Journal of Applied Biology & Biotechnology Vol. 11(5), pp. 59-69, Sep-Oct, 2023
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2023.11507

Population structure and genetic diversity analysis of *Dalbergia latifolia* and *Dalbergia sissoides* using RAPD markers

R. Vasudevan[1]*, D. Thangamani[1], S. Lalitha[1], K. R. Sasidharan[2]

[1]Department of Forest Genetic Resource Management, Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, India.
[2]Department of Forest Ecology and Climate Change, Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamil Nadu, India.

ARTICLE INFO

Article history:
Received on: March 13, 2023
Accepted on: June 24, 2023
Available online: August 10, 2023

Key words:
Cryptic sister species,
Bombay blackwood,
Indian rosewood,
Malabar blackwood,
PCR based dominant DNA marker,
Binary data analysis.

ABSTRACT

Dalbergia latifolia and *D. sissoides* are economically important and slow growing timber species of India. Both are closely related leguminous species and genetic divergence of these species is still untapped in Kerala and Tamil Nadu states. A total of 56 *Dalbergia* accessions from 20 forest divisions of Kerala and Tamil Nadu regions were used in the present study. Based on the Evanno method, Bayesian model of population structure analysis divides the tree accessions into 3 genotypic groups, namely *D. sissoides* group-1, *D. sissoides* group-2 and *D. latifolia* group. The tree accessions were also divided into 2 distinct clusters (*D. latifolia* and *D. sissoides* clusters) according to Neighbour joining clustering and principal co-ordinates analysis. AMOVA, allelic frequency analysis and Mantel test were performed for both species. The results indicated that *D. latifolia* and *D. sissoides* were genetically distinct without any recent intermixing. The results also expressed that *D. sissoides* cluster consists of 2 genotypes which contained recently admixed individuals. The present work was proved that *D. latifolia* is primitive than *D. sissoides* and both are considered as a monophyletic sibling species. 32 out of 40 *D. sissoides* accessions showed *D. latifolia*-like leaflet apex in the Western Ghats of Kerala and Tamil Nadu. It was formed a species complex which caused misidentification of *D. sissoides* accessions as *D. latifolia*. The study will be useful for the species identification, mapping of populations, species conservation and further genetic improvement programs.

1. INTRODUCTION

Population structure and genetic diversity analyses are important ways to find out the genetic relationship and evolutionary history among the species. Studies on population structure and genetic diversity provide a framework to explore the ecological and conservation issues for species management. Details of population structure and genetic diversity are essential and invaluable to understanding the gene flow, genetic drift, and natural selection processes among populations [1]. Molecular markers are useful for assessing the genetic variation within and among species. Random Amplified Polymorphic DNA (RAPD) markers are dominant DNA markers and were commonly used to study population structure and genetic diversity [2,3].

Dalbergia latifolia Roxb. and *Dalbergia sissoides* Wight and Arn. are valuable and precious timber species of the family *Fabaceae*. *D. latifolia* is known as "Indian Rosewood" or "Bombay Blackwood" and distributed in the sub-Himalayan tract from Oudh eastwards to Sikkim, Bihar, Orissa, Central, Western, and Southern India. *D. sissoides* is commonly known as "Malabar Blackwood" and distributed in Western Ghats of Karnataka, Tamil Nadu, and Kerala. These tree species are found in the semi-evergreen and deciduous forests of the above areas. The timber of these species was used for making furniture, carvings, decorative plywood, and veneers [4,5].

D. latifolia and *D. sissoides* are genetically closer species and have a wide range of habitat-preferring morphological characteristics which caused many difficulties in species identification using herbarium specimens [6]. Therefore, earlier taxonomical studies considered *D. sissoides* as a variety of *D. latifolia* and mentioned it as *D. latifolia* var. *sissoides* (Wight and Arn.) Baker whereas, later studies separated it as a distinct species [7,8]. Hiremath and Nagasampige (2004) showed a high jaccard similarity index (0.37) between these species was noted using RAPD markers in the Western Ghats of Karnataka and supports the independent species status of *D. sissoides* [9]. Yulita *et al*. reported the genetic diversity of five populations of *D. latifolia* from Yogyakarta and Lombok Island, West Nusa Tenggara, Indonesia, using sequence random amplified polymorphism (SRAP) markers [10]. Yulita *et al*. also revealed the population structure and genetic diversity study of *D. latifolia* in Java and West Nusa Tenggara using SRAP markers [11].

Many natural factors and illegal logging have affected the reproduction and establishment of these tree species. Therefore, the natural populations of these species have been declining in their habitats [5]. Both rosewood

*Corresponding Author:
R. Vasudevan,
Division of Forest Genetic Resource Management, Institute of Forest Genetics and Tree Breeding, Coimbatore - 641 002, Tamil Nadu, India.
E-mail: vasubot @ gmail.com

species were listed in Appendix 2 of CITES since 2017. The species appearing in Appendix 2 of CITES were banned from international trade without an import and export license or re-export certificate [10]. Both rosewood species have also been categorized as "Vulnerable" in the Red Data Book of IUCN [13]. Hence, population structure and genetic diversity studies of these species are essential for developing conservation strategies and further tree improvement programs.

2. MATERIALS AND METHODS

2.1. Establishing of Germplasm Assemblage

Field surveys were undertaken in Gudalur, Coimbatore, Erode, Salem, Dharmapuri, Theni, Tirunelveli forest divisions of Tamil Nadu and Kannur, Wayanad, Nilambur, Mannarkad, Palakkad, Chalakkudy, Nemmara, Malayattor, Munnar, Ranni, Konni, and Thiruvanandapuram forest divisions of Kerala and altogether 173 morphologically superior *D. latifolia* and *D. sissoides* trees were selected. The species were differentiated mainly based on the morphological characters of leaflets [Figure 1]. However, many trees showed a wide range of variations in leaflet morphology which makes them very difficult to classify. The root cuttings were collected from the selected trees, vegetatively propagated and used to establish the clonal germplasm assemblage [14]. *Dalbergia* clones (56 Nos) from the above assemblage were used for the present study and listed in Table 1. Indian Rosewood seedlings (KFRI-1 and KFRI-2) bought from Kerala Forest Research Institute, Peechi were used as check or control trees for *D. latifolia*.

2.2. Extraction and Purification of Genomic DNA

The young leaves of 56 *D. latifolia* and *D. sissoides* samples [Table 1] were collected from the germplasm assemblage and used for genomic DNA extraction following the CTAB method developed by Ginwal and Maurya with some modifications [15]. The extracted DNA samples were purified by RNase treatment (0.5 µg of RNase A used for 1 µg of DNA). The purified DNA was quantified using a spectrophotometer (NanoDrop™ Lite, Thermo Scientific). The quality of DNA ranged between 1.75 to 1.96 (A260/A280 value). The quantity of DNA ranged from 143 to 457 ng/µL. However, all samples were diluted to 50 ng/µL. The purified DNA samples were stored in a freezer at −20°C and used as template DNA in thermal cycler reactions.

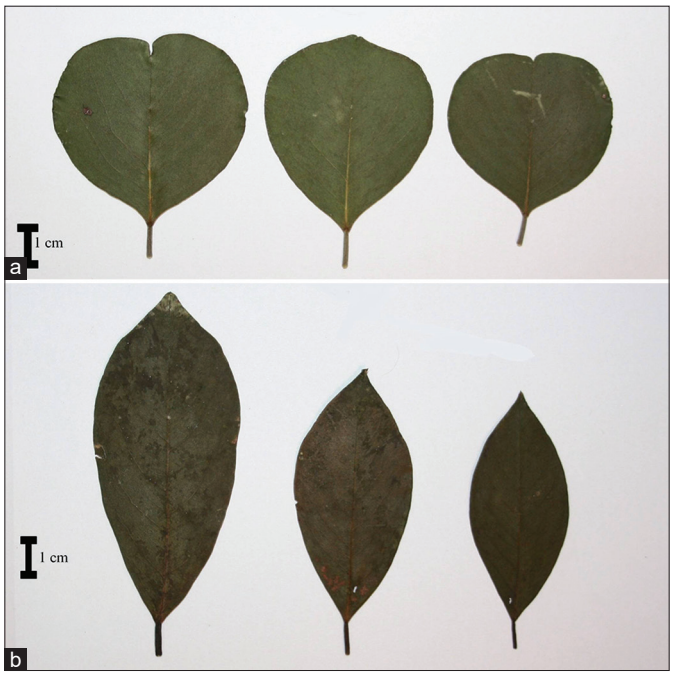

Figure 1: Leaflets of *Dalbergia latifolia* and *Dalbergia sissoides*. (a) *D. latifolia* leaflets with retuse and obtuse apex. (b) *D. sissoides* leaflets with acute apex.

2.3. Polymerase Chain Reaction and Gel Documentation

Each reaction mixture (15 µL of final volume) consisted of 7.5 µL of sterile water, 1.5 µL of 10X Taq buffer, 1.5 µL of 25 mM $MgCl_2$, 1.5 µL of 10 mM dNTPs, 1.5 µL of 10 µM of RAPD Primer, 0.5 µL of Taq DNA polymerase (3U/µL), and 1 µL of template DNA. A thermal cycler (BIO-RAD T100™) was set up for DNA amplification according to the program with an initial denaturation at 94°C for 1 min, 40 cycles of denaturation at 94°C for 20 s, annealing at optimized temperature for 1 min, extension at 72°C for 2 min, then final extension at 72°C for 5 min and infinite hold at 12°C.

After completion of the above steps, the amplified PCR products were measured for their success through horizontal electrophoresis of 1.5% agarose gel mixed with ethidium bromide (0.5 µg/mL). The electrophoresis was carried out with 80 Voltage power for 3 h. The gel was documented using BIO-RAD Gel Doc™ XR^+ Gel documentation system. The base pairs length of each band was estimated using Image Lab™ software (version 5.2.1).

2.4. Selection of High Polymorphic RAPD Markers

The randomly selected DNA of *D. latifolia* sample (TNCBBOL-1) was used to find out the best annealing temperature (T_a). The melting temperature (T_m) of RAPD primers ranged from 31.1 to 39.1°C. Therefore, RAPD markers were tested with different annealing temperatures viz., 27, 31, 35, 39, and 43°C. The amplified products of different annealing temperatures were run in agarose gel and documented. The best and most suitable annealing temperature was determined based on the band clearness [16].

The extracted DNA of nine randomly selected clones (KLNMNEL-1, TNCBBOL-1, KLNWBEG-10, TNSLSHR-1, TNEDBAR-11, KLKNTAL-2, KLPKWAL-1, TNTVCOR-2, and TNGDCHR-3) was used to select the high polymorphic RAPD primers. A total of 19 primers were tested with their respective optimized annealing temperatures. The high polymorphic primers were selected based on the high percentage of polymorphic bands.

2.5. Preparation of Binary or Dominant Marker Data

Each high polymorphic RAPD marker was separately amplified with 56 *Dalbergia* samples and the amplified products of eight RAPD markers were run in agarose gel and then digitally imaged [Figure 2]. The binary data were manually scored for the presence and absence of bands with 1 and 0, respectively, according to band size.

2.6. Estimation of Discriminatory Power of Selected RAPD Markers

The binary data were used to calculate the discriminatory power of selected RAPD markers [Table 2]. Genotypic gene diversity or expected heterozygosity, polymorphic information content, and effective multiplex ratio were computed using the formulas described by Sornakili *et al*., Chesnokov and Artemyeva, and Ismail *et al*., respectively [17-19]. Marker index and resolving power were calculated by using the formulas described by Dobhal *et al*., in Table 2 [20]. The relationships between the above parameters were

Table 1: Geographical locations of *Dalbergia latifolia* and *Dalbergia sissoides* populations in Kerala and Tamil Nadu.

S. No.	Clone name	Forest Range	Forest division/population	Region/state	Latitude	Longitude	Elevation (masl)
1	KLPKWAL-1	Walayar	Palakkad	Kerala	10° 51'20.6"	76° 48' 34.0"	360.4
2	KLPKWAL-5	Walayar	Palakkad	Kerala	10° 49' 49.4"	76° 47' 43.6"	360.3
3	KLNMNEL-1	Nelliyampathy	Nemmara	Kerala	10° 26' 44"	76° 42' 35"	353.7
4	KLNMNEL-3	Nelliyampathy	Nemmara	Kerala	10° 31' 16"	76° 37' 51"	329.4
5	KLNMNEL-6	Nelliyampathy	Nemmara	Kerala	10° 31' 39"	76° 37' 47"	326.8
6	KLNMNEL-8	Nelliyampathy	Nemmara	Kerala	10° 31' 38"	76° 37' 46"	326.8
7	KLCHPAL-1	Palappally	Chalakudy	Kerala	10° 26' 21"	76° 23' 28"	211.9
8	KLCHPAL-6	Palappally	Chalakudy	Kerala	10° 26' 27"	76° 23' 51"	213.2
9	KLCHVEL-2	Vellikulangara	Chalakudy	Kerala	10° 23' 10"	76° 24' 22"	221.3
10	KLCHVEL-5	Vellikulangara	Chalakudy	Kerala	10° 22' 54"	76° 24' 42"	224.3
11	KLMKAGL-4	Agali	Mannarkad	Kerala	11° 02' 02.2"	76° 37' 56.8"	289.1
12	KLMKATP-1	Attappady	Mannarkad	Kerala	11° 09' 59.2"	76° 38' 26.0"	271.3
13	KLMYKUT-1	Kuttampuzha	Malayattoor	Kerala	10° 10' 33.9"	76° 47' 04.7"	462.5
14	KLMYKUT-3	Kuttampuzha	Malayattoor	Kerala	10° 09' 27"	76° 46' 13"	458.8
15	KLMYKUT-7	Kuttampuzha	Malayattoor	Kerala	10° 09' 26"	76° 45' 35"	458.8
16	KLKNKAN-1	Kannavam	Kannur	Kerala	11° 47' 46.9"	75° 44' 29.8"	395.1
17	KLKNKAN-5	Kannavam	Kannur	Kerala	11° 47' 46.9"	75° 44' 29.8"	395.1
18	KLKNTAL-2	Thaliparamba	Kannur	Kerala	12° 16' 03.0"	75° 26' 06.0"	375.7
19	KLKNTAL-3	Thaliparamba	Kannur	Kerala	12° 16' 03.0"	75° 26' 06.0"	375.7
20	KLMUADI-2	Adimali	Munnar	Kerala	10° 00' 56.4"	76° 54' 01"	570.9
21	KLMUADI-3	Adimali	Munnar	Kerala	10° 00' 55.3"	76° 53' 58.4"	570.9
22	KLMUNER-2	Neriyamangalam	Munnar	Kerala	10° 05' 59.9"	76° 51' 00.8"	513.0
23	KLMUNER-4	Neriyamangalam	Munnar	Kerala	10° 05' 01.9"	76° 51' 03.0"	513.0
24	KFRI-1*	KFRI	Thrissur	Kerala	-	-	-
25	KFRI-2*	KFRI	Thrissur	Kerala	-	-	-
26	KLTMPLO-4	Palode	Thiruvanantha-puram	Kerala	8° 42' 16.1"	77° 06' 41.8"	46.3
27	KLTMPLO-5	Palode	Thiruvanantha-puram	Kerala	8° 42' 16"	77° 06' 41.4"	46.3
28	KLKOKON-1	Konni	Konni	Kerala	9° 13' 54.1"	76° 54' 54.3"	205.7
29	KLRAVAD-2	Vadasserikkara	Ranni	Kerala	9° 17' 41.5"	76° 57' 27.9"	243.7
30	KLRAVAD-5	Vadasserikkara	Ranni	Kerala	9° 17' 38.2"	76° 57' 31.5"	243.2
31	KLNLKAR-2	Karulai	Nilambur	Kerala	11° 16' 36.7"	76° 19' 23.8"	339.8
32	KLNLKAR-4	Karulai	Nilambur	Kerala	11° 16' 24.9"	76° 21' 52.9"	344.3
33	KLSWMEP-4	Meppadi	Wayanad	Kerala	11° 33' 46.5"	76° 04' 22"	509.2
34	KLSWMEP-7	Meppadi	Wayanad	Kerala	11° 33' 36.6"	76° 04' 09.3"	508.7
35	KLNWBEG-3	Begur	Wayanad	Kerala	11° 52' 20"	76° 03' 24"	740.7
36	KLNWBEG-7	Begur	Wayanad	Kerala	11° 52' 18.4"	76° 03' 29"	740.6
37	KLNWBEG-10	Begur	Wayanad	Kerala	11° 52' 17.2"	76° 03' 28.8"	740.6
38	TNCBBOL-1	Boluvampatty	Coimbatore	Tamil Nadu	10° 56' 28.2"	76° 42' 24.1"	307.7
39	TNCBBOL-4	Boluvampatty	Coimbatore	Tamil Nadu	10° 56' 28.2"	76° 42' 24.1"	307.7
40	TNCBBOL-6	Boluvampatty	Coimbatore	Tamil Nadu	10° 57' 45.4"	76° 40'53.8"	295.4
41	TNEDBAR-2	Bargur	Erode	Tamil Nadu	11° 50' 55"	77° 34' 26"	507.2
42	TNEDBAR-6	Bargur	Erode	Tamil Nadu	11° 48' 55"	77° 33' 6"	507.2
43	TNEDBAR-8	Bargur	Erode	Tamil Nadu	11° 48' 15"	77° 32' 55"	517.8
44	TNEDBAR-11	Bargur	Erode	Tamil Nadu	11° 51' 17"	77° 31' 01"	517.8
45	TNTHCUM-1	Cumbum	Theni	Tamil Nadu	9° 37' 24"	77° 11' 33"	374.3
46	TNTHCUM-6	Cumbum	Theni	Tamil Nadu	9° 37' 18"	77° 11' 35"	374.0
47	TNDHHAR-4	Harur	Dharmapuri	Tamil Nadu	11° 51' 57"	78° 27' 37.4"	285.3

(Contd...)

Table 1: *(Continued)*.

S. No.	Clone name	Forest Range	Forest division/population	Region/state	Latitude	Longitude	Elevation (masl)
48	TNDHHAR-8	Harur	Dharmapuri	Tamil Nadu	11° 51’ 51.5”	78° 27’ 28.7”	285.3
49	TNSLSER-1	Shervarayan	Salem	Tamil Nadu	11° 46’ 16.3”	78° 11’ 20.6”	338.9
50	TNSLYER-2	Yercaud	Salem	Tamil Nadu	11° 49’ 49.1”	78° 16’ 46.2”	328.3
51	TNSLYER-3	Yercaud	Salem	Tamil Nadu	11° 49’ 56.8”	78° 16’ 51.6”	328.3
52	TNSLDAN-2	Danishpet	Salem	Tamil Nadu	11° 50’ 28.6”	78° 10’ 00.3”	337.4
53	TNTVCOR-2	Courtallam	Tirunelveli	Tamil Nadu	8° 55’ 38.1”	77° 16’ 04.6”	74.2
54	TNTVCOR-10	Courtallam	Tirunelveli	Tamil Nadu	8° 55’ 55.0”	77° 16’ 00.2”	74.6
55	TNGDCHR-3	Cherambady	Gudalur	Tamil Nadu	11° 35’ 03.0”	76° 21’ 42.7”	492.3
56	TNGDBIT-10	Bitherkad	Gudalur	Tamil Nadu	11° 31’ 28.4”	76° 16’ 33.0”	534.4

*KFRI-1 and KFRI-2 are *Dalbergia latifolia* seedlings bought from Kerala Forest Research Institute, Peechi, Thrissur.

Table 2: Discriminatory powers of selected RAPD markers are used in the present study.

S. No.	Marker name	Sequence (5’ to 3’)	TNB	MB	PB	ABS (bp)	PPB	Hg	PIC	EMR	MI	RP
1	RAPD 1	GGGAATTCGG	12	1	11	210–3100	91.66	0.23	0.28	10.12	2.83	0.82
2	RAPD 4	CTGCTGGGAC	12	0	12	226–2478	100	0.17	0.18	12	2.16	0.55
3	RAPD 12	GTGACGTAGG	17	2	15	200–3850	88.23	0.13	0.18	13.2	2.38	0.53
4	RAPD 13	GGGTAACGCC	12	1	11	320–1850	91.66	0.14	0.21	10.12	2.13	0.47
5	RAPD 14	TCGGCGATAG	14	0	14	200–1780	100	0.18	0.23	14	3.22	0.49
6	RAPD 15	TCTGTGCTGG	15	0	15	260–2048	100	0.2	0.23	15	3.45	0.52
7	RAPD 16	AGCCAGCGAA	14	0	14	230–1754	100	0.18	0.22	14	3.08	0.43
8	RAPD 17	GAC CGCTTGT	17	0	17	320–1950	100	0.13	0.19	17	3.23	0.31
	Total		113	4	109			1.36	1.72	105.44	22.48	4.12
	Average		14.1	0.5	13.6		96.44	0.17	0.22	13.18	2.81	0.52

TNB: Total number of bands, MB: Monomorphic bands, PB: Polymorphic bands, ABS: Amplicon band size, PPB: Percentage of polymorphic band, H_g: Genotypic gene diversity or expected heterozygosity, PIC: Polymorphic information content, EMR: Effective multiplex ratio, MI: Marker index, RP: Resolving power.

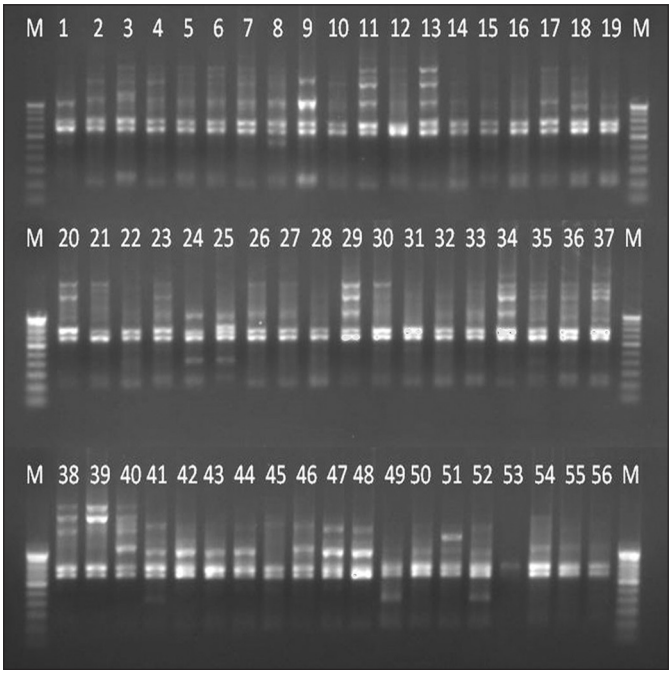

Figure 2: A gel image of the RAPD 12 marker with 56 accessions of *Dalbergia latifolia* and *Dalbergia sissoides*. M – 100 bp DNA ladder, the numbers adherent at the top of each lane were represented the serial numbers of Table 1.

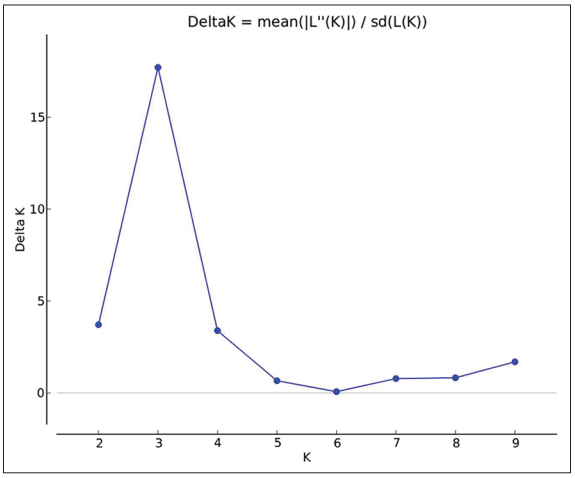

Figure 3: Evanno plot with cluster numbers (k) in X axis and delta K values in Y axis. Cluster numbers 3 (K = 3) showed the high value of delta K (delta K =17.07) than other cluster numbers.

calculated by the Pearson correlation coefficient method using IBM Statistical Package for the Social Sciences software (version 20).

2.7. Statistical Analysis

The RAPD data were also used for population structure and genetic diversity analysis. Population structure analysis was used to detect the subsets

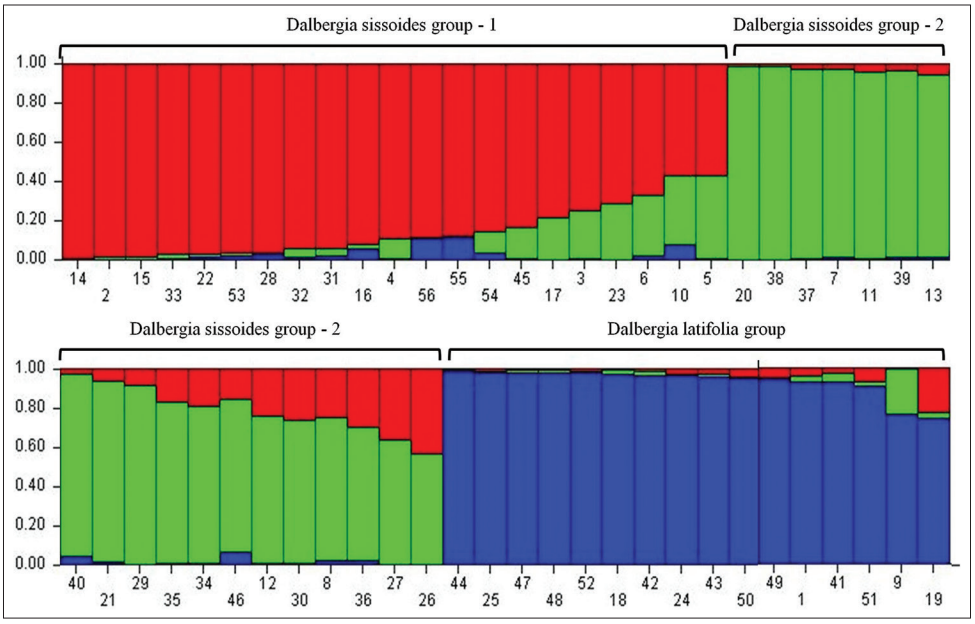

Figure 4: The population structure chart showed 56 Dalbergia individuals which were categorized into 3 genotypic groups (*Dalbergia sissoides* group-1, 2 and *Dalbergia latifolia* group). The numbers in X axis were similar to the serial numbers of Table 1.

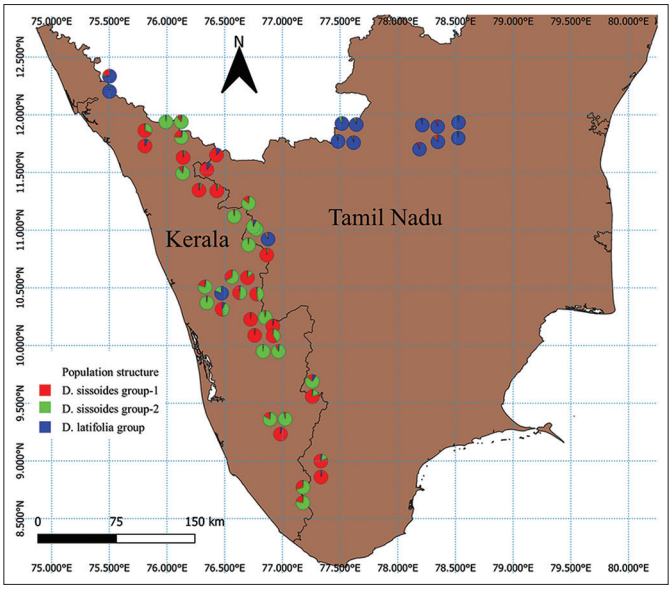

Figure 5: Population structure map of *Dalbergia sissoides* and *Dalbergia latifolia* in Kerala and Tamil Nadu of India.

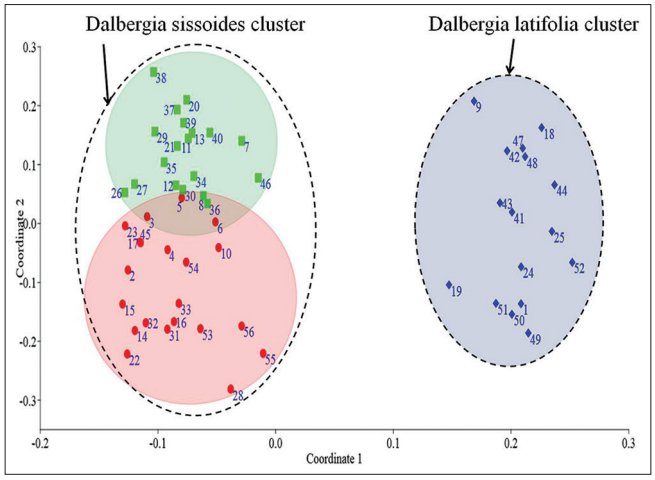

Figure 6: Principal coordinates analysis showed *Dalbergia latifolia* and *Dalbergia sissoides* clusters. Blue diamond points – *D. latifolia* individuals, Red dot points – *D. sissoides* Group-1 individuals and Green square points – *D. sissoides* Group-2 individuals. The numbers adherent to each points were represented the serial numbers of Table 1.

(number of K groups) of the whole sample by detecting allele frequency differences and to assign individuals in respective K groups based on the analysis of likelihoods. This analysis was performed with STRUCTURE software (version 2.3.4) using the Bayesian model and a web-based program STRUCTURE HARVESTER using the Evanno method [21,22]. In Evanno plot, the K group showed the highest Delta K value detected as the best fit for the dataset [Figure 3]. The population structure chart was derived based on the best-fitted K group [Figure 4]. The population structure map [Figure 5] was created using QGIS software (version 3.16.15).

Principal coordinates analysis was a statistical method that converts RAPD data into distances between individuals and showed a map-based visualization of individuals [Figure 6]. This analysis was carried out with PAST software (version 4.03) using the Jaccard similarity coefficient [23].

Neighbor joining cluster analysis was a statistical method used to group an individual with other closely related individuals producing a dendrogram or phylogenetic tree as an outcome [Figure 7]. This analysis was performed by DAR win software (version 6.0.021) with unweighted neighbor joining method using the Jaccard dissimilarity coefficient along with 100 times bootstrapping [24].

Analysis of molecular variance (AMOVA) was a nonparametric analog method to detect molecular variance among individuals. AMOVA was performed based on Phi (л) statistics with 1000 permutations [Table 3]. Allele or gene frequency was the relative frequency of an

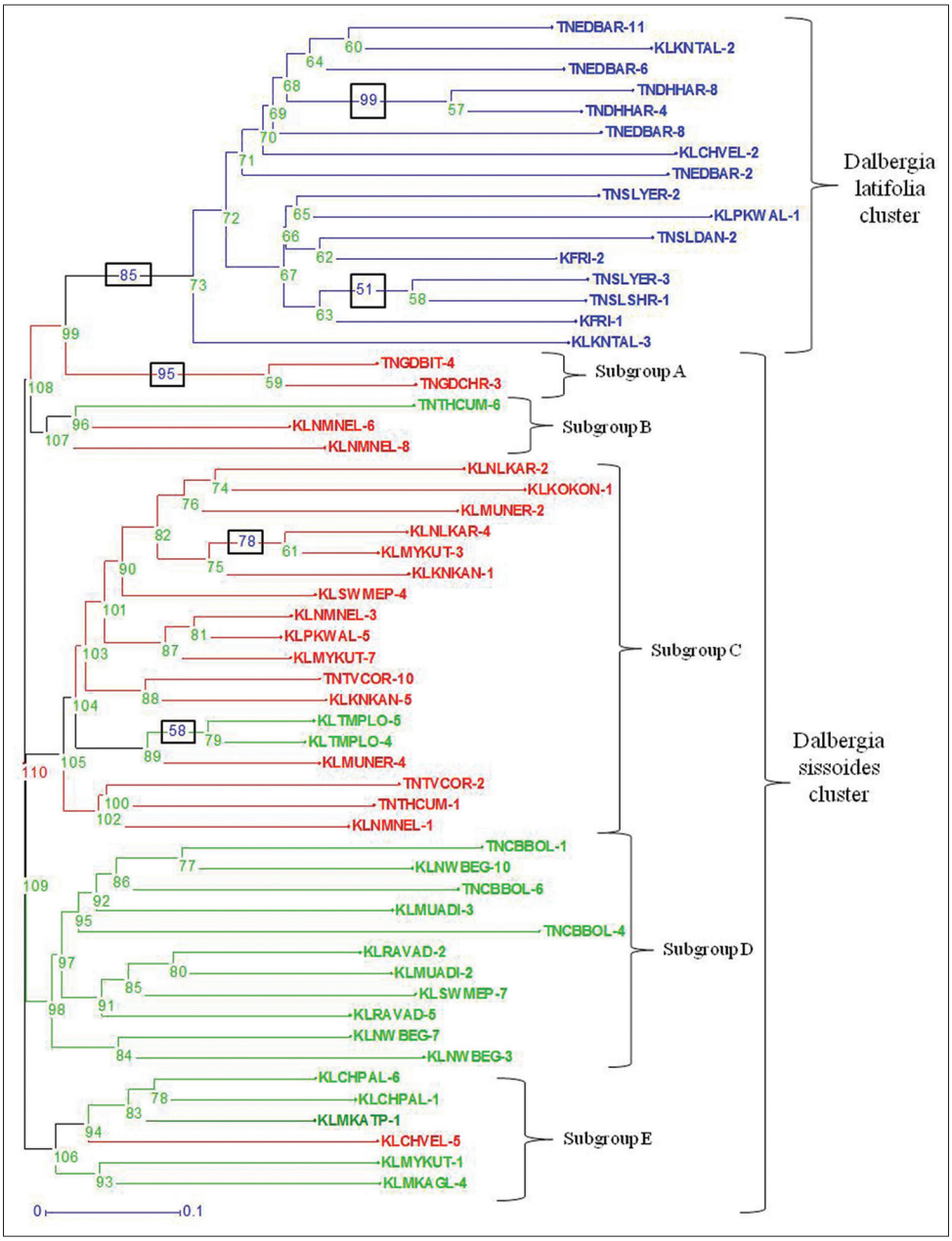

Figure 7: Dendrogram of *Dalbergia latifolia* and *Dalbergia sissoides* using Unweighted Neighbour Joining method with Jaccard dissimilarity coefficient supported by ≥50% bootstrap values (box in the middle of branches) and node numbers. Blue - *D. latifolia* individuals, Red - *D. sissoides* group-1 individuals, Green - *D. sissoides* group-2 individuals.

Table 3: AMOVA of *Dalbergia latifolia* and *Dalbergia sissoides* from Kerala and Tamil Nadu.

Species	Source	df	SS	MS	Est. Var.	PMV (%)	Stat	Value	*P*
Dalbergia latifolia	Among Kerala and Tamil Nadu	1	11.783	11.783	0.000	0	PhiRT	-0.045	0.903
	Among forest divisions	4	62.217	15.554	2.396	20	PhiPR	0.204	0.001
	Within forest divisions	10	93.250	9.325	9.325	80	PhiPT	0.169	0.003
	Total	15	167.250		11.721	100			
Dalbergia sissoides	Among Kerala and Tamil Nadu	1	19.378	19.378	0.604	6	PhiRT	0.061	0.004
	Among forest divisions	12	135.180	11.265	1.090	11	PhiPR	0.118	0.002
	Within forest divisions	26	211.967	8.153	8.153	83	PhiPT	0.172	0.001
	Total	39	366.525		9.846	100			

df: Degree of freedom, SS: Sum of squared observations, MS: Mean of squared observations, Est. Var.: Estimated variance, PMV: Percentage of molecular variance, PhiRT: Proportion of the total genetic variance between the Kerala and Tamil Nadu regions, PhiPR: Proportion of the total genetic variance among forest divisions within a region, PhiPT: Proportion of the total genetic variance among individuals within forest divisions, P: Probability is based on standard permutation across the full data set.

Table 4: Genetic diversity within the populations of *Dalbergia latifolia.*

S. No.	Forest division (s)	Region	*n*	Na±SE	Ne±SE	I±SE	He±SE	uHe±SE	PPL
1	Palakkad+Chalakudy	Kerala	2	0.796±0.086	1.238±0.032	0.203±0.027	0.139±0.018	0.186±0.025	33.63
2	Kannur	Kerala	2	0.611±0.078	1.156±0.028	0.134±0.024	0.092±0.016	0.122±0.022	22.12
3	KFRI	Kerala	2	0.389±0.063	1.075±0.021	0.064±0.018	0.044±0.012	0.059±0.016	10.62
4	Erode	Tamil Nadu	4	0.788±0.087	1.207±0.032	0.180±0.025	0.120±0.017	0.137±0.020	33.63
5	Dharmapuri	Tamil Nadu	2	0.469±0.062	1.063±0.019	0.054±0.016	0.037±0.011	0.049±0.015	8.85
6	Salem	Tamil Nadu	4	0.558±0.078	1.110±0.023	0.108±0.020	0.070±0.013	0.080±0.015	22.12
	Mean±SE			0.602±0.036	1.142±0.032	0.124±0.011	0.084±0.009	0.105±0.006	21.83±4.37

SE: Standard error, N: Number of accessions, Na: No. of different alleles, Ne: No. of effective alleles, I: Shannon's information index, He: Expected heterozygosity, uHe: Unbiased expected heterozygosity, PPL: Percentage of polymorphic loci.

Table 5: Genetic diversity within the populations of *Dalbergia sissoides.*

S. No	Forest Division (s)	Region	*n*	Na±SE	Ne±SE	I±SE	He±SE	uHe±SE	PPL
1	Nemmara	Kerala	4	0.522±0.073	1.109±0.024	0.097±0.020	0.065±0.014	0.074±0.016	17.70
2	Chalakudy	Kerala	3	0.504±0.072	1.118±0.027	0.098±0.021	0.067±0.015	0.080±0.017	16.81
3	Mannarkad+Palakkad	Kerala	3	0.664±0.079	1.121±0.022	0.121±0.021	0.078±0.014	0.094±0.016	23.89
4	Malayattoor	Kerala	3	0.584±0.077	1.119±0.024	0.112±0.021	0.074±0.014	0.088±0.017	21.24
5	Kannur	Kerala	2	0.434±0.066	1.088±0.022	0.075±0.019	0.051±0.013	0.068±0.017	12.39
6	Munnar	Kerala	4	0.752±0.086	1.187±0.030	0.167±0.024	0.111±0.017	0.126±0.019	31.86
7	Thiruvananthapuram	Kerala	2	0.336±0.053	1.031±0.014	0.027±0.012	0.018±0.008	0.024±0.011	4.42
8	Ranni+Konni	Kerala	3	0.673±0.084	1.177±0.030	0.157±0.024	0.105±0.016	0.126±0.020	28.32
9	Nilambur	Kerala	2	0.389±0.066	1.088±0.022	0.075±0.019	0.051±0.013	0.068±0.017	12.39
10	Wayanad	Kerala	5	0.876±0.087	1.179±0.028	0.174±0.023	0.112±0.016	0.124±0.017	37.17
11	Coimbatore	Tamil Nadu	3	0.788±0.086	1.219±0.033	0.187±0.026	0.126±0.018	0.152±0.021	32.74
12	Theni	Tamil Nadu	2	0.434±0.070	1.106±0.024	0.091±0.020	0.062±0.014	0.083±0.019	15.04
13	Tirunelveli	Tamil Nadu	2	0.372±0.064	1.081±0.021	0.070±0.018	0.048±0.012	0.064±0.017	11.50
14	Gudalur	Tamil Nadu	2	0.221±0.047	1.025±0.012	0.021±0.011	0.015±0.007	0.020±0.010	3.54
	Mean±SE			0.539±0.020	1.118±0.007	0.105±0.006	0.070±0.004	0.085±0.005	19.22±2.79

SE: Standard error, i: Number of accessions, Na: No. of different alleles, Ne: No. of effective alleles, I: Shannon's information index, He: Expected heterozygosity, uHe: Unbiased expected heterozygosity, PPL: Percentage of polymorphic loci.

Table 6: Genetic diversity among the populations of *Dalbergia latifolia.*

	1	2	3	4	5	6
1	-	0.896	0.917	0.917	0.82	0.932
2	0.109	-	0.906	0.938	0.83	0.944
3	0.086	0.099	-	0.95	0.847	0.949
4	0.087	0.064	0.052	-	0.883	0.947
5	0.198	0.186	0.166	0.124	-	0.847
6	0.07	0.058	0.053	0.055	0.166	-

1: Palakkad+Chalakudy, 2: Kannur, 3: KFRI seedlings, 4: Erode, 5: Dharmapuri, 6: Salem, below diagonal values are Nei genetic distances, above diagonal values are Nei genetic identities.

allele at a particular locus in a population. It was used to find the genetic diversity among and within the populations. Genetic diversity within populations was estimated as Shannon information index, unbiased heterozygosity, and percentage of polymorphic loci [Tables 4 and 5]. Genetic diversity among populations was estimated as Nei's genetic distance and genetic identity [Tables 6 and 7]. Similarly, genetic diversity between these species was estimated as Nei's genetic distance and genetic identity. AMOVA and Allele frequency analysis were performed using GenAlEx software (version 6.502) [25].

A mantel test was carried out to understand the correlation between the geographical and genetic distances of the *Dalbergia* species using GenAlEx software (version 6.502). The geographical distance was measured in Kilometers, whereas genetic distance was measured in Euclidean distance. The matrix table of geographical and genetic distances were created separately and used for this test [25].

3. RESULTS AND DISCUSSION

3.1. Estimation of Discriminatory Power of Selected RAPD Markers

Based on the banding patterns, RAPD markers were classified based on their efficiency to detect the polymorphism between the individuals and results were given in Table 2. A highly significant positive correlation between the expected heterozygosity and polymorphic information content (r = 0.857, P > 0.01) was observed. There is a significant positive correlation between the expected heterozygosity and resolving power (r = 0.733, P > 0.05) was noted. RAPD 1 marker showed high values of expected heterozygosity, polymorphic information content, and resolving power compared with other RAPD markers. Resolving power was the ability of a marker to detect the level of genetic variation between individuals. Hence, RAPD 1 marker showed a high level of genetic variation than other RAPD markers in the present study.

Table 7: Genetic diversity among the populations of *Dalbergia sissoides*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	-	0.944	0.985	0.968	0.949	0.948	0.935	0.966	0.937	0.969	0.907	0.959	0.966	0.931
2	0.057	-	0.969	0.931	0.919	0.939	0.9	0.927	0.902	0.925	0.913	0.95	0.921	0.893
3	0.015	0.032	-	0.977	0.967	0.964	0.955	0.96	0.943	0.963	0.917	0.959	0.97	0.922
4	0.032	0.071	0.023	-	0.991	0.974	0.946	0.976	0.972	0.966	0.919	0.95	0.967	0.935
5	0.052	0.085	0.033	0.009	-	0.96	0.928	0.961	0.977	0.942	0.893	0.93	0.956	0.93
6	0.053	0.062	0.036	0.027	0.041	-	0.942	0.993	0.969	0.972	0.943	0.952	0.957	0.916
7	0.067	0.105	0.046	0.056	0.075	0.06	-	0.921	0.907	0.936	0.9	0.917	0.917	0.866
8	0.034	0.076	0.041	0.024	0.04	0.007	0.083	-	0.98	0.975	0.919	0.952	0.976	0.925
9	0.065	0.103	0.059	0.028	0.023	0.032	0.098	0.02	-	0.955	0.899	0.924	0.95	0.926
10	0.031	0.078	0.038	0.035	0.06	0.028	0.066	0.025	0.046	-	0.936	0.948	0.946	0.923
11	0.098	0.091	0.087	0.085	0.113	0.058	0.105	0.084	0.106	0.066	-	0.937	0.896	0.883
12	0.042	0.052	0.042	0.051	0.073	0.049	0.087	0.05	0.079	0.054	0.065	-	0.955	0.934
13	0.034	0.083	0.03	0.034	0.045	0.044	0.087	0.025	0.051	0.056	0.11	0.046	-	0.946
14	0.071	0.113	0.081	0.067	0.073	0.088	0.144	0.078	0.077	0.08	0.125	0.068	0.055	-

1: Nemmara, 2: Chalakudy, 3: Mannarkad+Palakkad, 4: Malayattoor, 5: Kannur, 6: Munnar, 7: Thiruvananthapuram, 8: Ranni+Konni, 9: Nilambur, 10: Wayanad, 11: Coimbatore, 12: Theni, 13: Tirunelveli, 14: Gudalur; below diagonal values are Nei genetic distances, above diagonal values are Nei genetic identities.

The effective multiplex ratio was the number of polymorphic bands produced by polymorphic markers per assay. A significant negative correlation between the effective multiplex ratio and resolving power ($r = -0.708$, $P > 0.05$) was observed. RAPD 17 marker produced more polymorphic bands per assay. However, the produced bands were able to detect a low level of genetic variation than other RAPD markers.

The marker index was the overall usefulness of a marker or the product of an effective multiplex ratio and expected heterozygosity for the polymorphic bands in an assay. RAPD 15 marker showed high marker index value than the other markers. It means the RAPD 15 marker produced more polymorphic bands with high expected heterozygosity values per assay.

3.2. Population Structure Analysis of *D. latifolia* and *D. sissoides*

All samples were categorized into three genotypic groups based on Evanno method of population structure analysis using the Bayesian model. The most supported cluster number was K = 3 which showed a high delta K value (delta K = 17.07) in Evanno plot [Figure 3]. The population structure chart and map were derived based on the selected cluster number given in Figures 4 and 5.

There are 16 accessions coming under a unique genotypic or allelic group known as *D. latifolia* group. The accessions of *D. latifolia* group showed 1 to 11% of shared alleles with *D. sissoides* groups except for KLCHVEL-2 and KLKNTAL-3 which showed up to 24% of shared alleles. Similarly, *D. sissoides* groups showed 1–10% of shared alleles with *D. latifolia* group. It indicates the ancient admixture and further introgressive hybridization between *D. latifolia* and *D. sissoides* genotypic groups. It also indicates that both species were monophyletic groups. Similar results were reported in the subgroups of *D. cochinchinensis* in the Indochinese landscape [26].

There were 40 accessions coming under *D. sissoides* groups known as *D. sissoides* Group-1 and 2. *D. sissoides* Group-1 genotypic group has 21 accessions among them KLMYKUT-3 showed a low number of shared alleles whereas other accessions showed a wide range of shared alleles up to 50% (KLCHVEL-5, KLNMNEL-6) with Group-2. Similarly, *D. sissoides* Group-2 genotypic group has 19 accessions and among them, KLMUADI-2 showed a low number of shared alleles, whereas many other accessions showed a wide range of shared alleles up to 50% (KLTMPLO-4) with Group-1. It indicates a recent intermixing between *D. sissoides* Group-1 and Group-2. Hartvig *et al*. and Liu *et al*. reported similar results in the subgroups of *D. oliveri* and *D. odorifera* in the Indochinese landscape and Hainan Island of China, respectively [26,27].

3.3. Principal Coordinates Analysis of *D. latifolia* and *D. sissoides*

A total of 56 samples were divided into two clusters by the principal coordinates analysis [Figure 6]. *D. latifolia* cluster has 16 accessions from Palakkad, Chalakudy, Kannur Forest divisions of Kerala and Erode, Salem, Dharmapuri Forest divisions of Tamil Nadu along with check accessions (KFRI-1 and KFRI-2). *D. sissoides* cluster has 40 accessions from Palakkad, Nemmara, Chalakudy, Mannarkad, Malayattoor, Kannur, Munnar, Thiruvananthapuram, Konni, Ranni, Nilambur, Wayanad Forest divisions of Kerala and Coimbatore, Theni, Tirunelveli, and Gudalur Forest divisions of Tamil Nadu.

Many *D. latifolia* individuals (12 out of 16) were found only in Erode, Salem, and Dharmapuri forest divisions which come under the Eastern Ghats of Tamil Nadu and few of them were found in Palakkad, Chalakudy, and Kannur Forest divisions which fell under the Western Ghats of Kerala. All *D. sissoides* individuals were found only in the Western Ghats of Kerala and Tamil Nadu. Therefore, Palakkad, Chalakudy, and Kannur Forest divisions were considered as "Junctions" of *D. latifolia* and *D. sissoides*.

Principal coordinates analysis showed the distinct clusters of *D. latifolia* and *D. sissoides* without any intermediate individuals. It indicated that there was no recent gene flow between these species. It may be due to the phenological reproductive isolation between the two species [28]. This analysis also showed that *D. latifolia* cluster was formed with a single genotypic group whereas *D. sissoides* cluster was made up of two overlapping genotypic groups with some intermixed individuals. Therefore, *D. sissoides* individuals showed a wide range of morphological variations in leaves.

3.4. Neighbor Joining Cluster Analysis of *D. latifolia* and *D. sissoides*

The unweighted Neighbor Joining dendrogram showed the relationship between the 56 individuals of *D. latifolia* and *D. sissoides*. The high polymorphic RAPD markers divided the samples into two main clusters known as *D. latifolia* and *D. sissoides* clusters with a moderately significant bootstrap value (85%). The markers also showed strong significant bootstrap values between the individuals of Dharmapuri (99%) and Gudalur (95%) forest divisions whereas weak significant bootstrap values between the individuals of Thiruvananthapuram (58%) and Salem (51%) forest divisions.

The branch length of an individual in a dendrogram was directly proportional to the mutational changes of an individual. All individuals of *D. latifolia* cluster showed a high rate of mutation than the individuals of *D. sissoides* cluster. It indicated that *D. latifolia* was more primitive than *D. sissoides*. The genome size variation study of *D. latifolia* and *D. sissoides* showed similar results in Karnataka, India [4]. All accessions of *D. latifolia* cluster showed a single lineage. Palakkad accession (KLPKWAL-1) of *D. latifolia* showed a high rate of mutation compared to other individuals and therefore, it is proved as the oldest among the accessions.

The individuals of *D. sissoides* cluster were subdivided into five subgroups which might have evolved from different lineages at different periods of time. Some individuals of Subgroups D and C showed a high rate of mutation since they are older than Subgroups A, B, and E. In Subgroup D, the accessions of the Coimbatore Forest division (TNCBBOL-4, TNCBBOL-1 and TNCBBOL-6) showed a high rate of mutation and comes under the *D. sissoides* Group-2. Therefore, the Coimbatore Forest division can be considered as the point of origin of *D. sissoides* Group-2. Subgroup D members were further distributed to Wayanad, Munnar, and Ranni Forest divisions.

In Subgroup C, the accessions of Konni (KLKOKON-1), Nilambur (KLNLKAR-2), and Munnar (KLMUNER-2) forest divisions showed a high rate of mutation and comes under the Group-1 genotypic group of *D. sissoides* except for the accessions of Thiruvananthapuram. Therefore, the Group-1 genotypic group has multiple origin points on the western side of the Western Ghats. It may be due to seed dispersal by wind and/or water at the time of south-west monsoon in Kerala. This group was further distributed to Kannur, Malayattoor, Tirunelveli, Theni, Nemmara, and Walayar Forest divisions.

The accessions of Nemmara and Theni divisions came under Subgroup B which contains both genotypic groups of *D. sissoides*. Similarly, the accessions of Chalakudy, Malayattoor and Mannarkad forest divisions come under Subgroup E. However, the accessions of the Gudalur Forest division fall under Subgroup A which contains only the Group-1 genotypic group.

The accessions of Thiruvananthapuram (KLTMPLO-4), Nemmara (KLNMNEL-6), and Chalakudy (KLCHVEL-5) showed less mutation rate in the dendrogram with more shared alleles [Figure 3]. It indicates the recent admixture of *D. sissoides* groups in the above forest divisions. *D. sissoides* accession (KLPKWAL-5) of Palakkad Forest division showed a very less mutation rate. Interestingly, the oldest *D. latifolia* (KLPKWAL-1) and youngest *D. sissoides* (KLPKWAL-5) were found in the same forest division (Palakkad).

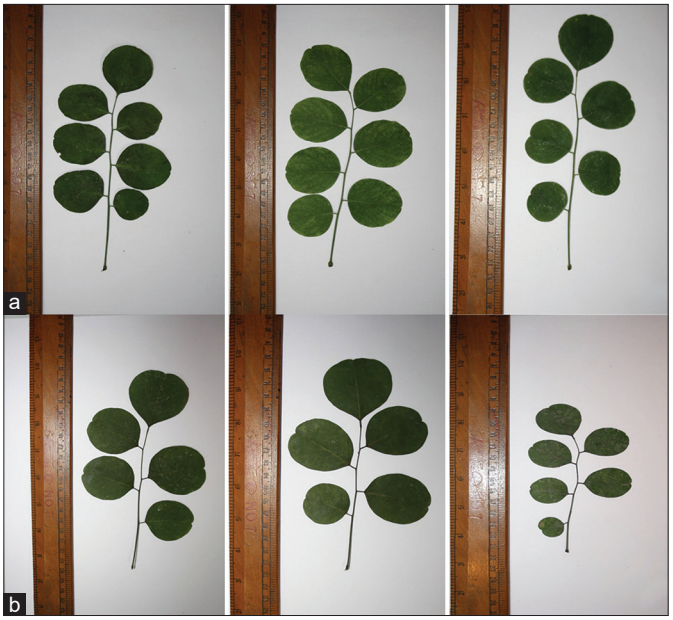

Figure 8: The leaves of *Dalbergia latifolia* and *Dalbergia sissoides* showed the species complex. (a) leaves of *D. latifolia* with retuse and/or leaflet apex. (b) leaves of *D. sissoides* with retuse and/or leaflet apex.

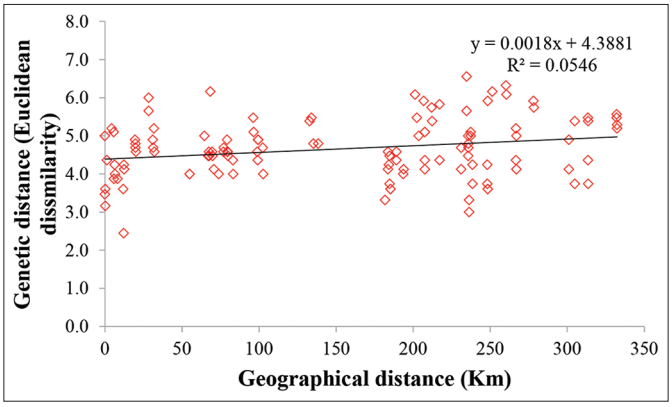

Figure 9: The correlation between the genetic and geographical distances of *Dalbergia latifolia* accessions.

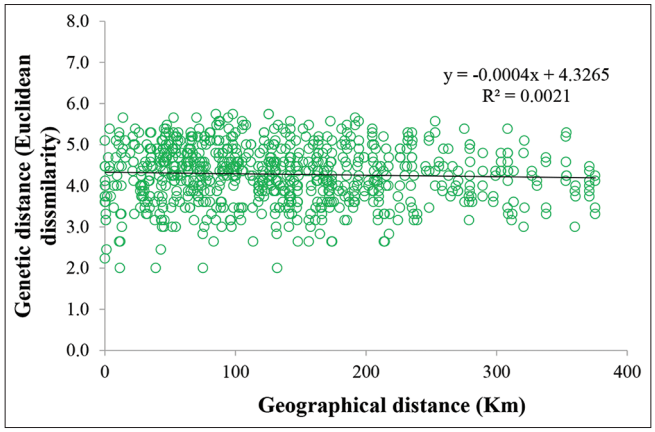

Figure 10: The correlation between the genetic and geographical distances of *Dalbergia sissoides* accessions.

3.5. AMOVA of *D. latifolia* and *D. sissoides*

Palakkad and Chalakudy Forest divisions had only a single individual of *D. latifolia*. Hence, both divisions were combined together and treated as "Palakkad + Chalakudy" Forest division in this analysis. Similarly, Konni and Palakkad Forest divisions had single individuals of *D. sissoides*. Hence, both divisions were combined with the nearby forest division and treated as "Konni + Ranni" and "Palakkad + Mannarkad" Forest divisions in this analysis.

D. latifolia samples of Kerala (six out of 16) and Tamil Nadu (10 out of 16) regions did not show any genetic variations. It indicated that *D. latifolia* accessions of Eastern Ghats and Western Ghats were genetically similar. However, *D. sissoides* showed a low genetic variation (6%) between the western side (Kerala) and eastern side (Tamil Nadu) of the Western Ghats. *D. latifolia* and *D. sissoides* showed low genetic variance (20 and 11%, respectively) among the forest divisions and expressed more genetic variance (80 and 83%, respectively) within the forest divisions [Table 3]. Yang *et al*. reported that cross-pollinated species show more genetic variation between individuals within populations than among populations and among regions [29]. *D. latifolia* and *D. sissoides* were preferred cross-pollination due to self-incompatibility which affected the seed formation [28]. Within and among populations, genetic diversity of *D. latifolia* and *D. sissoides* was presented in Tables 4-7, respectively. The Nei's unbiased genetic identity value between these species is 0.943 whereas Nei's unbiased genetic distance value between these species is 0.059. It indicated the close genetic relationship between *D. latifolia* and *D. sissoides.*

The geological and geochemical studies proved that the Eastern Ghats were older than the Western Ghats. Eastern Ghats were formed during the Columbia amalgamation period, between 1.64 and 1.57 Giga annum whereas Western Ghats were formed during the Cretaceous-Tertiary boundary period around 67 Mega annum [30,31]. Therefore, the absence of *D. sissoides* in the Eastern Ghats was another best evidence to presume that *D. latifolia* was more primitive than *D. sissoides*.

The leaflet apex of *D. latifolia* was retuse and/or obtuse in shape whereas the leaflet apex of *D. sissoides* was acutely shaped [Figure 1]. The acute leaflet apex was found in *D. sissoides* accessions of Tirunelveli, Gudalur Forest divisions of Tamil Nadu and Mannarkad, Malayattoor Forest divisions of Kerala. It may be due to the high rainfall in the Western Ghats [32]. However, 32 out of 40 *D. sissoides* accessions showed the retuse and/or obtuse leaflet apex in the Western Ghats. It formed a species complex with *D. latifolia* [Figure 8] and species identification based on the morphological characters becomes very difficult and caused misidentification.

3.6. Mantel test of *D. latifolia* and *D. sissoides*

Mantel test of *D. latifolia* showed a non-significant ($P > 0.05$) positive correlation ($r = 0.234$, $P = 0.09$) between the genetic and geographical distances in Figure 9. Similarly, Andrianoelina *et al*. reported a positive correlation between the genetic and geographical distances in *D. monticola* at Madagascar [33]. Mantel test of *D. sissoides* showed a non-significant ($P > 0.05$) negative correlation ($r = -0.045$, $P = 0.25$) between the genetic and geographical distances in Figure 10. *D. sissoides* showed a low level of geographical differentiation than its primitive species (*D. latifolia*). However, both species showed a high genetic diversity within a 50 km distance due to their self-incompatibility habit [28].

4. CONCLUSION

RAPD marker was basically the dominant DNA marker that cannot distinguish the homozygous and heterozygous alleles. However, it was a quick, simple and efficient method to detect species diversity and population structure since DNA probes and sequence information for the design of specific primers were not required. The present study has highlighted the fact that molecular markers play important role in the identification of a species and are highly needed to explore the population genetics in closely related species. Hence, the study will be useful for species identification, the mapping of populations, species conservation, and further genetic improvement programs for *D. latifolia* and *D. sissoides*.

5. ACKNOWLEDGMENT

The authors are thankful to The Director, Institute of Forest Genetics and Tree Breeding, Coimbatore, for providing the facilities and support during the study. The authors are also thankful to the Kerala and Tamil Nadu Forest Departments for granting permission to conduct the research in the forest areas and for their support rendered during the field visits.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

The present study was financially supported by Indian Council of Forestry Research and Education, Dehra Dun, Uttarakhand, India under the project NFRP-166.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

'The binary data and gel images used for this study were provided at https://doi.org/10.6084/m9.figshare.21723494.v3.

11. PUBLISHER'S NOTE

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How to cite this article:
Vasudevan R, Thangamani D, Lalitha S, Sasidharan KR. Population structure and genetic diversity analysis of *Dalbergia latifolia* and *Dalbergia sissoides* using RAPD markers. J App Biol Biotech. 2023;11(5):59-69.
DOI: 10.7324/JABB.2023.11507