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Gender-based Genetic Variability of *Ailanthus excelsa* Roxb, Populations Using, RAPD, ISSR and SCoT Markers

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Authors' contributions

All authors contributed to the experiment design. Authors SB, SA and MC conducted lab experiments. Author SB analyzed the data and wrote the first manuscript. Authors MC and UKT reviewed and editing to the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Ailanthus excelsa Roxb. is an economically important and multipurpose dioecious tree species of India, mainly used for fodder and timber. Gender-based genetic diversity of five populations of two sites (Jodhpur, Rajasthan and Deesa, Gujarat) of *A. excelsa* was assessed. A total of 42 RAPD, 20 ISSR and 23 SCoT primers were screened for DNA amplification of 232 individuals. Out of which only 25 primers (13 RAPD, 6 ISSR and 6 SCoT) were found polymorphic. The SCoT markers were showed the highest value for PIC, MI, Rp value, Nei's gene diversity and Shannon's index, as compared with the other two markers. Female individuals in all five populations had slightly higher genetic diversity as compared with male individuals. A high level of genetic diversity (55%) was detected within the populations of male and female individuals. High gene flow (6.70) and low genetic differentiation (0.069) values were found between Jodhpur and Deesa sites. Principal component analysis for all populations were accounted for 48.7% of the genetic variation. The Mantel test showed significant correlation (R = 0.178, *P* = .01) between genetic and geographic distances. The present study showed that SCoT markers were best for genetic diversity assessment in *A. excelsa* over RAPD and ISSR markers. High gene flow and low genetic differentiation in *A. excelsa* indicates its poor population fragmentation despite long geographic distances.

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1. INTRODUCTION

Ailanthus excelsa Roxb. (Ardu or Tree of Heaven) is an economically important, fast-growing tree. It has been reported as dioecious or polygamo-dioecious species with male, female and bisexual flowers [1,2]. It belongs to the family Simaroubaceae. It is widely distributed in Central India and grows in the semi-arid and semi-moist regions [3]. It is a priority species for the safety match industry in Tamilnadu and an excellent fodder material of semi-arid and semi-moist regions [4], as well as the use of stem bark in traditional medicines [5]. Being such an important species, the Indian Council of Forestry Research and Education (ICFRE), the Government of India has initiated research programs in recent years and constituted a task force to evaluate and draft DUS test guidelines on *A. excelsa*.

Advancement in molecular markers and sequencing technology changed the fate of plant sciences and various types of molecular marker techniques have been developed according to their advantages [6]. Molecular markers have been used successfully for the identification of genetic diversity in various crop plants [7,8]. Assessment of genetic diversity is essential for the improvement and conservation of valued species [9]. Molecular markers are a very useful tool for the identification of genotypes, assessing and exploiting the genetic variability in many tree species. Several molecular marker techniques such as randomly amplified polymorphic DNA (RAPD), amplified length polymorphism (AFLP), inter simple sequence repeats (ISSR), simple sequence repeats (SSR), start codon targeted (SCoT) markers, were used to assess genetic diversity in dioecious tree species. Kulhari et al. [10] studied genetic variability in 52 accessions of *Commiphora wightii* using 37 RAPD and 43 ISSR markers. They found that RAPD markers (74.16%) were more efficient for detecting polymorphism than ISSR markers (62.52%) and indicated the existence of wide genetic variability in this species. Similarly, Kumar and Agrawal [11] studied genetic diversity in dioecious *Trichosanthes dioica* Roxb. A total of 22 ISSR and 22 SCoT markers were used to analyze genetic diversity and population structure. ISSR marker was found to be more informative than SCoT markers. The male plants of the *T. dioica* were more diverse than female plants. In the case of *A. excelsa* only a single publication is available in which the genetic diversity from eight seed sources of five states was assessed by using thirteen RAPD markers [12]. They have not estimated genetic diversity in male and female individuals/groups. Dioecious trees showed different levels of genetic diversity in male and female individuals. The present study focuses on the male and female genetic diversity of *A. excelsa*, in five populations using RAPD, ISSR and SCOT markers and their genetic relationship.

2. MATERIALS AND METHODS

Two sites namely, Jodhpur, Rajasthan and Deesa, Gujarat, located about 400 km apart were selected due to the non-availability of natural populations of *A. excelsa*. Five plantations, two at Rajasthan and three at Gujarat were selected from these two sites. Each population was a mixture of male and female individuals. It was also noticed during this study that no hermaphrodite individuals were recorded. Each individual was marked for gender as identified by the flowers before collecting the samples for DNA analysis. The details of different populations, the number of female and male individuals and their codes are given in Table 1. The GPS locations of individual genotypes were recorded for analysis of the correlation between geographic and genetic distances. A total of 42 RAPD, 20 ISSR and 23 SCoT primers were screened for DNA amplification of male and female individuals.

Young leaves were collected from these sampled individual trees and washed, dried and then crushed in liquid nitrogen for DNA extraction. Genomic DNA was extracted from 500 mg leaf samples by the CTAB method [13] with an additional one purification step of Phenol: Chloroform: isoamyl alcohol (25:24:1). After purification DNA pellet was dissolved in 50 µl of TE buffer and stored at -20°C for further use. The yield and purity of isolated DNA were determined using Nano-spectrophotometer (Denovix). The purified DNA, 30 ng/µl concentration was used for amplification with RAPD, ISSR and SCOT primers in a polymerase chain reaction (PCR). Amplification was carried out in 25 µl reaction mixture containing 16.8 µl of molecular grade water (Hi-media), 2.5 µl of 1X PCR buffer with $MgCl_2$ (Sigma Aldrich),2 µl of 2mM dNTPs mix (Thermo Scientific), 2.5 µl of 0.1 µM primer, 0.2 µl of 5U Taq DNA polymerase (Sigma Aldrich) and 1 µl of 30 ng/µl of template

DNA. The amplification reaction was carried out in a thermocycler (Agilent Biotech, sure cycler 8800). PCR cycling conditions for amplification of RAPD, ISSR and SCOT primers for 35 cycles were consisted of an initial denaturation at 94°C for 4 min followed by 1 min denaturation at 94°C, 1 min annealing at 35°C for RAPD, 46-52°C for ISSR and 50°C for SCOT, 1 min elongation at 72°C and finally single extension cycle of 5 min at 72°C to all the three markers. Agarose gel electrophoresis was performed using 1.4% agarose gel according to the protocol developed by Sambrook and Russell [14] and Thermo Scientific Gene Ruler 100 bp Plus DNA ladder was used as a size marker.

Each amplified DNA band was treated as a unit character and this was scored 1 as present or 0 as absent. The data matrix was constructed using this binary data. The discriminative power of RAPD, ISSR and SCoT primers were evaluated using three parameters, i.e., Polymorphic information content (PIC), Marker index (MI) and Resolving power (Rp) according to Roldan-Ruiz et al. [15], Powell et al. [16], Prevost and Wilkinson [17], respectively. The POPGENE software version 1.31 [18] was used to calculate the observed number of alleles (Na), the effective number of allele (Ne), Nei's genetic diversity (h), Shannon's information index (I), coefficient of gene differentiation (Gst) and the estimate of gene flow (Nm). Data were also analyzed for the Analysis of molecular variance (AMOVA), Principal Coordinates Analysis (PCOA) and Mantel test using the program GenAlEx version 6.503 [19].

3. RESULTS AND DISCUSSION

RAPD, ISSR and SCOT primers generated data were analyzed for marker polymorphism. Only thirteen RAPD, six ISSR and six SCoT primers were found polymorphic. All three combined markers generated a total of 201 loci. Out of which, 182 loci revealed 90.5% polymorphism for all five populations. The sizes of the amplified products generated by all primers were varied from 200 to 2000 bp. The average number of polymorphic loci per primer was 7. The mean PIC, MI and Rp values observed for combined data of all the 25 primers were 0.44, 7.6 and 8.7, respectively. Out of three markers, the SCoT marker showed better result for genetic diversity assessment and gave the highest value for PIC, MI and Rp. A total number of bands (TB), total numbers of polymorphic bands (PB), percentage of polymorphism (%P), PIC, MI and Rp is estimated by PCR products data generated by selected primers given in Table 2. The SCoT marker resulted in the highest values for polymorphism (91.7%), PIC (0.45), MI (8.17) and Rp value (9.11). Gorji et al. [20] also studied the efficiency of SCoT, ISSR and RAPD markers for tetraploid potato species and found the highest PIC value for the SCoT marker (0.4) as compared with ISSR (0.34), then RAPD (0.28). In another study on *L. esculentum* Mill. [21] revealed that the SCoT marker produced higher polymorphism (36.14%), PIC (0.142) and Rp (1.88) value as compared with the ISSR marker polymorphism (23-25%), PIC (0.088) and Rp value (1.55). On contrary, Osman and Ali [22] found slightly higher polymorphism (96.81%) and PIC (0.305) for the ISSR marker as compared with SCoT marker (polymorphism; 96% PIC;0.302) and RAPD marker (polymorphism; 94.2%, PIC;0.301). However, Collard and Mackill [23] suggested the use of SCoT markers being highly reproducible and more useful for targeted quantitative trait loci (QTL) mapping.

In the present study, combined data of all three markers revealed that the two populations of Jodhpur were more diverse (J1F: h=0.256, I=0.372; J1M: h=0.241, I=0.352 and J2F: h= 0.236, I=0.342 J2M: h=0.211, I=0.306) as compared with three populations of Deesa (D1F: h=0.218, I=0.321and D1M: h=0.195, I=0.292; D2F: h=0.229, I=0.338 and D2M: h=0.212, I=0.309; D3F: h=0.225, I=0.329 and D3M: h=0.167, I=0.245). In all three-markers tested, female individuals were showed slightly higher genetic diversity as compared with male individuals for all five populations (Table 3 a and

Table 1. Sampling location details of different populations of *Ailanthus excelsa*

Sites	Populations	Longitude	Latitude	Number of trees				
				Total	Female	Code	Male	Code
Jodhpur, Rajasthan	J 1	26.46528	72.35750	52	26	J1F	26	J1M
	J 2	26.43250	72.25667	30	15	J2F	15	J2M
Deesa, Gujarat	D 1	24.29563	72.20821	72	38	D1F	34	D1M
	D 2	24.47806	72.30694	40	20	D2F	20	D2M
	D 3	24.47806	72.30694	38	20	D3F	18	D3M
Total				232	119		113	

Table 2. Selected primers detail and primer parameters calculated for all three markers (RAPD, ISSR and SCOT) using five populations of *A. excelsa*

S. No	Primers	Sequence	TB	PB	% P	PIC	MI	Rp
1	OPD-11	AGCGCCATTG	6	5	83.3	0.40	8.99	6.73
2	OPE-07	AGATGCAGCC	11	11	100.0	0.33	5.55	17.47
3	OPB-05	TGCGCCCTTC	4	3	75.0	0.37	10.50	4.02
4	OPE-04	GTGACATGCC	5	4	80.0	0.34	9.82	5.81
5	OPC-05	GATGACCGCC	8	8	100.0	0.49	9.48	8.81
6	OPC-10	TGTCTGGGTG	8	7	87.5	0.47	7.30	7.84
7	OPN-06	GAGACGCACA	9	9	100.0	0.42	7.88	12.54
8	OPN-16	AAGCGACCTG	10	10	100.0	0.48	6.86	11.73
9	OPA-02	TGCCGAGCTG	8	7	87.5	0.49	7.24	7.32
10	OPW-05	GGCGGATAAG	10	10	100.0	0.50	6.12	9.26
11	OPU-19	GTCAGTGCGG	7	6	85.7	0.49	8.40	6.11
12	OPG-05	CTGAGACGGA	8	7	87.5	0.49	7.78	7.33
13	OPN-13	AGCGTCACTC	7	5	71.4	0.47	4.45	4.74
	Mean		8	7	89.0	0.44	7.72	8.43
1	ISSR-3	GCG(GT)6	10	8	90.0	0.50	5.23	11.47
2	ISSR-4	(GA)7GAT	7	6	75.0	0.49	4.89	5.27
3	ISSR-8	(TG)8GC	10	10	100.0	0.50	6.87	10.69
4	ISSR-11	GGG(GT)6	8	7	87.5	0.42	7.11	9.22
5	ISSR-13	(GACA)4	8	8	100.0	0.50	9.23	9.54
6	ISSR-16	GC(TC)7T	9	7	77.7	0.38	5.15	9.42
	Mean		8	7	88.4	0.44	6.58	8.83
1	SCOT-1	$(CAA)_2TGGCTA(CCA)_2$	6	6	100.0	0.49	11.33	6.74
2	SCOT-11	$AAGCAATGGCTA(CCA)_2$	7	6	85.7	0.39	7.95	8.31
3	SCOT-14	ACGACATGGCGACCACGC	6	5	83.3	0.50	7.07	4.51
4	SCOT-15	ACGACATGGCGACCGCGA	7	7	100.0	0.46	10.47	9.06
5	SCOT-18	$ACCATGGCT(ACC)_2GCC$	13	12	92.3	0.44	4.94	15.41
6	SCOT-21	ACGACATGGCGACCCACA	9	8	88.8	0.42	7.30	10.65
	Mean		8	7	91.7	0.45	8.17	9.11

b). The t-values for Nei's genetic diversity (h) and Shannon index (I) were 2.011 and 2.056, respectively at $P = .07$ significant level. Similar to our result in *Myrica rubra* [24] and *Salix vimnalis* [25] genetic diversity was slightly higher in females than male individuals. However, some of the male individuals such as *Hippophae rhamnoides* L. [26] and *Pistacia atlantica* [27] had higher genetic diversity than female individuals. The genetic diversity of a species is influenced by many factors, such as geographical range, seed dispersal mechanism, pollination method, life forms, breeding method, etc. The reproductive system and species history have a direct impact on the distribution of genetic diversity, genetic divergence and genetic structure [28]. Therefore, the genetic diversity of any tree species is dependent on its species structure and their climatic conditions in which they grew. During our study, we observed that the number of female individuals were relatively slightly higher than males as it appears that they were better adapted to our experimental arid zones. This may be one of the reasons that we have slightly higher genetic diversity in female individuals. A similar reason was found in male individuals of *Pistacia atlantica* [27], who had higher genetic diversity than females because they also had higher numbers of male individuals.

Analysis of molecular variance (AMOVA) for the pooled data of RAPD, ISSR and SCoT markers revealed that out of the total observed variances, 45% was related to the variation between population and 55% was related to the variation within the populations of male and female individuals (Table 4). Similarly, the high values of within-population genetic diversity were also found in *M. rubra* [24], *S. vimnalis* [25], *Juniper thurifera* [29]. A high value of within-population genetic diversity in *A. excelsa* is expected because dioecious species have low genetic differentiation and high intrapopulation genetic diversity due to cross-pollination nature [28].

The coefficient of genetic differentiation (G_{ST}) and gene flow (Nm) amongst five populations of male and female individuals were estimated (Table 3 a and b). The G_{ST} value in J1 and J2 populations were 0.036, 0.096 and Nm values 13.04, 4.68, respectively, while the G_{ST} values in the three populations of D1, D2 and D3 were 0.074, 0.087, 0.109 and Nm values 6.22, 5.25

and 4.07, respectively. The value of G_{ST} between both sites (Jodhpur and Deesa) was low (0.069) and the Nm value was high (6.70). Similarly, in other dioecious tree species such as *Trichosanthes dioica* (G_{ST}-0.05; Nm-9.32), *S. viminalis* (F_{ST}-0.08; *h*-0.75) and *Antennaria dioica* (F_{ST}-0.11, *h*-0.18-0.22) high levels of genetic diversity and low genetic differentiation value were reported [11, 25, 30]. Hamrick and Godt [28] suggested that the obligate cross-pollination nature of dioecious species exhibits higher gene flow rates and lower genetic differentiation values. High gene flow in *A. excelsa* indicates its poor population fragmentation in spite of the long geographical distances. High genetic diversity and low genetic differentiation value in the species can also arise from population history [30].

A principal component analysis (PCoA) was performed to provide spatial representation of the relative genetic distances among individuals of each population using pooled data of all three markers. The first three principal coordinate components were accounted for 30.4, 11.5 and 6.7% of the total variation, respectively (cumulative value = 48.7%). Results based on these three components showed that populations J1, J2 and D1 formed a separate cluster whereas, D2 and D3 populations were partially overlapped. There was no separation of individuals based on gender in any population (Fig. 1). The Mantel test also support and showed a significantly positive correlation (R = 0.178, *P* = .01) between genetic and geographic distances in the individuals of five populations of *A. excelsa*.

Table 3. Genetic diversity, coefficient of genetic differentiation (G_{ST}) and gene flow (Nm) in the male and female individuals of *A. excelsa* using three molecular markers for both site populations

a) Jodhpur, Rajasthan

Population	Marker	Gender (No.)	Na	Ne	h	I	% P	G_{ST}	Nm
J 1	**RAPD**	Female (26)	1.574	1.406	0.228	0.332	57.43	0.04	10.32
		Male (26)	1.544	1.377	0.216	0.317	54.46		
		Both sexes (52)	1.594	1.407	0.233	0.342	59.41		
	ISSR	Female (26)	1.538	1.416	0.224	0.323	53.85	0.04	11.30
		Male (26)	1.538	1.385	0.216	0.315	53.85		
		Both sexes (52)	1.557	1.422	0.230	0.332	55.77		
	SCoT	Female (26)	1.854	1.627	0.351	0.510	85.42	0.02	24.93
		Male (26)	1.791	1.566	0.320	0.467	79.17		
		Both sexes (52)	1.854	1.609	0.342	0.500	85.42		
	Combine all marker	Female (26)	1.631	1.461	0.256	0.372	63.18	0.03	13.04
		Male (26)	1.602	1.424	0.241	0.352	60.20		
		Both sexes (52)	1.646	1.459	0.258	0.377	64.68		
J 2	**RAPD**	Female (15)	1.524	1.388	0.218	0.317	52.48	0.10	4.38
		Male (15)	1.475	1.338	0.193	0.283	47.52		
		Both sexes (30)	1.574	1.404	0.229	0.336	57.43		
	ISSR	Female (15)	1.576	1.425	0.239	0.346	57.69	0.12	3.56
		Male (15)	1.538	1.403	0.225	0.326	53.85		
		Both sexes (30)	1.673	1.465	0.264	0.388	67.31		
	SCoT	Female (15)	1.625	1.496	0.273	0.391	62.50	0.06	8.15
		Male (15)	1.520	1.438	0.234	0.333	52.08		
		Both sexes (30)	1.625	1.374	0.221	0.331	62.50		
	Combine all marker	Female (15)	1.562	1.423	0.236	0.342	56.22	0.09	4.68
		Male (15)	1.502	1.378	0.211	0.306	50.25		
		Both sexes (30)	1.616	1.439	0.248	0.362	61.69		

Na- observed number of alleles, Ne- number of effective alleles, h-Nei's genetic diversity, I-Shannon's index, %P-percentage of polymorphism

b) Deesa, Gujarat

Population	Marker	Gender (No.)	Na	Ne	h	I	% P	G_{ST}	Nm
D 1	**RAPD**	Female (38)	1.594	1.380	0.215	0.318	59.41	0.06	6.87
		Male (34)	1.544	1.325	0.191	0.286	54.46		
		Both sexes (72)	1.623	1.370	0.219	0.329	62.38		
	ISSR	Female (38)	1.519	1.330	0.191	0.283	51.92	0.094	4.80
		Male (34)	1.473	1.272	0.164	0.248	47.37		
		Both sexes (72)	1.596	1.336	0.205	0.312	59.62		
	SCoT	Female (38)	1.645	1.442	0.251	0.369	64.58	0.068	6.78

Population	Marker	Gender (No.)	Na	Ne	h	I	% P	G_{ST}	Nm
		Male (34)	1.604	1.373	0.219	0.326	60.42		
		Both sexes (72)	1.687	1.433	0.253	0.377	68.75		
	Combine all marker	Female (38)	1.587	1.382	0.218	0.321	58.71	0.074	6.22
		Male (34)	1.552	1.329	0.195	0.292	55.22		
		Both sexes (72)	1.631	1.376	0.224	0.336	63.18		
D 2	**RAPD**	Female (20)	1.555	1.375	0.213	0.313	55.45	0.101	4.40
		Male (20)	1.505	1.348	0.197	0.289	50.50		
		Both sexes (40)	1.613	1.396	0.228	0.339	61.39		
	ISSR	Female (20)	1.480	1.363	0.202	0.293	48.08	0.096	4.68
		Male (20)	1.461	1.395	0.210	0.299	46.15		
		Both sexes (40)	1.538	1.416	0.229	0.330	53.85		
	SCoT	Female (20)	1.812	1.472	0.291	0.439	81.25	0.053	8.85
		Male (20)	1.666	1.424	0.246	0.365	66.67		
		Both sexes (40)	1.833	1.465	0.284	0.431	83.33		
	Combine all marker	Female (20)	1.597	1.395	0.229	0.338	59.70	0.087	5.25
		Male (20)	1.532	1.378	0.212	0.309	53.23		
		Both sexes (40)	1.646	1.418	0.242	0.358	64.68		
D 3	**RAPD**	Female (20)	1.514	1.364	0.208	0.305	51.49	0.077	5.99
		Male (18)	1.435	1.308	0.177	0.259	43.56		
		Both sexes (38)	1.574	1.355	0.210	0.313	57.43		
	ISSR	Female (20)	1.596	1.436	0.246	0.357	59.62	0.173	2.38
		Male (18)	1.384	1.286	0.158	0.229	38.46		
		Both sexes (38)	1.692	1.422	0.247	0.369	69.23		
	SCoT	Female (20)	1.604	1.423	0.238	0.348	60.42	0.097	4.64
		Male (18)	1.395	1.275	0.157	0.230	39.58		
		Both sexes (38)	1.625	1.374	0.221	0.331	62.50		
	Combine all marker	Female (20)	1.554	1.397	0.225	0.329	55.72	0.109	4.07
		Male (18)	1.412	1.294	0.167	0.245	41.29		
		Both sexes (38)	1.616	1.377	0.222	0.332	61.69		

Na- observed number of alleles, Ne- number of effective alleles, h-Nei's genetic diversity, I-Shannon's index, %P-percentage of polymorphism

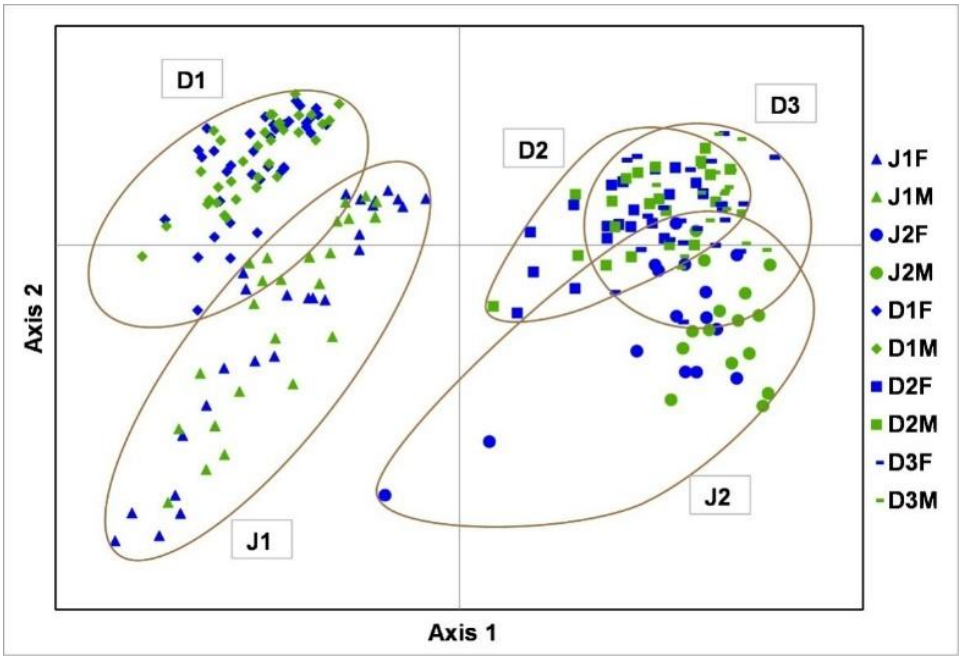

Fig. 1. Principal component analysis for the axis 1 vs 2 based on pooled data of RAPD, ISSR and SCoT for the *Ailanthus excelsa* five populations

Table 4. Summary of analysis of molecular variance (AMOVA) based on combined data of RAPD, ISSR and SCoT primers of 232 individuals of *A. excelsa*

Source	Df	SSD	Estimated variance	Total variance (%)	*P*= value
Among population	9	3466.39	15.94	45	.001
Within population	222	4329.73	19.50	55	
Total	231	7796.12	35.44		

4. CONCLUSION

The present study showed that the SCoT markers gave better results for genetic diversity assessment in *A. excelsa* over RAPD and ISSR markers. The genetic diversity was slightly higher in females than male individuals of all populations of *A. excelsa* under the studied arid conditions. High gene flow in this species indicates poor population fragmentation in spite of the long geographical distance. The Mantel test also support and showed a significant correlation between geographical and genetic distances among five populations of *A. excelsa*. Being a multipurpose and economically important tree species genetic improvement programs may be initiated with the strategy required for a dioecious species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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