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Molecular profiling and genetic relationship among *ber* (*Ziziphus* sp.) genotypes using RAPD markers

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Abstract

Genetic relationship study was performed with RAPD markers among 50 *ber* genotypes representing *Z. mauritiana*, *Z. nummularia* and *Z. spina-christi*. Out of 120 primers initially tested, 46 were highly reproducible and generated 368 RAPD markers with 86.2% polymorphism (316 polymorphic bands). The number of amplification product per primer ranged from 2 (OPF-9) to 17 (OPD-3) with an average of 8 bands per primer. The resolving power (Rp) for different primers ranged from 0.48 (OPE-4) to 9.37 (OPD-3) and polymorphic information content (PIC) ranged from 0.12 (OPD-20) to 0.82 (OPE-9). Nineteen primers distinguished at least one genotype that would prove to be highly useful for identification of genotype and designing future breeding strategy. Genetic relationships between the accessions were established based on Jacquard's similarity coefficient and it ranged from 26.3% to 78.9% suggesting that the *Ziziphus* germplasm is genetically diverse. UPGMA cluster analysis generated dendrogram with six clusters separating two wild genotypes, *Z. nummularia* (collection 1) and *Z. spina-christi* from rest of the genotypes. Cluster-I and II comprised of two genotypes each, whereas, the biggest cluster, cluster VI comprised of 20 out of remaining 44 genotypes. In cluster II to cluster-VI, genotypes were separated from each other at different similarity levels in successive branching. The present study has proved that *ber* genotype earlier reported to be similar based on morphology are genetically different. The degree of genetic variation detected in *Ziziphus* species with RAPD analysis in the present study suggests that it is an efficient marker technology for delineating genetic relationships among genotypes and estimating genetic diversity, thereby enabling the formulation of appropriate strategy for conservation and improvement programmes.

Key words: *Ziziphus* sp., genetic diversity, PIC, resolving power

Introduction

Ber (*Ziziphus mauritiana* L.) is cultivated all over the arid parts of Indian subcontinent. The tree is endowed with a high degree of edaphic and climatic adaptability and plays a major role in preventing soil erosion and desertification [1]. Its fresh fruits are rich in vitamins mainly C, A and B-complex [1]. South and Southeast Asia is considered as the centre for both evolution and distribution of the genus *Ziziphus* [2].

To assess genetic diversity in different plant species a large number of methodologies have been reported such as isozymes [3], allozymes [4] and seed storage proteins [5]. However, such traits are influenced by environmental factors and the developmental stage of the plant. Hence, the results elucidated based on such studies do not provide a true measure of the genetic diversity. Molecular markers based on Polymerase Chain Reaction (PCR) method offer several advantages over the conventional morphological markers. Random Amplified Polymorphic DNA (RAPD) uses arbitrary 10-base primers to amplify the random portions of the genome [6]. The fragments produced are easily visualized on ethidium bromide stained gel and polymorphism can be detected between the amplified products of different individuals. Although genetic diversity analysis and cultivar identification by RAPD and other molecular markers have been performed in many fruits [7, 8], its application in *Ziziphus* species and its relatives with exception of *Z. celata* [9] has not been carried out. Long juvenile phase of *Ziziphus* (up to 7 years) would make DNA markers an extremely useful tool for the identification of cultivars during propagation and growth. Cultivar identification using molecular markers would also aid in the management of germplasm collections of *Ziziphus*, as authenticity of many *ber* (*Z. mauritiana*) cultivars is unclear and the subject of some controversy. Moreover, because of the existence of a high level of morphological variability, morphological data can lead to ambiguous interpretations. We report here the RAPD markers to evaluate the genetic relationship among the 47 cultivars belonging to *Z. mauritiana*, two wild collections of *Z. nummularia* (designated as collection 1 and 2) and a single collection of *Z. spina-christi*. To our knowledge,

this is the first report on genetic diversity analysis of Indian *ber* using RAPD markers.

Materials and Methods

Plant material : Juvenile leaves of fifty genotypes (Table 1) were harvested in the month of May from single tree. The germplasm was maintained at Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi and Central Institute for Tropical Horticulture, Bikaner, India. Out of these 50 genotypes, *Z. nummularia* collection-2 was taken from Uttar Pradesh. The harvested leaves were immediately stored at –80°C until total genomic DNA was isolated using Cetyl-trimethyl Ammonium Bromide (CTAB) protocol [10] by adding PolyVinyl-Pyrrolidone (PVP).

Primer screening : One hundred and twenty RAPD primers (A, B, C, D, E, F series) from Operon Technology (Alameda, California, USA) were tested on three cultivars to identify primers that were giving good, scorable and polymorphic products. In order to check the reproducibility, two independent amplifications were carried out and finally 46 RAPD primers that showed clear and reproducible bands were chosen for further study.

PCR analysis and gel electrophoresis : PCR amplification was performed in a 25µl reaction volume, containing 1X reaction buffer (10mM Tris HCl pH 8), 1.5mM $MgCl_2$, 200µM of each dNTP, 1 unit of Taq polymerase, 30µM of primer and approximately 25ng genomic DNA template. Each genotype primer combination was amplified twice to check the reproducibility. Pre-screened primers were selected for the study of genetic relationships. The thermocycler (Perkin Elmer 480) was programmed for an initial step of 4 min at 94°C followed by 45 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C. A final extension step at 72°C was programmed for 7 minutes. The amplified products were stored at 4°C until loading.

Amplification products were separated on 1.4% agarose gels with 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA pH 8.0) at 3 V/cm for 4 hour. A 1 kb DNA ladder (MBI, Fermentas, USA) was used as a molecular size standard. Agarose gel was stained with 0.5µg/ml ethidium bromide solution and visualized under ultraviolet light and photographed with gel documentation.

Table 1. *Ziziphus* genotypes used for DNA fingerprinting and genetic relationship study

Species	Genotypes	No. of genotypes	Main areas of cultivation
Z. mauritiana Lam.	Sindhura, Seb, Vilayati, Sindhura Narwal, Kala Gola, Bawal Selection-1, Bawal Sel-2, Zg-3, Ilaichi Jhajhar, Rohtak Safeda, Kaithali, Sua, Govind Garh Selection	13	Haryana
	Katha, Sanauri, Chonchal, Pownda, Umaran, Lakhan, Pathan, Sanauri-3, Noki, Reshmi	10	Punjab
	Ilaichi, Muria Mehrara, Thornless, Banarasi Kadaka, Tass Bataso, Kishmish, Jogia, Hesang Tsaon, Kheera	9	Uttar Pradesh
	Desi Alwar, Nazuk, Bagwadi, Tikadi, Dandan, Katha Rajasthan	6	Rajasthan
	Narma, Popular Gola	2	Delhi
	Katha Bombay	1	Gujarat
	Gola	1	Rajasthan/Haryana
	Akhrota	1	Punjab/Haryana
	Chhuhara	1	Rajasthan/Punjab
	No-A	1	Haryana/Gujrat
	Kathi	1	Punjab/Gujrat/Rajsthan
	Wild collection	1	Delhi
Z. spina-christi Lam.	*Z. spina-christi*	1	Wild
Z. nummularia (Burm.f.) Willed.	*Z. nummularia* Collection 1	1	Wild
	Z. nummularia Collection 2	1	Wild (collected from Uttar Pradesh)

Data analysis : DNA bands in the profile were scored visually and recorded as present (1) or absent (0) and the binary qualitative data matrix was constructed. Data analysis was performed using the NTSYS-pc software (version 2.1). The genetic similarities were calculated using Jaccard's similarity coefficient.

The ability of a primer to distinguish large number of genotypes i.e. Resolving Power of the primer (Rp) = ΣI_b. Where, I_b is band informativeness = $1-|2 \times (0.5-p)|$ [11], where p being the proportion of the 50 genotypes possessing the band. The Rp of 46 RAPD

primers were determined in this way. The basic information about molecular markers that determines their application in genetic mapping was calculated for each marker using Polymorphism Information Content (PIC). PIC expresses the discriminating power of the locus by taking into account not only the number of alleles that are expressed but also the relative frequencies of alleles per locus, expressed as: $PIC = 1 - \Sigma pi^2$, where pi is the frequency of the ith (presence of band) allele [12].

Results and discussion

PCR based markers have been used to characterize a wide range of plant species, however, no such reports are available with *Z. mauritiana*, *Z. nummularia* and *Z. spina - christi*. In the present report, RAPD primers were used to study the variation in cultivars of *Z. mauritiana* and their relationship within themselves and also with *Z. nummularia* and *Z. spina-christi*. Genomic DNA samples extracted from different trees of the same varieties demonstrated the reproducibility of the RAPD technique. This contrasts with the reports demonstrating low level of reproducibility of RAPD analysis [13]. The present study suggests that selection of the primers and strict standardization of the protocol are crucial for reproducibility of results.

RAPD markers revealed considerable genetic diversity of *Ziziphus* species with genetic similarity ranging from 26.3% (between Chhuahara and Thornless) to 78.9%. (Narma and Vilayati). Nineteen primers produced genotype specific bands that would prove to be highly useful in identification of genotype and designing future breeding strategy. Thirteen out of these 19 primers were specific for *Z. nummularia* (collection 1) and/or *Z. spina-christi* (Table 2). While four primers *viz*., OPF-7, OPD-4, OPE-20 and OPF-13 has produced unique bands for Kala Gola, Thornless, Sua and Rohtak Safeda, respectively (Table 2). Two other primers, OPE-11 and OPE-13 amplified unique bands for Tikadi. These nineteen primers have also contributed significantly for high level of polymorphism (86.2%) in the fifty genotypes (Table 2). The results suggest that RAPDs are powerful markers and can be used for varietal identification and purification. Detail information about primers used, total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), resolving power (Rp), polymorphism information content (PIC) and size range of bands obtained are summarized in Table 2.

The resolving power (Rp) was calculated for all the 46 RAPD primers which ranged from 0.48 (OPE-4) to 9.37 (OPD-3) with an average of 3.20 per primer. Besides OPD-3 (9.37), the other RAPD primers *viz*., OPC-15, OPF-8, OPD-11 and OPE-6 also posses high Rp values of 6.08, 6.01, 5.76 and 5.68, respectively (Table 2). The primers with high Rp values were able to distinguish more number of *ber* genotypes. Polymorphism information content (PIC) was also calculated and it ranged from 0.12 (OPD-20) to 0.82 (OPE-9) with an average of 0.499 per primer. The primers with high PIC values OPE-13 (0.73), OPC-15 (0.72), OPF-2 (0.71) and OPF-8 (0.71) are presented in Table 2. The primers with high Rp and PIC values are more suitable for diversity analysis and fingerprinting of the *ber* genotypes. Graphical relationship between Rp (number of genotypes resolved by individual primer) and PIC (number of alleles analyzed per reaction) values of the primers (Fig. 1) showed linear relationship. The results are in accordance to Huang *et al.* [14] but in contrast to Prasad *et al.* [15] based on their study on wheat.

RAPD profiles : The 46 chosen primers generated a total of 368 fragments (316 polymorphic). Out of 46 polymorphic primers identified, eighteen primers showed 100% polymorphism (Table 2), whereas, the minimum level of polymorphism was shown by OPD-20 (42.86%) with average of 86.2% per primer indicating high marker index. The similar range of polymorphism has been reported by Singh *et al.* [16] based on their study using AFLP. The number of amplification product per primer ranged from 2 (OPF-9) to 17 (OPD-3) with an average of 8 bands per primer (Table 2). A representative DNA profile is presented in figure 2a and 2b.

Genetic relationship based on RAPD markers : The determination of genetic relationships between genotypes is important for *Ziziphus* improvement because till date no effort has been made in this direction. Initial selection and development of varieties has been done with vegetatively propagated material from one orchard to other and from one region to another. The establishment of volunteer seedlings from fallen fruits, consequent selection under differing environments and the lack of qualitative morphological markers has made confusion regarding varietal identity and trueness-to-type. These genetic variants can confound the evaluation of environmental and genetic factors in *ber* research and also can have serious economic ramification for *ber* growers. The taxonomic identity of the common cultivated types of *ber* is confusing [17] and the widely cultivated varieties are very similar with each other [18]. As a consequence, it is difficult to separate them merely on botanical informations. Mixing of the two phenotypically similar genotypes occurs during propagation/nursery stage and disposal of the samples.

The UPGMA dendrogram obtained from the cluster analysis using Jaccard similarity coefficient showed four

Table 2. List of RAPD primers used in the study and their properties on *ber* genotypes

Sl No.	Primer designation	Sequence	TNB*	PNB	P%	Genotype distinguished	Rp	PIC	Size range (bp)
1	OPC-2	5'-GTGAGGCGTC-3'	8	4	50.00	-	1.20	0.27	200-700
2	OPC-3	5'-GGGGGTCTTT-3'	9	7	77.78	-	3.40	0.54	200-900
3	OPC-4	5'-CCGCATCTAC-3'	6	4	66.67	-	2.96	0.45	180-350
4	OPC-6	5'-GAACGGACTC-3'	8	7	87.50	-	3.76	0.51	190-800
5	OPC-7	5'-GTCCCGACGA-3'	7	7	100.00	-	2.52	0.42	500-1500
6	OPC-8	5'-TGGACCGGTG-3'	7	6	85.71	-	3.24	0.48	250-1500
7	OPC-9	5'-CTCACCGTCC-3'	3	3	100.00	-	0.68	0.41	700-1500
8	OPC-10	5'-TGTCTGGGTG-3'	6	5	83.33	*Z. nummularia,Z. spina-christi*	2.24	0.35	450-2000
9	OPC-11	5'-AAAGCTGCGG-3'	10	6	60.00	*Z. nummularia* (2), *Z. spina-christi*	1.44	0.29	450-2000
10	OPC-13	5'-AAGCCTCGTC-3'	5	5	100.00	-	2.88	0.56	500-3000
11	OPC-14	5'-TGCGTGCTTG-3'	6	6	100.00	-	1.52	0.53	500-1000
12	OPC-15	5'-GACGGATCAG-3'	11	11	100.00	*Z. spina-christi*	6.08	0.72	250-1500
13	OPC-16	5'-CACACTCCAG-3'	8	6	75.00	*Z. nummularia* (2)	2.92	0.39	250-1500
14	OPC-18	5'-TGAGTGGGTG-3'	10	8	80.00	*Z. nummularia*	3.64	0.37	150-750
15	OPC-20	5'-ACTTCGCCAC-3'	6	5	83.33	-	2.04	0.49	500-800
16	OPD-3	5'-GTCGCCGTCA-3'	17	16	94.12	-	9.37	0.68	500-5000
17	OPD-4	5'-TCTGGTGAGG-3'	10	9	90.00	Thornless	4.60	0.64	450-1500
18	OPD-7	5'-TTGGCACGGG-3'	14	9	64.29	-	3.51	0.33	350-3000
19	OPD-8	5'-GTGTGCCCCA-3'	7	7	100.00	-	3.24	0.56	450-1100
20	OPD-11	5'-AGCGCCATTG-3'	12	12	100.00	-	5.76	0.53	250-1500
21	OPD-12	5'-CACCGTATCC-3'	6	6	100.00	-	2.08	0.61	550-3000
22	OPD-20	5'-ACCCGGTCAC-3'	7	3	42.86	-	1.00	0.12	200-750
23	OPE-2	5'-GGTGCGGGAA-3'	10	8	80.00	-	3.32	0.53	400-3000
24	OPE-4	5'-GTGACATGCC-3'	6	4	66.67	*Z. nummularia*	0.48	0.38	500-1800
25	OPE-6	5'-AAGACCCCTC-3'	9	9	100.00	-	5.68	0.59	300-3000
26	OPE-7	5'-AGATGCAGCC-3'	3	2	66.67	-	0.92	0.25	450-750
27	OPE-9	5'-CTTCACCCGA-3'	4	4	100.00	-	2.80	0.82	400-1031
28	OPE-11	5'-GAGTCTCAGG-3'	5	3	60.00	Tikadi	2.04	0.46	400-1250
29	OPE-13	5'-CCCGATTCGG-3'	8	7	100.00	Tikadi	3.80	0.73	600-2000
30	OPE-14	5'-TGCGGCTGAG-3'	8	6	75.00	-	3.16	0.63	200-1200
31	OPE-19	5'-ACGGCGTATG-3'	8	6	75.00	-	4.40	0.49	400-1800
32	OPE-20	5'-AACGGTGACC-3'	8	8	100.00	Sua	3.88	0.44	300-1500
33	OPF-1	5'-ACGGATCCTG-3'	11	8	72.73	*Z. nummularia* (3)	4.81	0.42	400-3000
34	OPF-2	5'-GAGGATCCCT-3'	14	13	92.85	-	4.12	0.71	250-3000
35	OPF-3	5'-CCTGATCACC-3'	9	8	88.89	*Z. nummularia*	4.20	0.49	500-3000
36	OPF-4	5'-GGTGATCAGG-3'	7	7	100.00	*Z. nummularia,Z. spina-christi*	1.64	0.35	600-1400
37	OPF-6	5'-GGGAATTCGG-3'	7	5	71.43	-	3.72	0.61	400-3000
38	OPF-7	5'-CCGATATCCC-3'	6	6	100.00	Kala Gola	2.52	0.58	900-3000
39	OPF-8	5'-GGGATATCGG-3'	11	11	100.00	-	6.01	0.71	200-3000
40	OPF-9	5'-CCAAGCTTCC-3'	2	2	100.00	-	1.00	0.41	500-900
41	OPF-10	5'-GGAAGCTTGG-3'	6	5	83.33	-	2.20	0.56	600-2000
42	OPF-11	5'-TTGGTACCCC-3'	12	10	83.33	*Z. nummularia,Z. spina-christi*	4.20	0.62	400-1250
43	OPF-12	5'-ACGGTACCAG-3'	9	8	88.89	*Z. spina-christi*	2.48	0.55	400-2000
44	OPF-13	5'-GGCTGCAGAA-3'	5	4	80.00	Rohtak Safeda	1.76	0.29	400-2000
45	OPF-15	5'-CCAGTACTCC-3'	10	10	100.00	*Z. nummularia*	4.24	0.64	600-3000
46	OPF-16	5'-GGAGTACTGG-3'	7	7	100.00	*Z. spina-christi*	3.92	0.48	400-2000
	Total		368	316	-		-	-	
	Average		-	7.83	86.20		3.20	0.499	

*TNB = Total number of bands; PNB = Number of polymorphic bands; P% = Polymorphism percentage; Rp = Resolving power; PIC = Polymorphic information content.

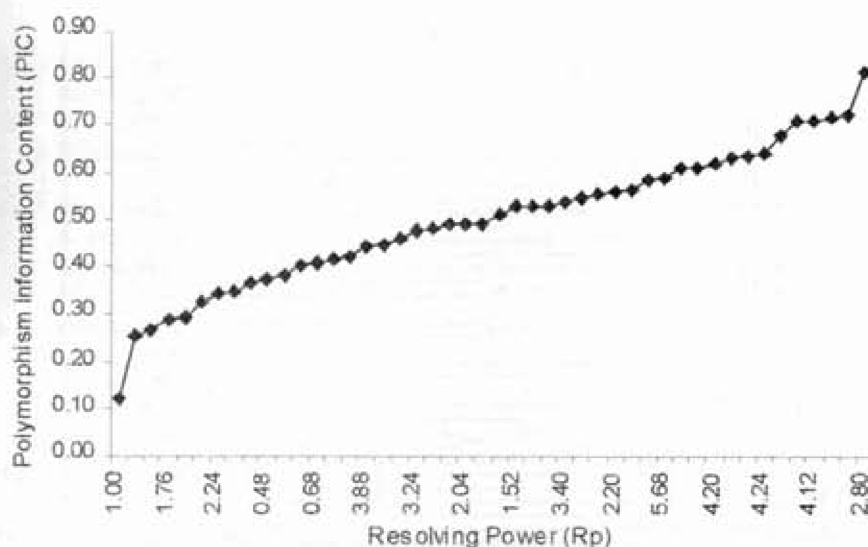
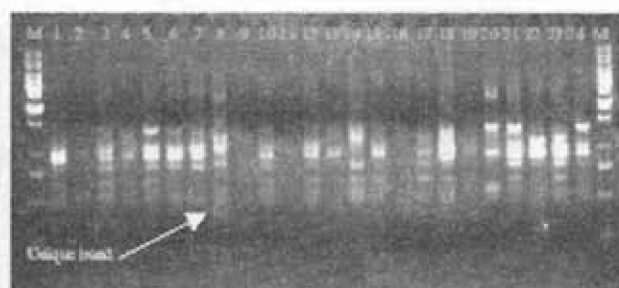
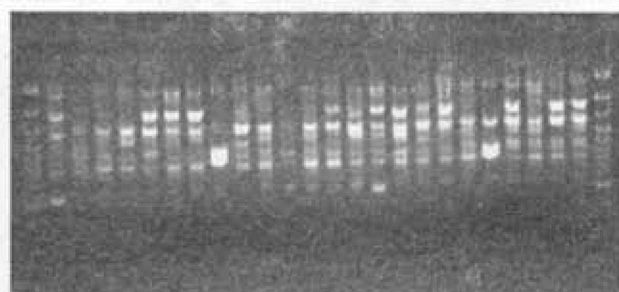


Fig. 1. Graphical relationship between resolving power and polymorphism information content



a: OPD-4 (1-24)



b: OPF-3 (1-24)

Fig. 2. RAPD profile of *ber* accessions generated by primer OPD-4 and OPF-3 (M = 1kbp DNA ladder from MBI Fermentas, 1-24 = *ber* genotypes)

major and two minor clusters (Fig. 3), while *Ziziphus nummularia* collection-1 and *Z. spinachristi* does not fall in any cluster. The cluster-I and cluster-II, both comprised of two genotypes, Seb and Chhuahara and Thronless and Tikadi, respectively. The major clusters (Cluster III to Cluster VI) comprised of remaining 44 genotypes. In cluster-III six genotypes, Rohtak Safeda, Sanauri-3, Bagwadi, Chonchal, Sindhura Narwal and Sanauri were grouped. In cluster IV eleven genotypes (Umran, Tass Bataso, Ilaichi, No-A, Noki, Hesang Tsaon, Jogia, Akhrota, Katha Bombay, Dandan and Gola), in cluster-V, seven genotypes (Ilaichi Jhazzar, Zg-3, Kheera, Kishmish, Nazuk, Reshmi and Pathan), whereas, in the biggest cluster VI, 20 genotypes (Katha Rajasthan, Sua, Popular Gola, *Z. nummularia* collection 2, Pownda, Katha, Muria Mehrara, Bawal Sel-2, Lakhan, Kala Gola, Bawal Sel-1, wild collection of *Z. mauritiana*, Kathi, Govindgarh Sel., Kaithali, Desi Alwar, Narma, Banarasi Karaka and Vilayati) grouped together. The two *Z. nummularia* genotypes were collected from different geographical regions, collection-1 was from Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi, whereas, collection-2 was from Uttar Pradesh, this may be apparent reason of their different grouping. The analysis of grouping pattern for all other genotypes did not correspond to their geographical regions. For instance, cultivars from Rajsthan, Punjab, Uttar Pradesh and Haryana remained together, e.g. Sindhura (from Haryana), Banarasi Karaka (from Uttar Pradesh), Narma (from Delhi) and Desi Alwar (from Rajasthan) grouped in cluster-VI. In contrast the genotypes from the same geographical region were grouped into different clusters e.g. Rohtak Safeda (cluster III) and Kaithali (cluster V) are both from Haryana. This may be explained by the fact that human interventions have played a major role in distribution of *ber* followed by cross-pollination between local and introduced materials. The groupings of samples in one cluster collected from different sub-zones have been reported in neem by Deshwal *et al.* [19].

Our study revealed that the phenotypically similar genotypes could be reliably distinguished using RAPD markers. Morphologically similar genotypes; Katha, Katha Bombay, Katha Rajasthan and Umaran were clustered into two different clusters Katha and Katha Rajasthan (cluster VI) and Katha Bombay and Umraan (cluster IV) and shared 65.03% and 74.72%, respectively. That can be one of the possible reasons for high morphological and flavonoid pattern similarity between these genotypes [1]. The results are in accordance to the suggestions made by Chadha *et al.* [18]. Moreover,

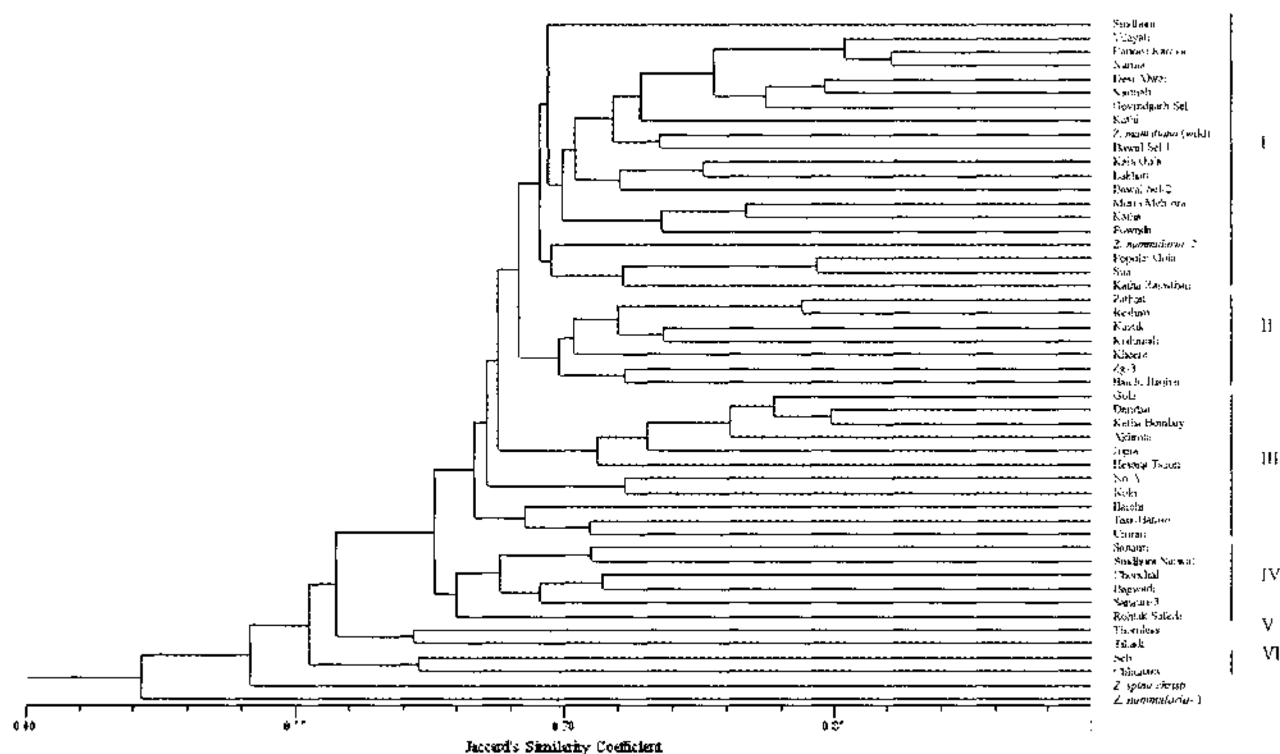


Fig. 3. Genetic relationships among 50 *Ziziphus* genotypes based on Jaccard's smilarity

all the primers except OPC-2, OPC-8, OPC-13, OPC-20, OPE-4 and OPF-7 amplified at least one different band to distinguish the four genotypes namely, Katha, Katha Bombay, Katha Rajasthan and Umran. The genotypes Gola, Akhrota, Seb and Nazuk were reported to be different morphologically, but in the present study Gola and Akhrota grouped together in subcluster IV with 79.39% genetic similarity, whereas, Seb and Nazuk were grouped with the genotypes in subclusters I and V, respectively. Genotype, Seb showed 61.86% genetic similarity with the genotype Chhuhara in the cluster-I. The four genotypes, namely Gola, Nazuk, Akhrota and Seb were distinguishable with all the RAPD primers tested except OPC-4, OPC-13, OPC-16, OPE-4 and OPF-4. The genotype Popular Gola that is morphologically similar to Gola (meaning round in shape) fell into different subclusters IV and VI, respectively. These two genotypes were distinguishable with all the RAPD primers tested except OPC-10, OPE-4, OPE-7, OPE-11 and OPF-12. The result suggests that RAPD markers are able to differentiate and fingerprint different genotypes that are phenotypically similar. The present study has revealed a broad genetic basis in *Ziziphus species* and also suggests that breeding programme can be assisted with the RAPD markers for purification and the identification of a particular genotype.

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