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Assessment of Genetic Diversity in *Ziziphus mauritiana* Using Inter-Simple Sequence Repeat Markers

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Genetic diversity among 47 *ber* accessions belonging to cultivated species (*Ziziphus mauritiana* Lam) and one wild accession of *Ziziphus nummularia* (Burm F) Willed was investigated using Inter-Simple Sequence Repeat (ISSR) markers. A total of 167 amplification products were detected with 18 ISSR primers of which 152 (89.96%) were polymorphic. Most of the primers that produced distinct bands (14 primers out of 18) contained dinucleotide repeats. Primers based on $(AC)_n$ and $(AG)_n$ repeats produced more polymorphic bands. Genetic similarity ranging from 43.07% to 90.30% suggested that the 48 *Ziziphus* genotypes used in the study were divergent. Cluster analysis based on UPGMA method and Bootstrap analysis separated all the 48 genotypes in four distinct clusters. The present study has successfully distinguished morphologically similar genotypes that emphasize the use of molecular markers to the taxonomists. Morphologically similar but genetically distinct genotypes, identified using ISSR markers could be potential sources for genotype identification and to resolve controversies over misnomination of *ber* genotypes. Present study is the first report on the exploitation of ISSR markers in *ber* for genetic diversity analysis.

***Key words*:** *Ziziphus mauritiana*, genetic diversity, cluster analysis, ISSR, UPGMA.

The genus *Ziziphus* commonly known as *ber* belongs to the buckthorn family Rhamnaceae. It is a genus of about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world (1). Some species like *Z. mauritiana* and *Z. jujuba*, occur in nearly every continent, whereas, other species like *Z. nummularia*, *Z. spina-christi* and *Z. mucronata* are restricted in their distribution. These species can grow either as trees and shrubs (*Z. mauritiana, Z. rotundifolia, Z. jujube and Z. mucronata*) or exclusively as small shrubs or bushes (*Z. nummularia, Z. lotus, Z. spina-christi and Z. obtusifolia*). The Indian *ber*, *Ziziphus mauritiana* Lam, a beautiful evergreen tree is an example of extremely drought-hardy species and is a dominant component of the natural vegetation in the Indian desert. The scions of improved varieties are routinely grafted on to the rootstocks of wild species to provide a reasonable cash crop on lands unsuitable for other forms of cultivation (2). Improved Indian cultivars like Gola and Seb have been introduced in Israel and some countries of Africa, where they have been grafted onto native rootstocks of *Z. spina-christi* and *Z. abyssinica*, respectively (3). The same technique was successfully used in Zimbabwe to propagate high-quality Indian selections on the popular Z. *nummularia* rootstocks (4).

The wide geographical and climatic distribution is indicative of the fact that there exists a tremendous genetic diversity in *ber* which needs to be identified and catalogued. South and Southeast Asia is the centre of both evolution and distribution of the genus *Ziziphus* (5). Diversity among and within breeding material and elite germplasm is key to successful breeding programs (6). 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 (7), allozymes (8) and seed storage proteins (9) 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. Molecular markers based on PCR method offer several advantages over the sole use of conventional morphological markers.

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Abbreviations: ISSR- Inter Simple Sequence Repeat; UPGMA- Unweighted Paired Group Method of Arithmetic averages; RAPD- Random Amplified Polymorphic DNA; AFLP- Amplified Fragment Length Polymorphism.

The ISSR markers are very useful tool to detect genetic polymorphism (10). ISSR markers are inexpensive and readily adaptable technique for routine germplasm fingerprinting and evaluation of genetic relationship between accessions or genotypes (11) and construction of genetic linkage maps (12). Although genetic diversity analysis and cultivar identification by RAPD and ISSR markers have been performed in many fruits (13-15), its application in *Ziziphus* species and its relatives (with exception of *Z. celata*) has not been carried out (16). AFLP has been used to document genetic diversity in limited accessions of *Ziziphus mauritiana* and *Z. nummularia* (17). 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. Although there exists a high level of morphological variability but the similar genotype is known by different names in different regions of cultivation, it is possibly due to influence of environment on morphology (18) and therefore morphological appearances are not reliable measure of diversity among *ber* genotypes. The genotypes of *ber* used in the present study represent only a subset of the existing natural variation in the species (*Ziziphus mauritiana*) and to date no systematic attempt has been made to understand the level of genetic variation that is of utmost need for designing further breeding strategies in this fruit crop. Therefore, the present study using ISSR markers aimed to asses the genetic diversity among *ber* genotypes grown in different geographical region of India.

Materials and Methods

Plant material and DNA Isolation — Leaf sample of forty-seven accessions of *ber* representing *Ziziphus mauritiana* and one accession of *Z. nummularia* already collected from different states of India and maintained in the orchard of Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi, India were used in present investigation (Table 1).Young leaves were collected from single tree of each cultivar and frozen in liquid nitrogen immediately. Leaf samples were stored at -80 °C till DNA was isolated. Total genomic DNA was extracted from green leaves using the cetyltrimethyl ammonium bromide (CTAB) method with some

Table 1. *Ber* genotypes analysed for diversity analysis using ISSR markers

Sl No.	Accessions	Area(s) of cultivation
Z. mauritiana Lam genotypes		
1	Sindhura	Haryana
2	Seb	Haryana
3	Vilayati	Haryana
4	Ilaichi	Uttar Pradesh
5	Gola	Rajasthan / Haryana
6	Muria Mehrora	Uttar Pradesh
7	Katha	Punjab
8	Thornless	Uttar Pradesh
9	Sanauri	Punjab
10	Banarasi Kadaka	Uttar Pradesh
11	Chhuahara	Rajasthan/Punjab
12	Narma	Delhi
13	Chonchal	Punjab
14	Bagwadi	Rajasthan
15	Pownda	Punjab
16	Sindhura Narwal	Haryana
17	Kala Gola	Haryana
18	Tass Bataso	Uttar Pradesh
19	Tikadi	Rajasthan
20	Dandan	Rajasthan
21	Umaran	Punjab
22	Lakhan	Punjab
23	Katha Bombay	Gujarat
24	Bawal Selection -2	Haryana
25	Pathan	Punjab
26	Akhrota	Punjab/Haryana
27	Rohtak Safeda	Haryana
28	Sanauri-3	Punjab
29	Jogia	Uttar Pradesh
30	Desi Alwar	Rajasthan
31	Nazuk	Rajasthan
32	Hesang Tsaon	Uttar Pradesh
33	No-A	Haryana/Gujrat
34	Kathi	Punjab/Gujrat/Rajasthan
35	Kaithali	Haryana
36	Noki	Punjab
37	Popular Gola	Delhi
38	Kheera	Uttar Pradesh
39	Sua	Haryana
40	Katha Rajasthan	Rajasthan
41	Reshmi	Punjab
42	Wild collection	Delhi
43	Govind Garh Selection	Haryana
44	Kishmish	Uttar Pradesh
45	Bawal Selection-1	Bawal, Haryana
46	Zg-3	Haryana
47	Ilaichi Jhajhar	Haryana
Z. nummularia (Burm F) Willed		
48	Z. *nummularia*	Uttar Pradesh

modifications (19). DNA was quantified in a TKO 100 Fluorometer (Hoefer, San Fransisco, CA).

Primers and PCR amplification conditions — Forty ISSR primers (15-16mer oligos, UBC: University of British, Columbia) were synthesized (Microsynth Gmbh, Switzerland) and were tested for PCR amplification on 12 randomly selected genotypes to identify primers that were giving good, scorable and polymorphic amplification products. In order to estimate experimental reproducibility, two independent amplifications were carried out for the selected primers over the same set of genotypes and the primers that showed a clear and reproducible band pattern were chosen for further study on 48 genotypes of *ber* (Table 2). Amplifications were carried out with 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM dNTPs, 0.1% Triton X-100, 2% formamide, 200 nM primer, 1 unit of *Taq* DNA polymerase (Operon Technologies, Almada, CA, USA) and 25 ng of genomic DNA. Amplifications were carried out using a 96 thermal cycler (Perkin-Elmer, USA) programmed for 35 cycles as follows: an initial denatuartion was for 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, 2 min at 72 °C and final extension for 7 min at 72 °C. The amplification products were stored at 4 °C until loading. PCR-products were resolved at 60 Volts for 3 h on 1.6% agarose gel prepared in 1x TBE buffer. Gel was photographed using Gel-Documentation system (Gel Doc Mega, Biosystematica, UK).

Data analysis — The band profiles of each gel were scored visually and recorded as presence (1) or absence (0) of bands and binary quantitative data matrix was constructed. A pair-wise difference matrix between genotypes was determined using Jaccard Similarity coefficient (20). Data analysis was performed using the NTSYS-pc (Numerical Taxonomic System, 21) version 2.11 computer programme package and Winboot software. A dendrogram was constricted by UPGMA method to measure the resulting phenetic groups and the original matrix was bootstrapped 1000 times by employing Winboot to group the genotypes into discrete clusters.

The ability of a primer or technique to distinguish between large numbers of genotypes, i.e. Resolving Power of the primer (Rp) of selected ISSR primers were determined as described by Prevost and Wilkinson (22).

Table 2. ISSR primers along with their sequences and some characteristics of amplification products in the *ber* genotypes

UBC[1] Primer	Sequence (5′ - 3′)	TNB	NPB	P%	Rp	PIC
808	AGAGAGAGAGAGAGAGAGC	11	9	81.82	4.04	0.34
809	AGAGAGAGAGAGAGAGG	11	10	90.91	4.75	0.37
814	CTCTCTCTCTCTCTCTA	10	10	100.00	4.54	0.42
825	ACACACACACACACACT	9	6	66.67	2.34	0.23
829	TGTGTGTGTGTGTGTGC	12	10	83.33	4.21	0.51
840	GAGAGAGAGAGAGAGACTT	10	10	100.00	2.50	0.40
841	GAGAGAGAGAGAGAGACTC	10	10	100.00	5.29	0.53
848	CACACACACACACACAAGG	7	7	100.00	3.67	0.44
850	GTGTGTGTGTGTGTGTCTC	8	8	100.00	4.71	0.64
854	TCTCTCTCTCTCTCTCAGG	9	9	100.00	2.50	0.38
855	ACACACACACACACACCTT	10	9	90.00	3.79	0.60
856	ACACACACACACACACCTA	4	4	100.00	3.67	0.36
876	GATAGATAGACAGACA	9	9	100.00	3.71	0.36
880	GGAGAGGAGAGGAGA	9	7	77.78	5.63	0.54
889	AGTCGTAGTACACACACACACAC	7	4	57.14	2.04	0.24
890	ACGACTACGGTGTGTGTTTGTGT	9	9	100.00	1.00	0.56
894	TGGTAGCTCTTGTCAGGCAC	12	11	91.67	5.17	0.43
900	ACTTCCCCACAGGTTAACACA	10	8	80.00	2.63	0.27
	Total	167	152	-	66.19	7.62
	Average	9.28	8.44	89.96	3.68	0.42

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

The Polymorphism Information Content (PIC) expresses the discriminating power of the locus taking into account not only the number 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 (23).

Results and Discussion

Initial screening of 40 ISSR primers on 12 genotypes showed that 22 of these were monomorphic. These 22 monomorphic primers were tested on the remaining 36 genotypes in 3 bulks of 12 genotypes each to check for any polymorphism. The bulk samples were prepared by pooling equal amount of purified genomic DNA from three different sets of 12 genotypes and finally the 18 polymorphic primers were used to analyse the diversity among 48 genotypes. Details regarding the polymorphic primers, the number and size of the polymorphic fragments revealed by each primer are presented in Table 2. The primers amplified 4 fragments with UBC-856 and 12 fragments with UBC-829 and UBC-894 (Table 2). Only reproducible fragments were considered for data analysis. The ISSR profile generated with primer UBC-855 is shown in Fig.1. A total of 167 scorable fragments were yielded by the 18 polymorphic primers with an average of 9.28 bands per primer ranging between 250-2500 bp. One hundred and fifty two fragments (89.96%) with an average of 8.44 per primer were polymorphic. Among the 18 polymorphic primers, 9 primers showed 100% polymorphic bands (Table 2). Out of 18 primers used, 14 were anchored; of which 12 were 3′-anchored and 2 were 5′-anchored. Most of the 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.

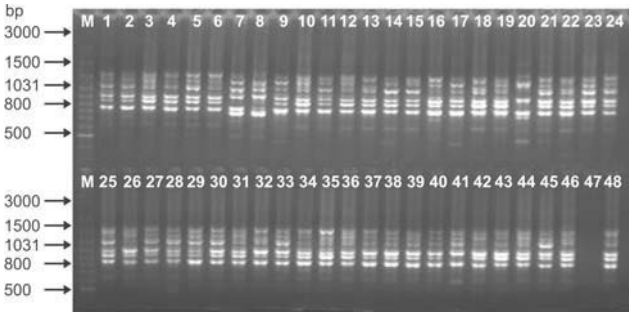

Fig. 1. ISSR profiles of 48 *ber* genotypes generated with primer UBC-855. M: 100bp DNA ladder, Lanes 1-48: correspond to *ber* genotypes listed in Table 1.

Resolving power (Rp) for each primer was calculated and it 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 are able to distinguish among most of the *ber* genotypes (Table 2). Polymorphism information content (PIC) ranged from 0.23 (UBC-825) to 0.64 (UBC-850) with high PIC values for UBC-855 (0.60) and UBC-890 (0.56).

Genetic similarity between 48 accessions based on Jaccard coefficient ranged from 43.07% (between Katha and Seb) to 90.3% (between Narma and Banarasi Karaka). Dendrogram based on genetic similarity values were constructed to reveal similarities between varieties and later it was bootstrapped (1000 replications) to confirm the grouping (Fig. 2). The bootstrapped method showed that all the genotypes (except Tikadi and Sindhura) grouped into four clusters (Fig. 2). Not much variation in the dendrogram was observed between UPGMA and bootstrapped version. Dendrogram showed highest similarity between Narma and Banarasi Karaka (90.3%). Cluster I contained 2 genotypes viz., Katha and Thornless with 70.14% genetic similarity, cluster II with Rohtak safeda and Bawal Sel-2 and the cluster IV was consisted of five genotypes Seb, Chhuhara, No-A, Sindhura Narwal and Sanauri. Cluster III was the largest with 37 out of 48 genotypes subgrouped into five subclusters (Fig. 2). In subcluster III-D the wild accession of *Z. nummularia* grouped with Bagwadi, however, the grouping was not significant as revealed by bootstrap analysis. In our earlier study on AFLP analysis of *ber* (17), 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 primers amplified approximately six times more number of loci (952 loci) than the present ISSR analysis (167 loci). The present study using 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 (18). However, in the present investigation we found that these four genotypes were genetically different thus shared different clusters. 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 III-C, whereas, Seb shared 72.41% (57.3% of the 1000 replications) genetic similarity with the genotype

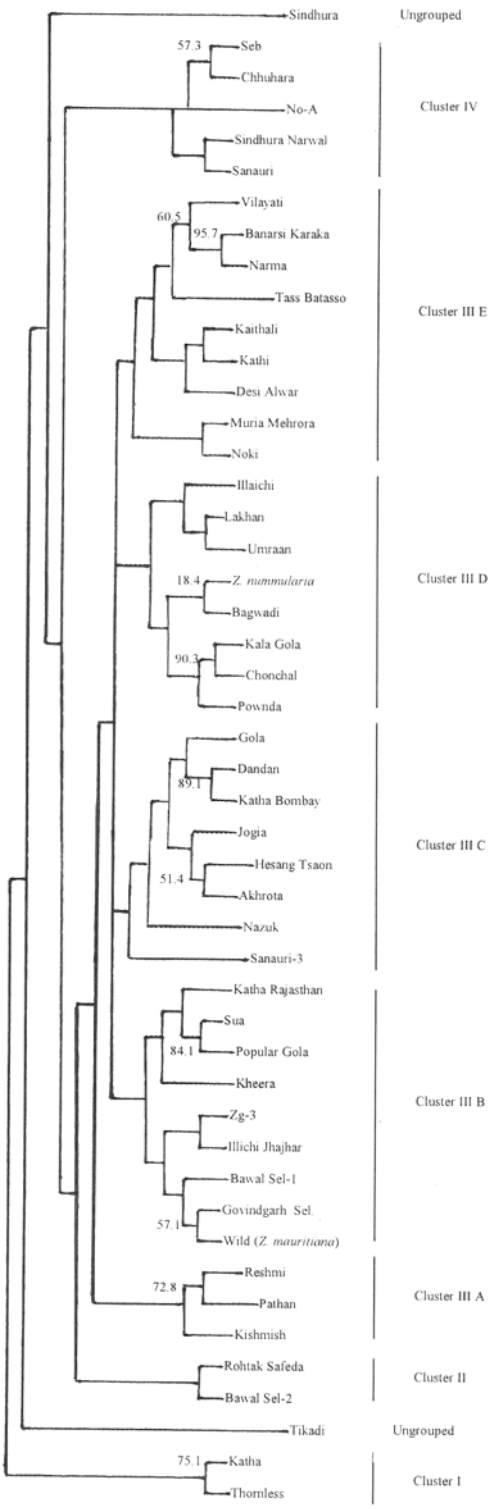

Fig. 2. Dendrogram generated through bootstrap analysis for 18 ISSR primers showing genetic relationships among the 48 *ber* genotypes. Names of the genotypes are given on the termini of branches. Numbers on the nodes are bootstrap values with 1000 replication.

Chhuhara**.** The genotypes Kala Gola, Popular Gola and Gola that are characteristically round in shape fell into different subclusters of cluster III sharing 76.03% genetic similarity between Popular Gola and Gola, 74.15% between Kala Gola and Gola, and 70.00% between Kala Gola and Popular Gola. Based on morphology, two genotypes Chhuhara and Reshmi are reported to be exactly same (18, 24) but based on the present study they share only 62.50% of genetic similarity and fall into different subclusters, IV and III-A, respectively. The results suggest that ISSR markers are able to genetically differentiate phenotypically similar genotypes. The present study has revealed a broad genetic base in *Ziziphus species* and suggests the taxonomist to review and resolve the mis-nomination of *ber* genotypes based not only morphologically but also using molecular markers. This study would help the consideration of molecular fingerprinting for germplasm conservation, rectification, purification and the identification of genotypes.

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