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RESEARCH ARTICLE

Molecular Markers Assisted Identification of Intraspecific Hybrids in *Ziziphus mauritiana*

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Abstract Breeding in *Ziziphus mauritiana* through hybridization is limited by its small sized flowers, cross incompatibility, low fruit set and poor retention. Correct identification of a hybrid is often difficult when it resembles more with one parent or when new morphological combination of characters arise from recombination of distinct genotypes. Under present study three varieties of *Z. mauritiana* viz., Tikadi, Seb and Katha were found cross compatible and resulted in improved hybrids viz., F_1 (Seb × Tikadi), F_1 × Seb and Seb × Katha. Ten RAPD primers revealed a total of 108 bands in the range of 150 bp to 3 kb. Among these, 69 bands were polymorphic and exhibited 33.33–90.9 % polymorphism in the banding patterns. The dendrogram clearly delineated variety Tikadi and Katha from Seb. All the three hybrids formed a single cluster with the variety Seb. The 5.8S gene region was found to be highly conserved (99.39 %) followed by ITS-2 (97.77 %). and ITS-1 (90.6–90.8 %). Heterozygous positions in ITS sequences clearly show their hybrid nature and could perform as highly polymorphic molecular markers. Despite SNPs, INDELS and total length polymorphism among parents and their hybrids, all were molecularly identified as *Z. mauritiana* and did not cause any phylogenetic error. The nucleotide polymorphism in ITS-1 region can serve as a bar code to detect and address genetic diversity within *Z. mauritiana*.

Keywords Indian jujube · Seb · Tikadi · Katha · Hybridization · ITS · RAPD

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Introduction

Indian jujube (*Ziziphus mauritiana* Lamk.) commonly known as Ber is mainly distributed in the tropic zone of southern Asia, Australia and Africa [1] including Taiwan and China. Endowed with a high degree of climatic adaptability, it is cultivated all over the arid parts of Indian subcontinent and plays a major role in preventing soil erosion and desertification [2]. The fruits of Indian jujube are rich in vitamins and are used as sedative, flavonoids, anticancer, tonic, wound healer and against asthma [2–6]. The extract from fruits [7], leaves [8], and seeds [9] have been reported to exhibit antioxidant activity.

Indian jujube germplasm characterization, evaluation and improvement are fundamentally based on morpho-physiological traits. The lack of break through has been due to under utilization of genetic variability for superior quality, resistance to biotic and abiotic stresses and high yield potential. Selection of promising plant types for widespread variability resulted in evolution of promising varieties. However, these cultivars lack one or more quality and/or productivity traits. Presently very little information is available in *Z. mauritiana* regarding genetic parameters to be helpful for the effective selection leading to an ideal genotype having high fruit yield. Saran et al. [10] assessed morphological diversity among *Z. mauritiana* genotypes and suggested that the most divergent genotypes could be selected for crop improvement.

Out of more than 90 cultivars, only 11 are commonly cultivated in different agro-climate regions of India [11] and show substantial variations in terms of their natural habitats, growth characteristics and aroma. The species demonstrates a rich genetic diversity mainly through natural cross pollination due to self incompatibility [12]. The elite plant types with desirable traits have been released as

varieties, mass multiplied and propagated through standard vegetative multiplication method for this crop. However, the authenticity of cultivar identification in *Z. mauritiana* is unclear and a subject of some controversy [13].

The variety Tikadi, which is resistant to fruit fly and also exhibits frost tolerance was used as a parent in a crossing programme with the popular commercial cultivars Seb, which shows drought tolerance, market acceptability, mid early fruit maturity, apple shape of fruit and tolerance to powdery mildew [14–16]. The F_1 hybrid (Seb × Tikadi) showed resistance to fruit fly but inferior in fruit quality in respect of low pulp stone ratio and smaller sized fruits. The other crosses were incompatible. Subsequently, the back cross of F_1 × Seb regained the desired fruit quality whilst retaining the fruit fly resistant trait. Katha fruit have better shelf life [17–19]. Therefore it was crossed with Seb to get hybrid with better shelf life. The F_1 hybrid (Seb × Katha) was found to be a promising precocious, prolific bearer and early maturing with good fruit set at high temperatures under arid conditions [20]. All these parental varieties and their hybrids are vegetatively propagated and are being maintained at Central Arid Zone Research Institute (CAZRI), Jodhpur.

Even if some morphological traits may help in hybrid recognition, a correct identification of a hybrid plant may often be difficult when it resembles one parent more than the other or when new morphological combination of characters arise from recombination of distinct genotypes or when only a few morphological traits are available for analysis/overlapping. It is in this context that the molecular approach may be useful tool for the assessment of hybrid status and for the correct identification of parental lineages [21].

Trees in general and *Z. mauritiana* in particular have a long juvenile phase when plants are raised through seeds (even up to 7 years). During this phase true elements of hybridism based on a few morphological descriptors cannot be validated due to the absence of fruiting stages. Molecular marker technologies circumvent this problem and hasten the breeding process by detecting coinheritance of both parental characters at very early juvenile stage.

Among the various molecular marker systems, the Randomly Amplified Polymorphic DNA (RAPD) is one of the most popular DNA based approaches [22]. It allows the analysis of individual and large number of markers in relatively short time, as only a few primers allow the generation of sufficient data to obtain a robust estimate of diversity index and have allowed the resolution of complex taxonomic relationships. This technique has recently been used in a number of fruit trees i.e., Citrus species [23] and *Punica granatum* [24]. The nuclear ribosomal Internal Transcribed Spacer (ITS) intron regions encompassing highly conserved 5.8S rRNA gene is considered as one of the most useful phylogenetic markers due to less functional constrains and comparatively higher evolutionary changes [25]. The ITS polymorphism might occur at generic, specific or individual level, making it useful for phylogenetic, evolutionary and bio-geographical diversity studies [26, 27]. Because of the similarities between the methods, both ISSR [28] and RAPD markers have been successfully used to assess genetic diversity in *Z. mauritiana* but switching from RAPD to ISSR requires purchasing new primers and re-optimizing PCR parameters. Currently, ITS and RAPD techniques have been used together and have shown complimentary results for genetic diversity studies [24, 29, 30].

The authors report the extent of genetic diversity using nuclear ribosomal DNA and RAPD polymorphism to validate successful intra-specific hybridization within *Z. mauritiana*.

Material and Methods

Field Experiment

Morpho-physiological and molecular characterization of three putative *Z. mauritiana* varieties Tikadi, Seb and Katha and their crosses were carried out from ber orchard maintained in field gene bank at horticulture block, CR Farm of CAZRI, Jodhpur. Observations of fruit weight (g), fruit volume (CC), fruit width (cm) and fruit length (cm) were recorded from the composite samples collected from ten randomly selected trees of each of the parent and hybrid during January–February 2012 and 2013 (Table 1). Whereas the observations from five randomly selected plants were recorded for plant height (m), canopy area (m^2), fruit yield (kg per plant), pulp: stone ratio, total soluble solids (TSS %) and specific gravity. The phenotypic correlation of all the morpho-physiological traits studied with fruit yield was calculated to identify possible phenotypic markers.

DNA Isolation and Quantification

The genomic DNA was extracted from the leaves of three putative varieties viz., Tikadi, Seb, and Katha and three intraspecific hybrids Seb × Tikadi (F_1), F_1 × Seb (BC) and Seb × Katha of *Z. mauritiana*. To eliminate polyphenols, mucilages and polysaccharides as glucans and cuticular wax on leaves, a hybrid protocol for genomic DNA isolation developed using initial steps of CTAB method and subsequently columns and solutions of the Plant Genomic DNA Purification kit recently developed by Singh et al. [24] was followed. The genomic DNA was dissolved in 200 µl of Tris–EDTA buffer and diluted to make a working concentration of 40–50 ng/µl for ITS amplification and RAPD analyses.

Table 1 Morpho-physiological characterization of *Z. mauritiana* parents and their hybrids during 2012–2013

Variety	Plant height (m)	Canopy area (m^2)	Fruit weight (g)	Fruit volume (cc)	Fruit breadth (cm)	Fruit length (cm)	Pulp: stone ratio	TSS (%)	Specific gravity	Fruit yield (kg $plant^{-1}$)
Tikadi	4.55	32.52	3.48	3.10	1.50	2.90	4.28	26.33	0.990	31.80
Seb	2.74	16.90	15.8	15.6	2.86	3.13	9.69	19.20	1.012	45.20
Katha	2.61	28.20	25.60	25.2	3.30	3.80	14.33	24.80	1.016	23.60
F_1 (Seb × Tikadi)	4.20	23.74	5.50	9.42	2.30	2.5	6.76	24.20	0.988	18.23
BC_1 (F_1 × Seb)	2.71	22.25	15.80	16.50	2.90	3.51	10.98	22.40	0.988	43.80
F_1 (Seb × Katha)	2.54	18.08	17.20	17.50	3.12	2.80	12.25	23.80	0.980	32.50
C.D. (5 %)	0.22	3.13	1.22	1.70	0.26	0.32	0.99	2.82	0.042	3.51
Phenotypic correlation with fruit yield	−0.44	−0.47	0.16	0.05	0.16	0.29	0.10	−0.69	0.04	1.0

RAPD Analysis

The random primers of OPA, OPB, OPP series (Operon Technologies) and a custom primer were used for initial screening of *Z. mauritiana* varieties and their hybrids. Based on the reproducibility of scorable bands, the RAPD was finally performed using 10 decamer arbitrary primers. Each amplification was performed in a total volume of 25 µl containing: decamer primer, 1 µl (50 pmol/µl); dNTP mix, 2 µl (2.5 mM/µl Bangalore Genei); *Taq* DNA polymerase, 0.4 µl (5 U/µl, Sigma Chem); $MgCl_2$, 1 µl (25-mM, Sigma Chem); 10× PCR buffer, 2.5 µl (100 mM, Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl), 14.1 µl of dH_2O and 4 µl of genomic DNA (50 ng/µl). RAPD-PCR amplifications in a thermal cycler (Corbett Research, USA) with initial denaturation step of 94 °C for 3 min followed by 38 amplification cycles at 94 °C for 40 s, 50 °C for 40 s and 72 °C for 2 min and final elongation at 72 °C for 7 min. Amplicons were separated on a 1.6 % agarose gel and stained with ethidium bromide in 1× TAE buffer. The gels were run for 3 h at 45 V. The size of the amplified fragments was determined using 1 kb ladder. RAPD amplified DNA fragments were examined by repeating PCR reactions as well as running on gel for three times.

PCR Amplification and Sequencing of ITS Region

The genomic DNA of the parents and hybrids of *Z. mauritiana* were used for amplification of 5.8S gene region. The Polymerase Chain Reaction (PCR) universal primer ITS-1 and ITS-4 developed by White et al. [31] were used to amplify ITS region of ribosomal DNA. Each PCR amplification was performed in a total volume of 50 µl containing: 1 U Taq DNA polymerase (Bangalore Genei), 2.5 mM $MgCl_2$, 160 µM dNTP mix (MBI Fermentas), 50 pmol of each of ITS-1 and ITS-4 primers (Bangalore Genei), 50 ng genomic DNA in dH_2O. The reactions were performed in a thermal cycler (Corbett Research, USA) with following conditions: 1 min denaturation at 95 °C, 30 s annealing at 50 °C, 80 s elongation at 72 °C, for 35 cycles with a final elongation step of 72 °C for 10 min. Agarose gel was stained with ethidium bromide and photographed under UV light using Syngene gel documentation system.

PCR amplified products were purified using single step ExoSAP PCR clean up enzyme at 37 °C for 15 min followed by enzyme inactivation at 80 °C for a further 15 min to remove unconsumed dNTPs and ITS primers. The primers were diluted to 10 pM concentration for sequencing. Amplified ITS regions were then sequenced with an ABI Prism DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) using ITS-1 and ITS-4 primers separately for labeling of each DNA by the BigDye terminator method (Applied Biosystems, Foster City, CA, USA). Comparison of nucleotide sequences was performed using the Basic Local Alignment Search Tool (BLAST) network services of the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The phylogenetic relationships of parents and their hybrids were established by multiple alignment of sequences using ClustalX 2.0.11 and generating phylogram depicting bootstrap values using NJ plot software [32] based on Single Nucleotide Polymorphisms (SNPs), insertions/deletions (INDELS), and or length polymorphism in the ITS and 5.8S nuclear rDNA regions. A composite phylogenetic tree with bootstrap values showing grouping was generated to measure phylogenetic accuracy.

Molecular Analysis of RAPD

Molecular data of RAPD profiles was entered into a binomial matrix and was used to determine Jaccard's similarity coefficient with NTSYS-pc software [33, 34]. The polymorphic information content (PIC) values for all the selected primers amplified by a particular primer pair was calculated for the RAPD markers to characterize the capacity of each primer to detect polymorphic loci using the formula derived by Smith et al. [35].

Results

Morpho-Physiological Characterization

The data on morpho-physiological characterization of 10 characters recorded during 2012–2013 are presented in Table 1. The plant height varied from 2.61 m (Katha) to 4.55 m (Tikadi). The canopy area varied from 16.9 m^2 (Seb) to 32.52 m^2 (Tikadi). The lowest fruit weight of 3.48 g was recorded from Tikadi and the highest of 25.6 g from Katha. Similarly the minimum fruit volume, fruit width, length and pulp stone ratio were recorded from the parent variety Tikadi and the maximum were recorded from the variety Katha. The gross fruit morphology with element of hybridity in parental varieties and hybrids are shown in Fig. 1(a–c). The minimum TSS% of 19.2 % was recorded from the variety Seb and the maximum of 26.33 % was recorded from the variety Tikadi. The specific gravity did not vary significantly among the parental varieties and their hybrids and were statistically at par with each other. The minimum fruit yield per plant varied from 18.23 kg [F_1 (Seb × Tikadi)] to 45.2 kg (Seb).

The analysis of phenotypic correlation vis-à-vis fruit yield revealed that plant height, canopy area and TSS% had negative and highly significant correlation, whereas, positive and significant phenotypic correlation was recorded in most of the fruit related traits for example, the highest correlation of fruit length (0.29) followed by fruit weight, fruit width (0.16) and pulp stone ratio (0.10) with fruit yield.

RAPD Analysis

A set of 20 random primers of OPA and OPB series and one custom primer were used for initial screening of parents (Tikadi, Seb and Katha) of *Z. mauritiana* and their hybrids [F_1(Seb × Tikadi), BC (F_1 × Seb) and F_1 (Seb × Katha)]. Based on reproducibility and intra-specific variation patterns, 10 primers revealed a total of 108 bands in the range of 150 bp–3 kb (Table 2). Among these, 69 bands were polymorphic and exhibited 33.33–90.9 % polymorphism in the banding patterns. The number of PCR amplified products ranged from seven (OPA-03) to 14 (OPA-16). Primer OPA-17 was recorded the most informative primer and exhibited the maximum polymorphism amounting to 90.9 % in banding patterns. The RAPD profile generated by OPA-16 exhibited the maximum PIC value of 90 % (Fig. 2a). The dendrogram obtained from cumulative binary analysis of 10 RAPD primers scorable fragments clearly delineated Tikadi and Katha from Seb.

Phylogenetic Analysis

All the parental varieties of *Z. mauritiana* and their hybrids exhibited a single prominent band upon gel electrophoresis containing partial sequence of 18S gene complete sequence (ITS-1, 5.8S gene, ITS-2) and partial sequence of 28S gene upon direct sequencing using universal primer ITS-1 and ITS-4 (Fig. 2b). All the novel gene sequences have been assigned GenBank accession numbers by National Centre for Biotechnology Information (NCBI), USA (Table 3). The 5.8S rDNA region was recorded with a uniform nucleotide length of 164 bp and ITS-2 with 179 bp. The ITS-1 region exhibited base pair length diversity from 255 to 260 which eventually resulted in variation in the total length of the gene from 598 to 603 bp. The multiple sequence alignment of all the three parental varieties with their hybrids resulted in detection of Single Nucleotide Polymorphism (SNPs) at 20, Insertions at 2 and deletions at 2 positions in ITS 1 region, SNP at one position in 5.8S rDNA region and at 4 places in ITS2 region. All the three hybrids formed a single cluster with the variety Seb (Fig. 3a). The phylogram generated based on multiple sequence alignment further delineated all the parents and their hybrids with significant bootstrap values (Fig. 3b).

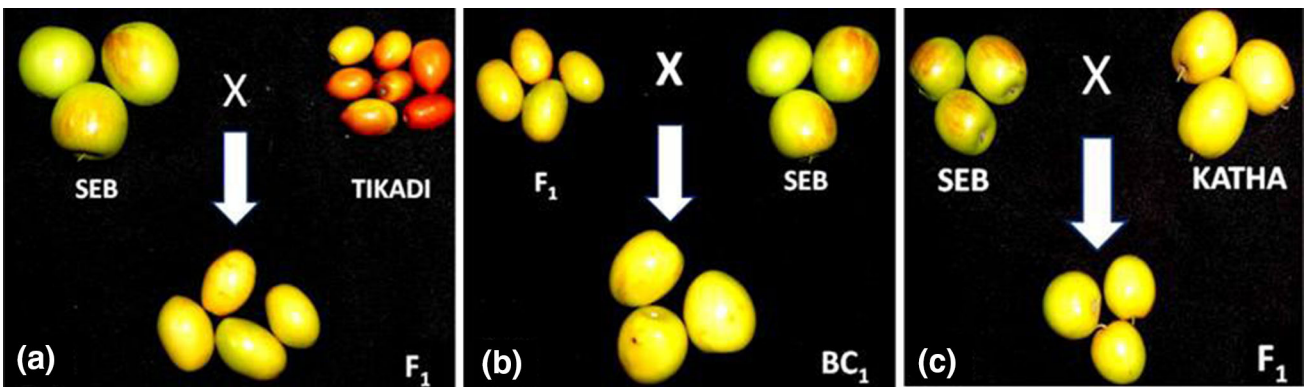

Fig. 1 **a** F_1 (Seb × Tikadi), **b** BC (F_1 × Seb) and **c** F_1 (Seb × Katha)

Table 2 Details of primer code, GC content, percent polymorphism and PIC values of RAPD primers used for molecular profiling of *Z. mauritiana* parents and hybrids

S. no.	Primer code	Primer sequence	GC (%)	No. of bands	No. of polymorphic bands	Polymorphism (%)	PIC values
1.	OPA-01	CAG GCC CTT C	70	12	8	66.66	88 %
2.	OPA-02	TGC CGA GCT G	70	12	6	50.00	89 %
3.	OPA-03	AGT CAG CCA C	60	7	5	71.43	83 %
4.	OPA-09	GGG TAA CGC C	70	8	4	50.00	84 %
5.	OPA-16	AGC CAG CGA A	60	14	9	64.29	90 %
6.	OPA-17	GAC CGC TTG T	60	11	10	90.90	87 %
7.	OPA-18	AGG TGA CCG T	60	13	11	84.61	89 %
8.	OPB-06	TGC TCT GCC C	70	9	3	33.33	88 %
9.	OPB-14	TCC GCT CTG G	70	10	6	60.00	87 %
10.	Custom primer	CGC ACC GCA G	80	12	7	58.33	89 %
	Total			108	69		
	Average					63.00	87.40

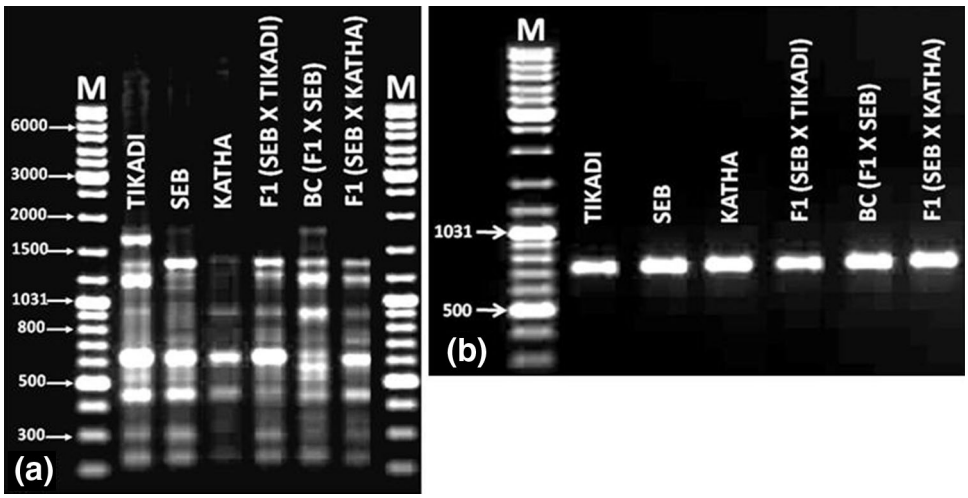

Fig. 2 **a** RAPD profiles (OPA-16) and **b** ITS profiles of *Z. mauritiana* parents and hybrids

Discussion

Hybrids offer an opportunity to recombine complementary quantitative and qualitative traits. Breeding in *Z. mauritiana* through hybridization is limited by its small sized flowers, cross incompatibility, low fruit set and poor retention [36]. Since nuclear markers are co-dominantly inherited, a putative hybrid plant must show the additive profile of the two parental sequences [37], otherwise the resultant cross may not be a hybrid but simply a morphological variant of one parental species.

Under present study all the three varieties of *Z. mauritiana* viz., Tikadi, Seb and Katha were found cross compatible and resulted in improved hybrids. For instance, the hybrid F_1 (Seb × Tikadi) exhibited element of hybridity with inheritance of fruit fly resistance and improvement in

Table 3 Nucleotide base pair lengths of nuclear ribosomal RNA gene of *Z. mauritiana* parents and hybrids

S. no.	Genotype	Gen accession number	ITS-1 (bp)	5.8 S (bp)	ITS-2 (bp)	Total (bp)
1.	Tikadi	JQ627029	255	164	179	598
2.	Seb	JQ627037	259	164	179	602
3.	Katha	JQ627045	257	164	179	600
4.	Tikadi × Seb (F_1)	KC155273	260	164	179	603
5.	Tikadi × Seb (BC)	KC155274	259	164	179	602
6.	Seb × Katha	KC155275	259	164	179	602

the fruit size in F_1 generation, back cross BC1 (F_1 × Seb) regained the desired fruit quality of variety Seb and Seb × Katha acquired the characters of early maturing and improved shelf life that validate the co-inheritance of both the parental characters and a novel component of hybridity which is different from both the parents. The acquired desirable traits of the successful hybrids have been perpetuated through vegetative propagation.

The phenotypic correlation of different morpho-physiological characters with fruit yield suggests that TSS, plant height and canopy area are inversely proportional to fruit yield. The maximum positive and significant correlation of fruit length followed by fruit weight and breadth with fruit yield suggest that these morphological traits are of significance in large scale screening of wild and cultivated germplasm for improving fruit yield in *Z. mauritiana.* It can be inferred that these traits are under the control of additive gene action and phenotypic selection for their improvement to be effective.

The methods of morphology or isozymes are insufficient to distinguish cultivars and the results are often liable to be influenced by the environment. It has been proved that ber genotypes earlier reported to be similar based on morphology are genetically different [13]. However the effect of hybridity on morphometric fruit characters and flovonoid spectrum facilitated characterization of varieties in *Z. mauritiana* to some extent [38–40] as well as in assessing the phylogenetic relationship between the parents and hybrids [41, 42]. By contrast, Tiwari et al. [43] attributed morphological variations within cultivar Banarsi of *Z. mauritiana* due to scion effect or clonal variation.

Molecular markers based on polymerase chain reaction (PCR) method offer several advantages over the conventional morphological markers. In general, the robustness of molecular marker technique depends on the amount of polymorphism it can detect. DNA markers have been often used for hybrid characterization in higher plants including *Ziziphus* [36, 44, 45]. Although genetic diversity analysis and cultivar identification by RAPD and other molecular markers have been performed in many fruit crops [24, 46], including *Ziziphus* [47], its application in identification of intraspecific hybrid within *Z. mauritiana* has not been carried out. AFLP and RAPD markers have been used to increase the diversity of the genetic linkage of *Z. jujube* [48, 49].

The cumulative analysis of all the ten informative RAPD primers detected an average of 63 % polymorphism in banding pattern with overall 87.4 % polymorphism indicating its efficacy for evaluating genetic diversity within species *Z. mauritiana*. All the three hybrid crosses clustered together with the parental variety Seb as all the three crosses had variety Seb as one of the parent. The test RAPD primers distinguished all the three parent DNA profiles from each other and exhibited the presence of distinct parental bands in the individual. Patterns of all the three hybrids, each of separate origin confirmed their hybrid status. The extent of co-inherited RAPD markers was high from 72 to 86 % indicating a substantial element of co-inheritance of parental characteristics into progenies. Intraspecific classification within *Z. jujuba* has been addressed using RAPD [50, 51] to distinguish or to reduce the number of varieties. Based on the presence of male parents specific DNA fragments produced by RAPD and ISSR markers, true hybrids within *Z. mauritiana* have been identified by Khan et al. [36].

The genetic variations ranging from 14 to 28 % (Fig. 3a) indicate that all the parents and hybrids were distinguished from each other. Co-inherited RAPD markers have also been reported from other plants [52]. Comparison of the RAPD band patterns between an F_1 hybrid, *Papaver bracteatum* and *Papaver pseudo-orientale* clearly showed that part of the bands of both parents were induced into the F_1 hybrid [53]. Lee et al. [54] investigated the segregation ratio of morphologic characters (4 qualitative and 15 quantitative) and RAPD marker in 55 intra-specific hybrids and their parents to obtain complex inheritance information in the genus *Dianthus*. Shasany et al. [52] experimentally demonstrated that through RAPD, hybridization patterns can be revealed as distinctly as by AFLP, and therefore RAPD analysis may be applicable to various plant species where polypoidy has played a role in evolution and genomic constitution.

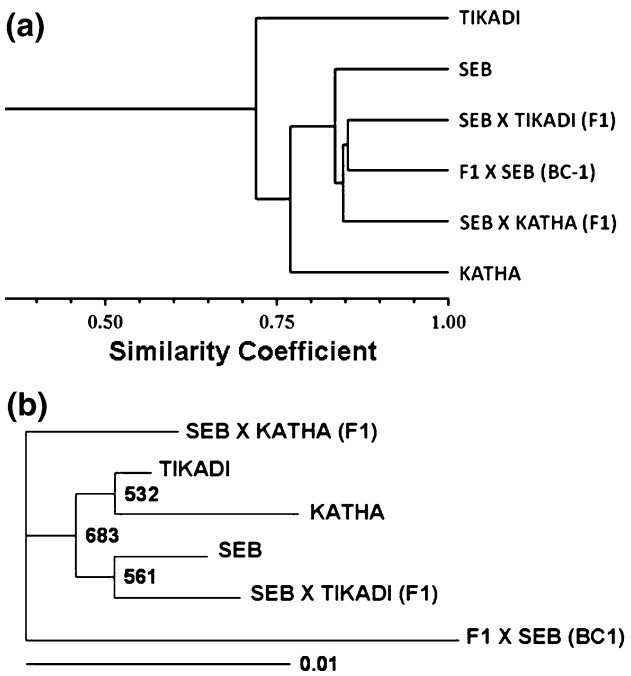

Fig. 3 **a** RAPD dendrogram and **b** phylogram of *Z. mauritiana* parents and hybrids

Sharma et al. [55] suggested that the origin of new compounds through hybridity can be explained on the basis of new gene combinations leading to novel expression. The disappearance of spots from the hybrids can be explained on the basis of epistatic phenomenon i.e. the masking effect of one gene over the other. In addition some compounds present in parents were not found in the hybrids [56].

Both the forward and reverse ITS primers amplified a bigger fragment which also included partial 18S and 28S sequences and therefore measured approximately plus 700 bp on gel electrophoresis (Fig. 2b). Total ITS region (ITS1-5.8S-ITS2) length of parents and hybrids was approximately 600 bp (Table 3). A uniform base pair length of 164 and 169 bp of all the 3 parents and their hybrids validate the conserve nature of 5.8S gene and ITS-2 regions. The total length polymorphism in the intron ITS-1 region indicates its non-coding and variable nature. The 5.8S region was found to be highly conserved (99.39 %) followed by ITS-2 (97.77 %) and ITS-1 (90.6–90.8 %). The multiple sequence alignment of nucleotide sequence of parents *vis-a-vis* their hybrids exhibited that ITS-1 region contains the highest per cent of parsimony informative sites with high divergence mainly due to SNPs and can serve as a genetic bar code to reveal genetic diversity within *Z. mauritiana*. The delineation of all the parents and hybrids into distinct sub-clad with significant bootstrap values was mainly because of nucleotide polymorphism in ITS-1 region that can serve as a bar code to detect and address genetic diversity within *Z. mauritiana*. Heterozygous positions in ITS sequences clearly show their hybrid nature and could serve as highly polymorphic molecular markers. Ma et al. [57] reported that the sequence specific microsatellites could efficiently reveal intra-specific variations within *Z. jujuba*. The ITS length variants and polymorphism have been reported in several plant species. Raturi et al. [30] reported heterogeneity in nuclear rDNA ITS region of *Vigna radiata* which did not cause any phylogenetic errors at species level. Barkley et al. [58] observed SNPs and suggested Eco-TILLING as a powerful genetic analysis tool for rapid identification of naturally occurring variations in plants. Under the present study, despite SNPs, INDELS and total length polymorphism among parents and their hybrids, all were molecularly identified as *Z. mauritiana* and did not cause any phylogenetic error using BLAST search of NCBI.

The phylogram generated based on multiple sequence alignment further delineated all the parents and their hybrids with highly significant bootstrap values. Recent phylogenetic studies carried out by using the sequences of the internal transcribed spacer (ITS 1 and ITS 2) of nuclear ribosomal DNA have shown a higher correlation between ITS nucleotide and species within genus [21, 59] and at intra specific levels of crop plants [29, 30] and tree species [24]. Since it is co-dominantly inherited, a putative hybrid plant must show the additive profile of the two parental sequences.

From molecular point of view, heterozygous positions in ITS sequences clearly showed the hybrid nature of the crosses. Together with RAPD, ITS facilitated the detection of maternal lineages in hybrid progenies because of similarity coefficients and polymorphism. These specific RAPD markers can be utilized to develop SCAR markers [60–62], adding a comparable precision for the probing of improved genotypes through hybridization programme. The nucleotide polymorphism in ITS-1 region can serve as a bar code to detect and address genetic diversity within *Z. mauritiana*. Consequently, variety specific restriction sites in the nucleotide sequences may be used as marker for identification of the varieties and their hybrids.

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