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Comparative evaluation of genetic relationships among *ber* (*Ziziphus* sp.) genotypes using RAPD and ISSR markers

A. K. Singh[1]*, Rakesh Singh[3] and N. K. Singh[2]

[1]Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi 110 012
[2]Rice Genome Project, National Research Center on Plant Biotechnology
[3]National Research Centre on DNA Fingerprinting, NBPGR, New Delhi 110 012

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Abstract

Genetic diversity among 48 ber genotypes from different eco-geographical regions of India was investigated using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. Forty-six RAPD and 18 ISSR primers generated a total of 368 and 167 amplification products of which 271 (74.15%) and 152 (89.94%) were polymorphic, respectively. The average num*ber*s of polymorphic fragments were 5.89 (RAPD) and 8.44 (ISSR) per primer with number of amplified bands ranging between 2 to 17 and 4 to 12 for RAPD and ISSR respectively. RAPD primer OPD-3 (9.00 Rp and 0.29 PIC) and ISSR primer UBC-880 (5.63 Rp and 0.29 PIC) were the most informative primers. Out of the 18 ISSR primers 14 were anchored, of which 12 were 3'-anchored and 2 were 5'-anchored. Fourteen ISSR primers produced distinct bands contained dinucleotide repeats. Primers based on $(AC)_n$ and $(AG)_n$ repeats produced the most polymorphic bands. Based on mantel matrix correlation between two marker systems it is suggested that the two markers amplify different genomic regions. The genetic similarity among 48 ber genotypes ranged from 47.62% (Thornless and Seov) to 88.97% (Narma and Banarsi Karaka) that suggested a wide genetic base for the ber germplasm collection. The study suggests superiority of ISSR marker system over RAPD for studying genetic diversity in ber.

Key words: Genetic diversity, *Ziziphus*, ber, RAPD, ISSR

Introduction

The genus *Ziziphus* is comprised of about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world [1]. South and Southeast Asia is the centre of both evolution and distribution of the genus *Ziziphus* [2]. The Indian *ber*, *Ziziphus mauritiana* Lam, commonly known as ber, a beautiful evergreen tree, is a dominant component of the natural vegetation in the Indian desert. The tree is endowed with a high degree of edaphic and climatic adaptability and can grow in different kind of soil types including the sub marginal lands. *Ziziphus* species are potentially important in preventing soil erosion and desertification and has therefore, been planted in India and several other countries [3]. However, indiscriminate felling of ber trees in the last 50 years has caused serious state of deterioration in their genetic resources. *Ziziphus* species are distributed between latitude 34°S and 51° N, up to 2,800 m a.m.s.l. The wide geographical and climatic distribution is indicative of a tremendous genetic diversity in ber, which needs to be identified and catalogued. The fruits of Indian ber have higher contents of protein, minerals and vitamins A and C than apples and citrus [4]. Because of high nutritional value, it has a great commercial importance. *Ziziphus* plants are highly cross-pollinated and as a result, the natural population exhibits a wide range of genetic variation. The commercial varieties of ber have been evolved through selection of promising types from this wide spread natural variability followed by budding on suitable rootstocks. A large number of methodologies exist for the assessment of genetic diversity in plant species. A combination of morphological traits and protein profiling methods such as isozymes [5], allozymes [6] and seed storage proteins [7] has conventionally been applied. However, such traits are influenced by environmental factors as well as the developmental stage of the plants. Hence, the result elucidated based on such studies do not provide a true measure of the genetic diversity. Long juvenile phase of *Ziziphus* (up to 7 years) would make DNA markers an extremely useful tool for the

*Corresponding author's e-mail: aksingh36@yahoo.com

identification of cultivars during propagation and growth. Molecular markers based on polymerase chain reaction (PCR) method offer several advantages over the sole use of conventional morphological markers. The PCR technology has led to the development of two simple and quick techniques *viz*., random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR). The former detects nucleotide sequence polymorphism with a primer of arbitrary nucleotide sequence [8] and the latter permits detection of polymorphism in inter-microsatellite loci with primer designed from dinucleotide or trinucleotide simple sequence repeats [9]. Both the techniques are inexpensive and readily adaptable for routine germplasm fingerprinting and evaluation of genetic relationship between accessions or genotypes and or construction of genetic linkage maps [10]. Although genetic diversity analysis and cultivar identification by RAPD and ISSR markers have been performed in many fruit crops [11], its application in *Ziziphus* species and its relatives (except *Z. celata*) has not been carried out [12]. AFLP has been used to document genetic diversity in *Ziziphus mauritiana* and *Z. nummularia* [13] but this technique either requires automation or long cumbersome procedure and also not very cost effective, whereas, RAPD and ISSR markers are easily available, relatively simpler and commonly used in all the laboratories. The genotypes of ber used in the present study represent only a subset of the existing natural variation in the species. We report here the genetic diversity assessed using RAPD and ISSR markers with the comparison of the two marker systems to evaluate the genetic relationship among the 47 cultivars belonging to *Z. mauritiana* and one accession of *Z. nummularia*

Material and methods

Plant materials

Plant material used for this study consisted 48 accessions of *Ziziphus* representing *Z. mauritiana* and *Z. nummularia*. The material had been collected from different states of India (Table 1) and maintained in the orchard of Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi, India.

DNA extraction

Young leaves were collected from a single tree of each accession and genomic DNA was extracted using the cetyl-trimethyl ammonium bromide (CTAB) method with some modifications [14]. DNA was quantified in a TKO 100 Fluorometer (Hoefer, San Fransisco, CA).

RAPD and ISSR analysis

Forty-six RAPD primers from Operon Technology (Alameda, California, USA) and eighteen ISSR primers (15-23 mer oligo-nucleotides) from University of British Columbia (UBC), Canada were used for this study. For both the marker systems, 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. The thermocycler (Perkin Elmer 480) was programmed for one initial step of 5 min at 94°C followed by 45 cycles (35 cycles for ISSR) of 1 min at 94°C, 1 min at 37°C (52°C for ISSR) and 2 min at 72°C. A final extension step at 72º C was programmed for 7 minutes. Amplification products were separated on 1.4% agarose gels prepared in 1 x TAE buffer (40 mM Tris-acetate and 1 mM EDTA pH 8.0) at 60 V for 3 h using a horizontal gel electrophoresis system (Bio-rad, Hercules, CA, USA). Gels were stained with EtBr. A 100 bp DNA ladder mix (MBI, Fermentas, Burlington, Ontario, Canada) was run alongside the amplified product to determine their approximate size. The amplified fragments were visualized under ultraviolet light and photographed with gel documentation Flourchem™ 5500.

Data analysis

Reproducible amplified fragments of RAPD and ISSR (i.e. those bands present in both repetitions of each sample) were scored manually. Weak bands of negligible intensity and smeared bands were excluded from the final data analysis. Band profiles were scored with 1 indicating the presence and 0 indicating the absence of a band to construct a binary qualitative data matrix. Pair-wise comparisons of genotypes were employed to calculate Jaccard's similarity coefficient (GS):

a / (n-d), where a = num*ber* of positive matches; d = num*ber* of negative matches and n = total sample size [15].

A dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) and computation for multivariate analysis was done using the computer programme NTSYS-pc Version 2.0 [16]. The correlation between RAPD and ISSR binary matrix was tested through Mantel matrix correspondence test with 100 random permutations.

The ability of a primer or technique to distinguish between large num*ber*s of genotypes, i.e. Resolving

Table 1. Ber genotypes used for genetic relationship study through RAPD and ISSR markers

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

Power of the primer (Rp) of RAPD and ISSR primers were determined as described by Prevost and Wilkinson (1999). The Polymorphism Information Content (PIC) expresses the discriminating power of the locus taking into account not only the num*ber* of alleles that are expressed, but also their relative frequencies and frequency of alleles per locus, expressed as: PIC = 1-Σp_i^2 as suggested by Lynch and Walsh [17], where p_i is the frequency of *i* th (presence) allele.

Results and discussion

Conservation of band profile for cultivars and species when replicate genomic DNA samples were extracted from different trees of the same varieties demonstrate the reproducibility of the technique. Out of 120 RAPD and 40 ISSR primers initially screened, 46 RAPD primers and 18 ISSR primers that showed a clear and reproducible band pattern were chosen for further study. Details regarding the polymorphic primers, the num*ber* and size of the polymorphic fragments revealed by each primer are presented for RAPD (Table 2) and ISSR (Table 3).

A total of 368 bands were amplified with 46 selected RAPD polymorphic primers (8 bands per primer), of which 271 (74.15%) were polymorphic (Fig. 1a) with an average of 5.89 band per primer ranging between 2 (OPF-9) to 14 (OPD-7 and OPF-2). Nine RAPD primers showed 100% polymorphism, whereas, OPD-20 (28.57%) was least polymorphic. Large genetic diversity (69.8% polymorphism) has been reported in other woody perennial species [18]. The resolving power (Rp) of the 46 RAPD primers ranged from 0.46 (OPE-4) to 9.00 (OPD-3) with an average of 3.06 per primer. RAPD primers *viz*., OPC-15, OPF-8, OPD-11 and OPE-6 possess high Rp values of 5.92, 5.75, 5.42 and 5.54 respectively (Table 2). The primers with the high Rp values were more informative as they were able to distinguish more number of ber genotypes. Polymorphism information content (for RAPD) ranged from 0.05 (OPC-11) to 0.41 (OPE-20) with an average of value of 0.25 per primer.

ISSR primers amplified a total of 167 scorable fragments (9.28 bands per primer) ranging between 4 (UBC-856) to 12 (UBC-829 and UBC-894) fragments (Fig. 1b). One hundred and fifty two fragments (89.94%) with an average of 8.44 per primer were polymorphic.

Table 2. RAPD primers with sequences and the properties of amplified products in ber genotypes

S.No.	Primer designation	Sequence	TNB	NPB	P%	Rp	PIC
1	OPC-4	5'-CCGCATCTAC-3'	6	4	66.67	3.00	0.29
2	OPC-3	5'-GGGGGTCTTT-3'	9	6	66.67	3.29	0.20
3	OPC-6	5'-GAACGGACTC-3'	8	7	87.50	3.67	0.34
4	OPC-7	5'-GTCCCGACGA-3'	7	5	71.43	2.33	0.24
5	OPC-13	5'-AAGCCTCGTC-3'	5	5	100.00	2.75	0.37
6	OPC-14	5'-TGCGTGCTTG-3'	6	6	100.00	1.50	0.27
7	OPC-2	5'-GTGAGGCGTC-3'	8	3	37.50	1.04	0.10
8	OPC-20	5'-ACTTCGCCAC-3'	6	4	66.67	1.83	0.19
9	OPC-8	5'-TGGACCGGTG-3'	7	4	57.14	3.08	0.19
10	OPC-9	5'-CTCACCGTCC-3'	3	3	100.00	0.58	0.28
11	OPC-11	5'-AAAGCTGCGG-3'	10	3	30.00	1.21	0.05
12	OPC-18	5'-TGAGTGGGTG-3'	10	6	60.00	3.50	0.25
13	OPC-16	5'-CACACTCCAG-3'	8	4	50.00	2.75	0.19
14	OPC-15	5'-GACGGATCAG-3'	11	9	81.82	5.92	0.24
15	OPC-10	5'-TGTCTGGGTG-3'	6	3	50.00	2.21	0.21
16	OPD-12	5'-CACCGTATCC-3'	6	6	100.00	1.96	0.31
17	OPF-1	5'-ACGGATCCTG-3'	11	6	54.55	4.67	0.21
18	OPD-11	5'-AGCGCCATTG-3'	12	10	83.33	5.42	0.29
19	OPD-3	5'-GTCGCCGTCA-3'	17	15	88.24	9.00	0.29
20	OPF-2	5'-GAGGATCCCT-3'	14	11	78.57	3.75	0.14
21	OPE-2	5'-GGTGCGGGAA-3'	10	7	70.00	3.29	0.20
22	OPF-11	5'-TTGGTACCCC-3'	12	9	75.00	3.83	0.17
23	OPD-8	5'-GTGTGCCCCA-3'	7	6	85.71	3.08	0.28
24	OPF-8	5'-GGGATATCGG-3'	11	11	100.00	5.75	0.35
25	OPF-7	5'-CCGATATCCC-3'	6	6	100.00	2.42	0.33
26	OPF-6	5'-GGGAATTCGG-3'	7	5	71.43	2.79	0.22
27	OPF-16	5'-GGAGTACTGG-3'	7	5	71.43	4.17	0.30
28	OPF-4	5'-GGTGATCAGG-3'	7	5	71.43	1.50	0.27
29	OP F-3	5'-CCTGATCACC-3'	9	7	77.78	4.04	0.28
30	OPE-14	5'-TGCGGCTGAG-3'	8	6	75.00	3.00	0.20
31	OPD-4	5'-TCTGGTGAGG-3'	10	9	90.00	4.54	0.29
32	OPE-11	5'-GAGTCTCAGG-3'	5	5	100.00	1.92	0.38
33	OPE-13	5'-CCCGATTCGG-3'	8	7	87.50	3.67	0.24
34	OPE-20	5'-AACGGTGACC-3'	8	8	100.00	3.75	0.41
35	OPE-19	5'-ACGGCGTATG-3'	8	6	75.00	4.33	0.32
36	OPE—4	5'-GTGACATGCC-3'	6	3	50.00	0.46	0.08
37	OPD-7	5'-TTGGCACGGG-3'	14	6	42.86	3.33	0.14
38	OPD-20	5'-ACCCGGTCAC-3'	7	2	28.57	0.92	0.13
39	OPE-6	5'-AAGACCCCTC-3'	9	8	88.89	5.54	0.36
40	OPE-9	5'-CTTCACCCGA-3'	4	4	100.00	2.83	0.33
41	OPF-15	5'-CCAGTACTCC-3'	10	9	90.00	3.96	0.28
42	OPF-13	5'-GGCTGCAGAA-3'	5	4	80.00	1.79	0.32
43	OPF-12	5'-ACGGTACCAG-3'	9	6	66.67	2.21	0.16
44	OPF-10	5'-GGAAGCTTGG-3'	6	4	66.67	2.13	0.19
45	OPE-7	5'-AGATGCAGCC-3'	3	2	66.67	0.92	0.28
46	OPF-9	5'-CCAAGCTTCC-3'	2	1	50.00	0.96	0.20
	Total		368	271	-	-	-
	Average		8	5.89	74.15	3.06	0.25

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

Table 3. ISSR primers with sequences and the properties of amplified products in ber genotypes

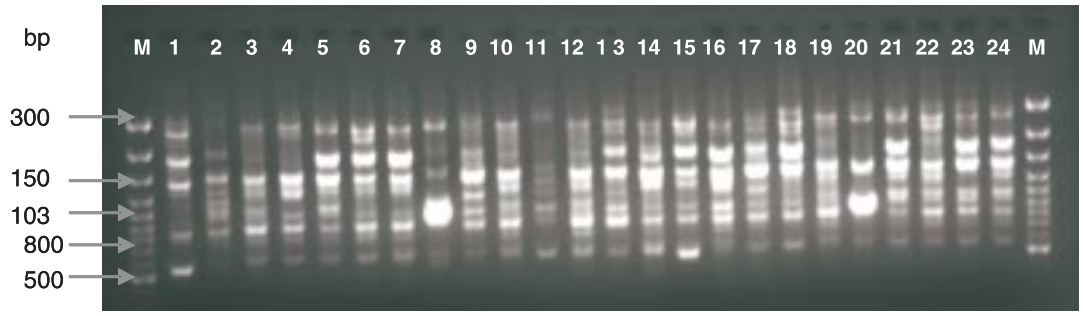
Sl. No.	UBC[1] Primer	Sequence (5′ - 3′)	TNB	NPB	P%	Rp	PIC
1	808	AGAGAGAGAGAGAGAGAGC	11	9	81.8	4.04	0.33
2	809	AGAGAGAGAGAGAGAGG	11	10	90.9	4.75	0.42
3	814	CTCTCTCTCTCTCTCTA	10	10	100.0	4.54	0.41
4	825	ACACACACACACACACT	9	6	66.6	2.34	0.31
5	829	TGTGTGTGTGTGTGTGC	12	10	83.3	4.21	0.26
6	840	GAGAGAGAGAGAGAGACTT	10	10	100.0	2.5	0.27
7	841	GAGAGAGAGAGAGAGACTC	10	10	100.0	5.29	0.41
8	848	CACACACACACACACAAGG	7	7	100.0	3.67	0.42
9	850	GTGTGTGTGTGTGTGTCTC	8	8	100.0	4.71	0.36
10	854	TCTCTCTCTCTCTCTCAGG	9	9	100.0	2.50	0.35
11	855	ACACACACACACACACCTT	10	9	90.0	3.79	0.30
12	856	ACACACACACACACACCTA	4	4	100.0	3.67	0.28
13	876	GATAGATAGACAGACA	9	9	100.0	3.71	0.43
14	880	GGAGAGGAGAGGAGA	9	7	77.8	5.63	0.29
15	889	AGTCGTAGTACACACACACACAC	7	4	57.1	2.04	0.27
16	890	ACGACTACGGTGTGTGTTTGTGT	9	9	100.0	1.00	0.43
17	894	TGGTAGCTCTTGTCAGGCAC	12	11	91.6	5.17	0.37
18	900	ACTTCCCCACAGGTTAACACA	10	10	80.0	2.63	0.30
	Total		**167**	**152**	**-**	**-**	**-**
	Average		**9.28**	**8.44**	**89.94**	**3.68**	**0.34**

*TNB = Total number of bands; NPB = Number of polymorphic bands; P% = Polymorphism percentage; Rp = Resolving power; PIC = Polymorphic information content ([1]-University of British, Columbia)

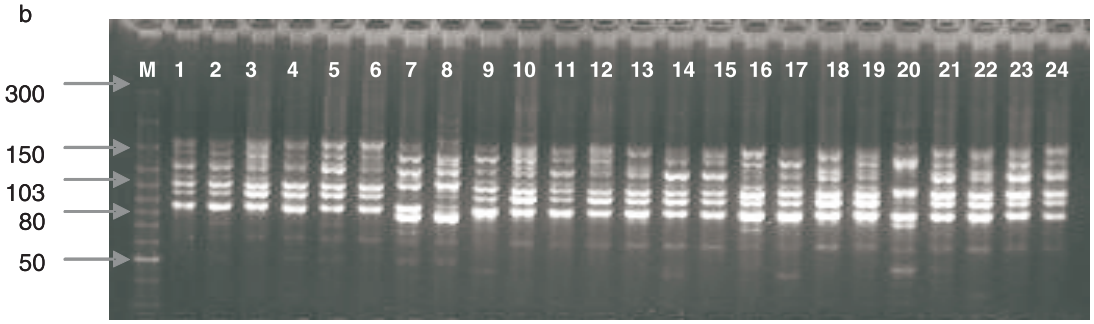
Nine ISSR primers showed 100% polymorphism, whereas, UBC-889 (57.1%) was least polymorphic. Out of the 18 selected primers 14 were anchored, of which 12 were 3'-anchored and 2 were 5'-anchored. Primers that produced polymorphic bands (14 primers out of 18) contained dinucleotide repeats. Primers based on $(AC)_n$ and $(AG)_n$ repeats produced more number of bands. The Rp values ranged from 1.0 (UBC-890) to 5.63 (UBC-880). The other primers with high Rp values were UBC-841 (5.29) and UBC-894 (5.17). The primers with high Rp values (Table 3) are able to distinguish most of the ber genotype. Polymorphism information content (PIC) ranged from 0.26 (UBC-829) to 0.43 (UBC-876 & 890).

The Mantel matrix correlation value (r) = 0.4196 (between RAPD and ISSR) suggests a poor correlation between the two marker systems. Negative correlations have been reported between RAPD and ISSR in Indian cashew [19]. The comparison between the two marker systems suggest that the ISSR markers are more efficient because of their capacity to reveal several informative bands in a single amplification (a mean of 8.44 informative bands per primers) as compared to 5.89 for RAPD and higher Rp (3.68 vs. 3.06) than RAPD primers (Fig. 2).

As the two markers differed for 58.04% of the amplified loci, present study was extended to the combined analysis of the RAPD and ISSR binary matrix data. A considerable genetic diversity was observed among ber genotypes ranging from 47.62% (between Thornless and Seov) to 88.97% (Narma and Banarsi Karaka). Such a wide range in similarity coefficient values suggests that the ber germplasm collection is genetically diverse. One of the major contributing factors to the high degree of genetic diversity observed in ber may be due to its evolutionary status as an outcrossing species. Similar observations have been made in coconut, neem [20] and ber [13] where the level of genetic diversity was shown to correlate with the breeding nature of the plants. The cluster dendrogram analysis revealed two major clusters, cluster I comprised of two genotypes, Thornless and Tikadi (63.72%), whereas, remaining 45 genotypes of *Z. mauritiana* and



(a)



(b)

Fig. 1. (a) RAPD profiles of 24 ber genotypes generated with primer OPF-3. M: 100bp DNA ladder, Lanes 1-24: correspond to 24 of the 48 ber genotypes; (b) ISSR profiles of 24 ber genotypes generated with primer UBC-855. M: 100bp DNA ladder, Lanes 1-24: correspond to 24 of the 48 ber genotypes

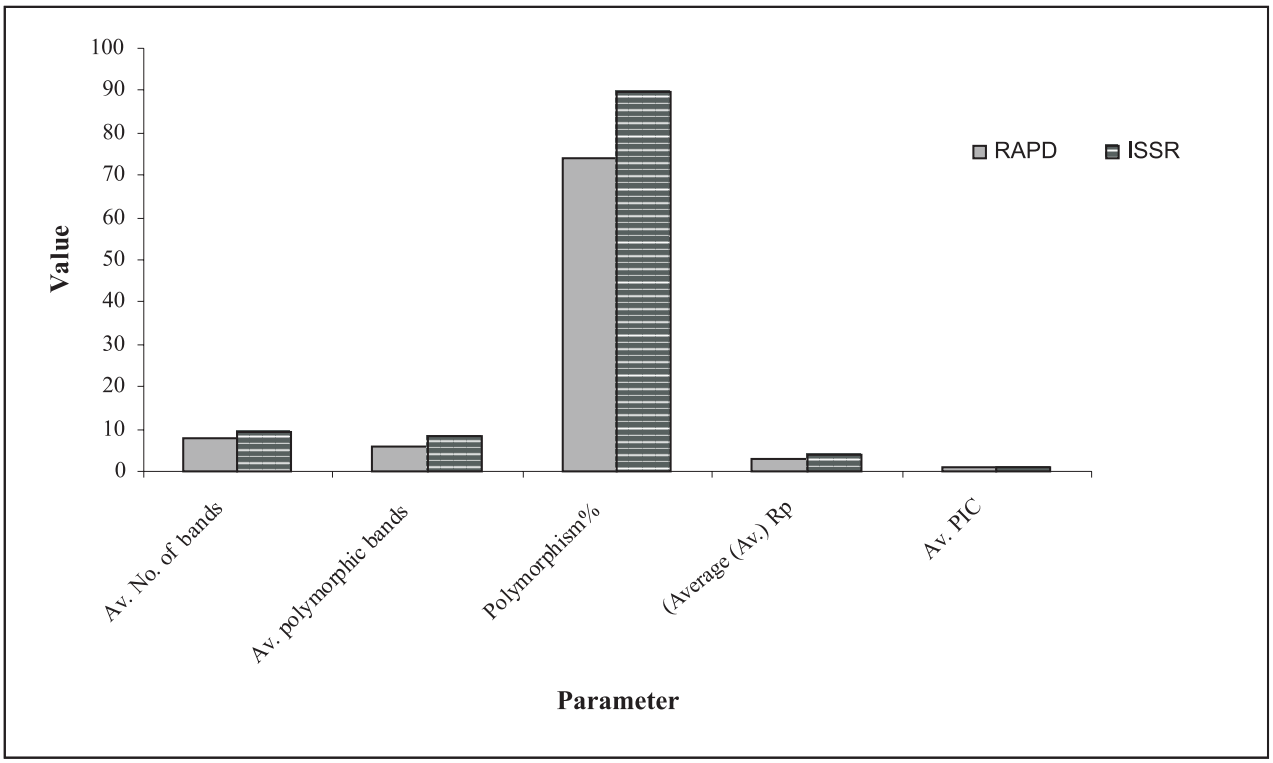


Fig. 2. Comparison between RAPD and ISSR marker systems

a single accession of *Z. nummularia* grouped into Cluster-II (Fig. 3). Cluster-II was further sub-grouped into 5 subclusters (II-A to II-E) at different similarity levels (Fig. 3). In subcluster II-C the wild accession of *Z. nummularia* grouped with Bagwadi and Chonchal. In our earlier study on AFLP analysis of ber [13], we have reported grouping of all the wild accessions of *Z. nummularia* together into a different sub-cluster away from *Z. mauritiana*. This is because AFLP is more robust marker system and amplified approximately twice the loci (952 loci) than the present analysis (535 loci). The present study using RAPD and ISSR markers reliably distinguishes morphologically similar genotypes e.g. based on morphological traits Katha, Katha Bombay, Katha Rajasthan and Umran have been reported to be different names of the same genotype in different regions [21]. However, in the present investigation we found that these four genotypes were genetically different thus shared different clusters. Similarly, Chuhara (at Bahadurgarh) and Reshmi (at Hissar) which are reported [22] to be exactly similar, grouped into different subclusters IV and III-A, respectively and shared 58.82% genetic similarity. The genotypes Gola, Akhrota, Nazuk and Seb were earlier reported to be different morphologically although with insignificant differences but in the present study the earlier three genotypes grouped together in subcluster II-D, whereas, Seb shared 67.09% genetic similarity with the genotype Chhuhara**.** The genotypes Kala Gola, Popular Gola and Gola that are characteristically round in shape fell into different subclusters of cluster II sharing 72.35% genetic similarity between Kala Gola and Gola (both grouped in subcluster II-D), 69.12% between Popular Gola and Gola and 70.60% between Kala Gola and Popular Gola. The groupings of the cultivars did not, however, correspond to their eco-geographical regions. For instance, cultivars from Rajsthan, Punjab, Uttar Pradesh and Haryana remained together. This may be explained by the fact that human interventions have played a major role in varietal distribution of ber followed by cross-pollination between local and introduced materials. Similar groupings of samples in one cluster collected from different sub-zones have been reported in neem [18, 23].

The present study revealed that the phenotypically similar genotypes could be reliably distinguished genetically using RAPD and ISSR markers. The study further displayed a broad genetic base in *Ziziphus species*. The above findings suggests the breeders to

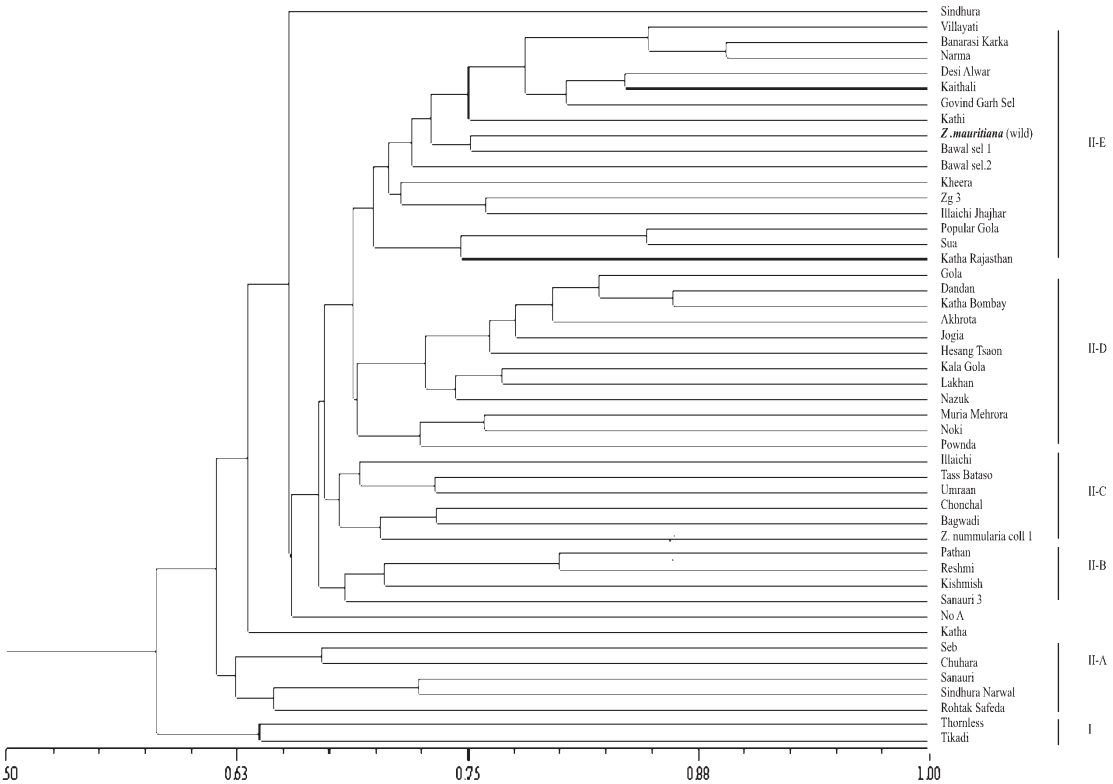


Fig. 3. Dendrogram generated through UPGMA cluster analysis for combined matrix data of 46 RAPD and 18 ISSR primers showing genetic relationships among the 48 ber genotypes. Names of the genotypes are given on the termini of branches

assess the parents for genetic improvement and taxonomists to review and resolve the mis-nomination of *ber* genotypes based not only on morphology but also molecular markers. The molecular profiling carried out in the present study could be utilized for germplasm conservation, authentication, purification and identification of genotypes. It is advocated that DNA markers such as ISSR and RAPDs could also be used effectively for genetic diversity analysis in ber.

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