. of Polymorphic products	% polymorphism
OPA-05	AGGGGTCTTG	25	100
OPA-08	GTGACGTAGG	22	96
OPA-09	GGGTAACGCC	17	100
OPA-12	TCGGCGATAG	27	100
OPA-14	TCTGTGCTGG	17	100
OPA-16	AGCCAGCGAA	15	100
OPA-17	GACCGTTGT	31	100
OPA-18	GACCGTTGT	22	100
OPA-19	CAAACGTCGG	19	95
OPA-20	GTTGCGATCC	27	100

22.4 bands per primer. The representative RAPD profiles of 10 species are shown in Fig. 1. Primer OPA-17 amplified highest number of bands (31), while OPA-16 amplified only 15 bands. All bands, except 2 monomorphic bands generated by primers OPA-08 and OPA-19 were polymorphic across species. The approximate size of the largest fragment was 5.0 kb, where as the smallest recognizable fragment was about 0.3 kb in size. Certain amplified bands were found to be specific to a given species *i.e.* they were present in only one species but absent from the remaining species. These bands could be used for species identification purposes.

The estimates of pair wise genetic distance between the 10 species of *Dalbergia* is given in Table 3. The similarity matrix was calculated based on Jaccard's Similarity Coefficient. The interspecific genetic similarity indices ranged from 0.05 between *D. lanceolaria* and *D. horrida* var. *concanensis* to 0.75 between *D. paniculata* and *D. lanceolaria*.

Cluster analysis of the genetic similarity estimates was performed using Jaccard's coefficient to generate a UPGMA dendrogram showing overall genetic relatedness among the *Dalbergia* species (Fig. 2). Two distinct clusters could be identified. The first cluster comprised, *D. latifolia*, *D. sissoides*, *D. horrida* and *D. melanoxylon*. The second cluster was formed by *D. lanceolaria*, *D. paniculata*, *D. volubilis*, *D. rubiginosa*, *D. malabarica* and *D. sissoo*. The Principle Coordinate Analysis supports the major clustering pattern (Fig. 3). Species placed within a cluster were more closely related to each other than to species present in different clusters. Thus, *D. latifolia* and *D. sissoides* were genetically closer to each other than other species in first cluster, while *D. lanceolaria* and *D. paniculata* were

Fig. 1. RAPD profiles of 10 *Dalbergia* species generated by primer (A) OPA-17 and (B) OPA-12. M indicates the DNA molecular size marker (*EcoRI-HindIII* double digest). Lane number corresponds to the species serial number given in Table 1.

Table 3. Similarity matrix based on the Jaccard's Similarity index

	1	2	3	4	5	6	7	8	9	10
1	1.00									
2	0.37	1.00								
3	0.11	0.12	1.00							
4	0.11	0.12	0.75	1.00						
5	0.10	0.11	0.15	0.13	1.00					
6	0.11	0.14	0.16	0.14	0.14	1.00				
7	0.09	0.09	0.10	0.10	0.12	0.17	1.00			
8	0.19	0.17	0.06	0.05	0.06	0.14	0.06	1.00		
9	0.13	0.09	0.08	0.09	0.06	0.12	0.09	0.12	1.00	
10	0.11	0.09	0.15	0.12	0.11	0.12	0.10	0.12	0.09	1.00

Fig. 2. Dendrogram showing the genetic relationship among 10 *Dalbergia* species generated by RAPD data using the UPGMA method.

Fig. 3. Relationship among 10 species of *Dalbergia* revealed by the Principal Co-ordinate Analysis (PCA) based on RAPD data. The numbers in the plot correspond to the species serial number given in Table 1.

genetically closer to each other than other species in the second cluster.

Our results show that RAPD markers successfully identified genetic variation in *Dalbergia*. In accordance with the sectional classification of *Dalbergia* by Thothathri (1987), the 10 *Dalbergia* species included in the present study were grouped into 2 main clusters, where cluster A corresponds to section: *Sissoa* and cluster B corresponds to section *Dalbergia*. However, 3 species viz. *D. sissoo*, *D. malabarica* and *D. rubiginosa* were grouped with species of cluster B. These results suggest that the classification of *Dalbergia* species based on morphological characters should be considered only after further investigation by molecular analysis.

UPGMA cluster analysis and Principle Coordinate Analysis reveals that *D. latifolia* is very closely related to *D. sissooides* with 0.37 similarity index in a cluster A. These 2 species are more or less similar morphologically. It is rather difficult to distinguish these 2 species in herbaria. This has led many of the earlier botanists to treat *D. sissooides* as a variety of *D. latifolia* Roxb. (Bentham 1860, Beddome 1869, Baker 1876). Our results of RAPD fingerprinting could easily distinguish these 2 species at molecular level and suggests that *D. sissooides* can be considered as a distinct species rather than a variety of *D. latifolia*. Cytologically these 2 species are distinct and exhibit different karyomorphology (unpublished data). This finding is in agreement with the treatment of *D. sissooides* as a distinct species by Prain (1904) and Thothathri (1987). *D. horrida* and *D. melanoxyton* are related to *D. latifolia* with 0.19 and 0.13 similarity values respectively.

Cluster B comprises *D. lanceolaria* and *D. paniculata* in a close sub cluster. These 2 species are very close to each other with a highest Jaccard's similarity index (0.75). These 2 species are morphologically similar to each other. Most of the RAPD bands in these 2 species are common. This finding agrees with the consideration of *D. paniculata* as a subspecies of *D. lanceolaria* by Thothathri (1987). *D. paniculata* has so far been treated as a distinct species in all the Indian and Burmese floras. The RAPD fingerprinting patterns of these 2 species in the present study suggest that, *D. paniculata* cannot stand as a distinct species, but can best be regarded as a subspecies of the *D. lanceolaria* (*D. lanceolaria* Linn.f. subsp. *paniculata* (Roxb.) Thoth.). *D. volubilis* is closely related to *D. paniculata* with 0.15 similarity index. *D. volubilis* is a climber and its flower and leaf characters are very much similar to *D. paniculata*. *D. rubiginosa* and *D. malabarica* grouped in a close sub cluster in Cluster B with 0.17 similarity index. Even though these 2 species are morphologically dissimilar, both are climbers and occupy the semi evergreen forests of Western ghats. However, all other species in the same cluster occupy deciduous forests. *D. sissoo* shows similarity (0.15) with *D. lanceolaria*. Even though these 2 species are morphologically dissimilar, they appear

to be genetically related to each other.

As many of the species of *Dalbergia* are used as potential forage and timber legumes, the presence of unique RAPD markers among the various *Dalbergia* species indicate the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications and includes the determination of cultivar purity, efficient use and management of a genetic resources particularly the establishment of property rights (Plant variety protection and patenting). This study represents only the first step in using RAPD markers as a tool to implement studies of Genetic relationships and molecular systematics in *Dalbergia*.

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