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ORIGINAL ARTICLE

Genetic diversity of Indian jujube cultivars using SCoT, ISSR, and rDNA markers

S. K. Singh[1] · Sidhika Chhajer[1] · Rakesh Pathak[1] · R. K. Bhatt[1] · Rajwant K. Kalia[1]

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Abstract Genetic variation and relationships among 37 cultivars of *Ziziphus mauritiana* (Lamk.) native of India were analyzed using start codon targeted (SCoT), inter-simple sequence repeats (ISSR), and ribosomal DNA (rDNA) markers. High level of polymorphism among SCoT (61.6%) and ISSR (61%) primers with higher PIC values ranging from 63.1 to 90.4% of SCoT and 47.3 to 88.8% of ISSR primers was recorded. SCoT and ISSR dendrograms revealed similarity coefficients ranging from 0.80 to 0.92 and 0.79 to 0.96, respectively, and clearly delineated all the cultivars of *Z. mauritiana* into well-supported distinct clusters. Greater Gst signifies higher amount of differentiation observed over multiple loci among seven *Z. mauritiana* populations. On the other hand, higher gene flow demonstrating a very high migration rate between *Z. mauritiana* populations indicated higher rates of transfer of alleles or genes from one population to another. The genetic diversity of population 1 (Rajasthan) was the richest among all the seven populations. The largest genetic distance was measured between Maharashtra and West Bengal and the least between Rajasthan and Punjab cultivars. Most of the genetic diversity exists within population rather than among populations. Substantial variation in the ITS-1 region signifies its phylogenetic utility specifically in assessing genetic diversity in *Z. mauritiana.* The clustering patterns using three molecular marker systems vis-à-vis place of origin exhibited no consistency in grouping of *Z. mauritiana* cultivars as cultivars from the same place of origin were genetically cataloged into different SCoT, ISSR, and ITS phylogram clusters indicating wide genetic diversity and distribution across agro-climatic zones validating the robustness of marker systems tested.

Keywords *Ziziphus mauritiana* · SCoT · ISSR · rDNA · Molecular markers · Indian jujube

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✉ S. K. Singh
sksingh1111@hotmail.com

Sidhika Chhajer
sid.chh29@gmail.com

Rakesh Pathak
pathakjodhpur@gmail.com

R. K. Bhatt
researcher.rkb@gmail.com

Rajwant K. Kalia
rajwantkalia@yahoo.com

[1] Central Arid Zone Research Institute, Jodhpur 342003, Rajasthan, India

Introduction

Indian jujube (*Ziziphus mauritiana* Lamk.), commonly known as Ber, is mainly distributed in the tropic zone of southern Asia, Australia, and Africa including Taiwan and China (Pasternak et al. 2009; Qu and Wang 1993). Due to a high degree of climatic adaptability, it is an important multipurpose fruit tree of arid and semi-arid regions and plays a major role in preventing soil erosion and desertification (Pareek 2001). *Z. mauritiana* is tolerant to drought, flooding, salinity, and withering (Grice 1997; Hooda et al. 1990). It is well known for its nutritional and medicinal benefits. The fruits are rich in vitamins and are used as sedative, flavonoids, anticancer, tonic, and wound healer and against asthma (Ashraf et al. 2015; Hudina et al. 2008; Mishra et al. 2011; Nyanga et al. 2013). The extract from fruits (Okala et al. 2014–2015), leaves (Dahiru and Obidoa 2007), and seeds (Bhatia and Mishra 2009) have been reported to exhibit antioxidant activity.

Most recently, Sadeghi (2015) demonstrated antibacterial activity and synthesis of gold nanoparticles from extracts of *Z. mauritiana*.

The lack of breakthrough has been due to underutilization of genetic variability for superior quality and high yield potential. The species demonstrates a rich genetic diversity mainly through natural cross-pollination due to self-incompatibility (Godara 1980). The elite plant types with desirable traits have been released as varieties, mass multiplied, and propagated through standard vegetative multiplication. However, the authenticity of cultivar identification in *Z. mauritiana* is unclear (Devanshi et al. 2007).

Different types of molecular markers based on DNA polymorphism have been used for the assessment of genetic diversity in the plant species. Recently, start codon targeted (SCoT) polymorphism has emerged as a new and promising marker technique for the genetic diversity assessment in plants (Collard and Mackill 2009) due to longer primer sequences and reproducibility. The SCoT marker is designed based on the conserved region surrounding the translation initiation codon, ATG (Sawant et al. 1999). It is used as a single primer for amplification of genomic DNA without prior genomic sequence information. Due to lesser recombination intensity between SCoT marker and the gene of interest, it can be used directly in marker-assisted breeding programs unlike random markers (Mulpuri et al. 2013). SCoT markers have been successfully used to assess genetic diversity and structure and identify cultivars and for quantitative trait loci (QTL) mapping and DNA fingerprinting in different plant species including orange (Jiang et al. 2011), date palm (Al-Qurainy et al. 2015), *Pistacia* species (Amirbakhtiar and Sorkheh 2015), mango (Leo et al. 2011), and jojoba (Heikrujam et al. 2015).

Inter-simple sequence repeats (ISSR) are inter-tandem repeats of short DNA sequences and are present within the microsatellite repeats. They exhibit specificity of sequence-tagged site markers showing the variation within unique regions of the genome at several loci. ISSR markers are an inexpensive and readily adaptable technique for routine germplasm fingerprinting and evaluation of the genetic relationship between accessions or genotypes (Agarwal et al. 2008) and the construction of genetic linkage maps (Sahu et al. 2015). The technique does not require sequence information for primer synthesis having the advantage of random markers. The ISSR markers have great potential to detect genetic polymorphism, determine intra- and inter-genomic diversity compared to other arbitrary primers, and simultaneously reveal variation within unique regions of the genome. The primers used in ISSR analysis are based on di-, tri-, tetra-, or penta-nucleotide motifs found at microsatellite loci and give a wide array of possible amplification products (Al-Turki and Basahi 2015). The technique is useful in examining genetic diversity, phylogenetic studies, gene tagging, genome mapping, and evolutionary biology studies in a wide range of crop species (Varshney et al. 2013). ISSR is a technique of choice for testing of genetic diversity in plant species for crop improvement (Dikshit et al. 2007; Pakseresht et al. 2013). Recently, its utility has been demonstrated in the genetic diversity analysis of *Eucalyptus* (Ballesta et al. 2015), chickpea (Bhagyawant et al. 2015), wild berry (Zoratti et al. 2015), *Z. mauritiana* (Singh et al. 2007), and rice (Al-Turki and Basahi 2015).

The nuclear ribosomal RNA (rRNA) complex is a tandem repeat unit having one to several thousand copies of genes that evolve at varying rates and have different phylogenetic utilities (Singh et al. 2014). The nuclear ribosomal internal transcribed spacer (ITS) evolves relatively faster and has been successfully used to determine inter-species (Chen et al. 2001; Hsiao et al. 1994; Singh et al. 2005) and intra-species relationships (Kakani et al. 2011; Raturi et al. 2012; Singh et al. 2013). The polymorphism in the nuclear rDNA region is usually observed at different levels, i.e., genus, species, or individual, and make it an efficient tool for the assessment of phylogenetic evolution and bio-geographical diversity (Andrew and Kohn 2009; Carvalho et al. 2009). The phylogenetic utility of ITS polymorphism has been successfully exploited in several crops (Kakani et al. 2011; Pathak et al. 2010) and tree species (Xiao et al. 2010) including *Z. mauritiana* (Singh et al. 2014).

The characterization, evaluation, and improvement of *Z. mauritiana* germplasm are primarily based on morpho-physiological traits. Earlier researchers have used morphological descriptors, i.e., growth habit, leaf, shoot, flower, and fruit morphology, to classify and discriminate different varieties of *Z. mauritiana* (Gupta et al. 2003; Saran et al. 2006; Vashishtha 2001). These morphological descriptors are quite homogenous and often found insufficient to distinguish varieties within morphological groups. Consequently, the utility of morpho-physiological traits used to separate cultivars within *Z. mauritiana* as well as the phylogenetic relationships among cultivars are difficult to assess. Further, the true elements of hybridism cannot be validated during the long juvenile phase of *Ziziphus*, due to the absence of fruiting stages on the basis of morphological descriptors. Molecular marker technologies circumvent this problem and hasten the breeding process by detecting the coinheritance of characters of both the parents at very early juvenile stage.

The choice of suitable cultivars is of paramount importance for its success. There is an urgent need for improvement and establishment of *Z. mauritiana* under different agro-ecological environments. The objective of this study was to estimate genetic diversity and relationships among native Indian Jujube using SCoT, ISSR, and nuclear rDNA polymorphisms for crop improvement.

Methodology

Thirty-seven cultivars of Indian jujube representing seven Indian states, namely, Rajasthan, Maharashtra, Punjab, Haryana, Gujarat, Uttar Pradesh, and West Bengal, are being maintained in randomized block design in a horticulture block under field GenBank program at the Central Arid Zone Research Institute (CAZRI), Jodhpur, Rajasthan, India (Table 1). Fresh leaves of these *Z. mauritiana* cultivars were collected in the month of February 2016 for genomic DNA extraction.

DNA extraction

Total genomic DNA was extracted using the modified CTAB method (Doyle and Doyle 1990). The yield and purity of the extracted DNA was determined electrophoretically on 0.8% agarose gel and spectophotometrically at 260 and 280 nm wavelength using BioPhotometer (D30, Eppendorf, Germany).

SCoT and ISSR analysis

A set of 36 SCoT markers (Integrated DNA Technologies (IDT) designed by Collard and Mackill (2009) were screened, and 17 primers producing unambiguous and scorable fragments were selected for assessment of genetic diversity among 37 varieties of *Z. mauritiana*. Similarly, a set of 22 ISSR primers were initially tested and finally ISSR analysis was performed with 14 primers. Each PCR amplification was performed in a total reaction volume of 25 μl containing primer, 50 pmol; dNTP mix, 0.25 mM, *Taq* DNA polymerase, 1 U (Sigma Chemicals); 10× PCR buffer, 5.0 mM (Tris–HCl, pH 8.3, 15 mM $MgCl_2$ 250 mM KCl); and genomic DNA, 50 ng in dH_2O. Amplicons were separated on 2% agarose gel prestained with ethidium bromide solution using 1× Tris–acetate–EDTA (TAE) buffer. The gels were run for 2 h at 110 V. The size of the amplified fragments was determined using 100 bp plus ladder (MBI Fermentas). All the reactions were performed twice to test the reproducibility of the amplicon profiles generated by both the marker systems.

SCoT–PCR amplification was performed in a gradient thermal cycler (Corbett Research, USA) with an initial denaturation step of 95 °C for 5 min followed by 40 amplification cycles of 95 °C for 45 s, annealing at 51 °C for 45 s and 72 °C for 1 min and 30 s, and final elongation at 72 °C for 10 min. On the other hand, the reaction mixture and PCR amplification conditions of ISSR were the same as that of SCoT amplification except the annealing temperature which was kept at 42 °C for ISSR.

Molecular analysis of SCoT and ISSR markers

Molecular data of SCoT and ISSR profiles were entered into a binomial matrix and were used to determine Jaccard's similarity coefficient with NTSYSpc software (Rohlf 1997; Sneath

Table 1 List and origin of 37 *Ziziphus mauritiana* cultivars

SN	Variety	State	SN	Variety	State
1	BC-1	Rajasthan	20	Kali	Rajasthan
2	Umran	Maharashtra	21	Jogia	Rajasthan
3	Seb	Rajasthan	22	Kaithli	Haryana
4	Illaichi	Punjab	23	Chhuhara	Maharashtra
5	Tikadi	Rajasthan	24	Dandan	Punjab
6	Gola	Haryana	25	Gola Gurgaon	Haryana
7	Reshmi	Haryana	26	Chonchal	Haryana
8	CAZRI-Gola	Rajasthan	27	Popular Gola	Haryana
9	Banarasi Karaka	Uttar Pradesh	28	Akrota	Haryana
10	Aliganj	Uttar Pradesh	29	Laddu	Uttar Pradesh
11	Katha	Rajasthan	30	Thar Bhubraj	Rajasthan
12	Z-G-3	Punjab	31	Ponda	Haryana
13	Mundia	Haryana	32	Wilayati	Punjab
14	Bagwadi	Punjab	33	Thar Sevika	Rajasthan
15	Maharwali	Rajasthan	34	Narikeli	West Bengal
16	Banarasi Pebandi	Uttar Pradesh	35	Sua	Haryana
17	S x K hybrid	Rajasthan	36	Vikas	Gujarat
18	Sanaur-5	Haryana	37	Babu	Gujarat
19	Thornless	Punjab			

and Sokal 1973). To perform molecular analysis, the 37 varieties of *Z. mauritiana* were divided into seven populations based on the state from which a variety was originally developed, viz., Pop 1 Rajasthan, Pop 2 Maharashtra, Pop 3 Punjab, Pop 4 Haryana, Pop 5 Gujarat, Pop 6 Uttar Pradesh, and Pop 7 West Bengal (Table 1). Analysis of molecular variance (AMOVA) and principal coordinate analysis via covariance matrix was calculated using GenAlEx 6 software (Peakall and Smouse 2006). On the other hand, diversity in the frequency of fragment size of SCoT and ISSR patterns was apportioned within and among *Z. mauritiana* varieties using Shannon's information index (*i*) (Lewontin 1972) and gene diversity index (*h*) following Nei (1973) using PopGen 32 program. Mantel statistic was used to compare the dissimilarity matrices as well as the dendrograms produced by the SCoT and ISSR techniques using NTSYS software.

ITS amplification

The genomic DNA of all the 37 varieties of *Z. mauritiana* was used for amplification of the 5.8S gene region. The universal primers ITS-1 (19 bp) and ITS-4 (20 bp) developed by White et al. (1990) were used to amplify the internal transcribed spacer (ITS) region of ribosomal DNA, which encompasses the 5.8S gene and both ITS-1 and ITS-2 regions. PCR amplification was performed in a total volume of 50 μl containing 1 U *Taq* DNA polymerase (Sigma Chemicals), 2.5 mM $MgCl_2$, 160 mM dNTP mix (MBI, Fermentas), 50 pmol of each ITS-1 and ITS-4 primers, and 200 ng genomic DNA in dH_2O. The PCR reactions were performed with 1 min. Denaturation was performed at 95 °C, 30 s annealing at 50 °C, and 1 min and 20 s elongation at 72 °C, for 36 cycles with a final elongation step of 72 °C for 7 min. The PCR products were run on 1.6% agarose gel pre-stained with ethidium bromide in 1× TAE buffer at 60 V for 100 min.

ITS sequence analysis

The PCR product of the ITS-amplified region containing ITS-1, 5.8S rDNA, and ITS-2 were directly sequenced using ITS-1 (forward) and ITS-4 (reverse) primers by the BigDye terminator method in an ABI PRISM DNA sequencer. Nucleotide sequence comparisons were performed by using the Basic Local Alignment Search Tool (BLAST) network services against the National Centre for Biotechnology Information (NCBI) databases. The molecular identification of 37 *Z. mauritiana* cultivars was done based on similarity with the best-aligned GenBank sequences. The complete rDNA sequences of ITS1 and ITS2 encompassing 5.8S of all the 37 varieties of *Z. mauritiana* have been assigned with GenBank accession numbers by the NCBI database, USA. The multiple sequence alignment of all the 37 cultivars using Clustal X 2.0.11 generated a phylogram depicting bootstrap values using the NJ plot software (Perrière and Gouy 1996). Based on single nucleotide polymorphisms (SNPs), insertions/deletions (INDELS), ITS length diversity, and/or 5.8S nuclear rDNA regions, phylogenetic relationships of *Z. mauritiana* cultivars were established.

Results

SCoT analysis

Out of 36 SCoT primers initially screened, 17 primers were finally used for assessment of genetic diversity based on reproducibility of scorable bands. Among the cultivars, 17 SCoT primers amplified a total of 125 clear and bright DNA fragments and their sizes ranged between 275 and 2500 bp. The number of amplified fragments varied from 1 (SCoT-23) to 12 (SCoT-13 and SCoT-31) with an average of 7.35 fragments per primer (Table 2). Of 125 amplified DNA fragments, 77 (61.6%) were found to be polymorphic. The detected polymorphism per primer among the tested cultivars ranged from 0 (SCoT-23) to 85.71% (SCoT-17). Except SCoT-23, all other primers exhibited higher polymorphic information content (PIC) ranging from 63.1 (SCoT-28) to 90.4% (SCoT-31). The profile generated by SCoT-13 is shown in Fig. 1 a, b.

All the 125 scored DNA fragments were used to calculate genetic similarity among cultivars of *Z. mauritiana*. A genetic similarity coefficient between pairs of accessions was obtained from the marker data based on similarity matrix coefficients. A dendrogram was constructed from cumulative cluster analysis of 17 SCoT primer scorable fragments which exhibited similarity of approximately 0.80 to 0.92 and clearly delineated all the test cultivars in to five well-supported distinct clusters and one outgroup (Fig. 2). Cluster I included five cultivars (BC-1, Seb, Vikas, S × K Hybrid, Sanaur-5). Cluster II contained 11 cultivars (Umran, Dandan, Jogia, Illaichi, Mundia, Gola, Banaras-Pebandi, CAZRI-Gola, Aliganj, Katha, Z-G-3). Cluster III had 10 cultivars (Tikadi, Kaithli, Babu, Banaras-Karaka, Narikeli, Thornless, Thar-Sevika, Sua, Wilayati, Chhuhara). Cluster IV grouped six cultivars (Kali, Ponda, Gola-Gurgaon, Popular-Gola, Laddu, Akrota). Cluster V comprised four (Bagwadi, Thar-Bhubraj, Maharwali, and Chonchal) cultivars whereas Reshmi did not cluster with other cultivars and was recorded as the most distinct outgroup. The highest genetic similarity value (0.923) was observed between Thar-Sevika and Sua.

ISSR analysis

Based on amplification of well-recorded, unambiguous, and reproducible DNA fragments, out of 22 ISSR primers tested, 14 were used for further analysis. Among the cultivars, 14 ISSR primers yielded a total of 100 clear and bright DNA

Table 2 Details of primer code, GC content, percent polymorphism, and PIC values of ScoT and ISSR primers used for molecular profiling of *Z. mauritiana* varieties

Primer code	Primer sequence	GC (%)	No. of bands	No. of polymorphic bands	Polymorphism (%)	PIC value (%)
SCoT						
SCoT-1	CAACAATGGCTACCACCA	50	7	4	57.14	82.9
SCoT-2	CAACAATGGCTACCACCC	55	7	5	71.42	83.4
SCoT-3	CAACAATGGCTACCACCG	55	9	7	77.77	87.4
SCoT-4	CAACAATGGCTACCACCT	50	8	2	25.00	86.9
SCoT-7	CAACAATGGCTACCACGG	55	5	3	60.00	77.1
SCoT-8	CAACAATGGCTACCACGT	50	3	2	66.66	65.2
SCoT-13	ACGACATGGCGACCATCG	61.1	12	8	66.66	90.4
SCoT-17	ACCATGGCTACCACCGAG	61.1	7	6	85.71	81.1
SCoT-18	ACCATGGCTACCACCGCC	66.7	9	5	55.55	88.1
SCoT-19	ACCATGGCTACCACCGGC	66.7	10	7	70.00	88.5
SCoT-22	AACCATGGCTACCACCAC	55	9	3	33.33	88.5
SCoT-23	CACCATGGCTACCACCAG	61.1	1	0	00.00	0.00
SCoT-28	CCATGGCTACCACCGCCA	66.7	6	3	50.00	63.1
SCoT-31	CCATGGCTACCACCGCCT	66.7	12	9	75.00	90.4
SCoT-32	CCATGGCTACCACCGCAC	66.7	6	4	66.66	78.8
SCoT-34	ACCATGGCTACCACCGCA	61.1	5	2	40.00	78.5
SCoT-35	CATGGCTACCACCGGCCC	72.2	9	7	77.77	85.5
ISSR						
ISSR-3	GTG + $(GT)_7$	53	7	7	100	83.7
ISSR-5	CCA + $(GTG)_4$	67	9	5	55.5	88.8
ISSR-12	GCAA + $(GACA)_3$	50	10	7	70.0	88.6
ISSR-19	$(GACA)_4G$	53	7	3	42.8	84.2
ISSR-24	$(GAA)_6$	33	7	3	42.8	84.6
ISSR-25	$[G + (AC)_2]_4$	60	3	2	66.6	66.6
ISSR-30	$(GT)_8C$	53	10	9	90.0	88.8
ISSR-39	$(CA)_8GC$	56	10	3	70.0	88.4
ISSR-40	$(AC)_8CA$	50	6	2	33.3	82.0
ISSR-42	$(TG)_8GC$	56	2	1	50.0	47.3
ISSR-43	$(AC)_8TT$	44	7	4	57.1	80.2
ISSR-44	$(AC)_8TA$	44	7	4	57.1	78.1
ISSR-47	$(AG)_8TA$	44	9	5	55.5	86.9
ISSR-50	$(AGTC)_4$	50	6	6	100	78.9

fragments and their sizes ranged between 275 and 3000 bp. The number of amplified fragments varied from 2 (ISSR-42) to 10 (ISSR-12, ISSR-30, and ISSR-39) with an average of 7.14 amplicons per primer (Table 2). Of 100 fragments, 61 (61.0%) were polymorphic. The detected polymorphism per primer among the tested cultivars ranged from 33.3 (ISSR-40) to 100% (ISSR-3 and ISSR-50). All the primers exhibited higher PIC ranging from 47.3% (ISSR-42) to 88.8% (ISSR-5, ISSR-30). The profile generated by ISSR-50 exhibiting 100% polymorphism is shown in Fig. 3a, b.

All the 100 scored amplicons were used to calculate genetic similarity among the test cultivars of *Z. mauritiana*. A genetic similarity coefficient between pairs of accessions was obtained from the marker data based on similarity matrix coefficients. A dendrogram was constructed from cumulative cluster analysis of scorable fragments generated by 14 ISSR primers which exhibited a similarity coefficient of approximately 0.79 to 0.96 and clearly delineated all the 37 cultivars of *Z. mauritiana* in to six well-supported distinct clusters and one outgroup (Fig. 4). Cluster I included six cultivars (BC-1, Seb, Umran, Illaichi, Gola, CAZRI-Gola). Cluster II contained 11 cultivars (Banaras-Karaka, Z-G-3, Aliganj, Katha, Thornless, Ponda, Thar-Bhubraj, Mundia, Bagwadi, Maharwali, Banaras-Pebandi,). Cluster III had six cultivars

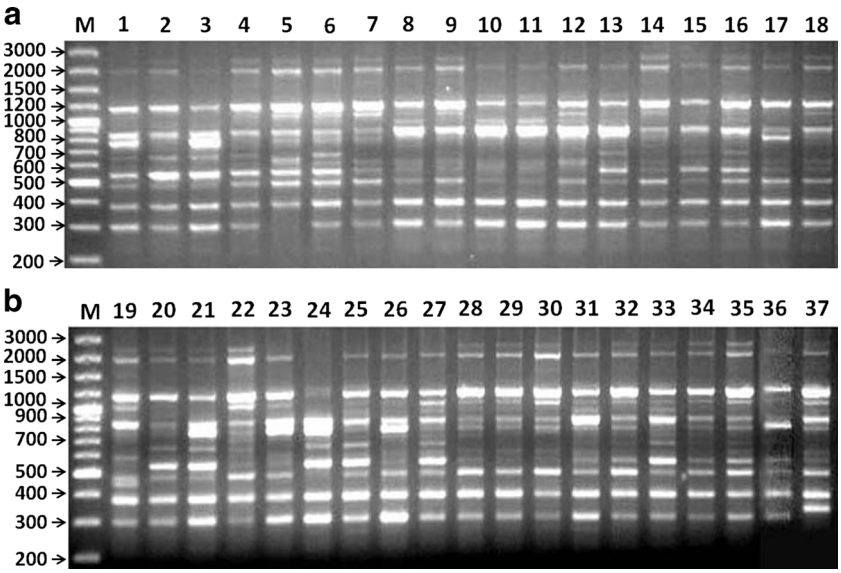

Fig. 1 **a** Profile of 1–18 cultivars of *Z. mauritiana* amplified by SCoT-13 primer. **b** Profile of 19–37 cultivars of *Z. mauritiana* amplified by SCoT-13 primer

(Gola-Gurgaon, Popular-Gola, Laddu, Jogia, Dandan, Thar-Sevika). Cluster IV grouped eight cultivars (Reshmi, S × K Hybrid, Chhuhara, Chonchal, Kali, Kaithli, Narikeli, Sua). Cluster V comprised three cultivars (Akrota, Vikas, Babu). Cluster VI separated two cultivars Tikadi and Sanaur-5 from others. The cultivar Wilayati did not cluster with any other cultivar and was recorded as the most distinct outgroup. The highest genetic similarity value (0.923) was recorded between Jogia and Dandan.

Correlation between SCoT and ISSR

The cophenetic coefficient was acceptable in both the molecular marker systems indicating good fit for clustering. The values of mantel test correlation showed a positive correlation between the SCoT and ISSR. The correlation coefficient (r) was 0.24 between SCoT and ISSR (significant $P > 0.05$) with an approximate t value of 3.57.

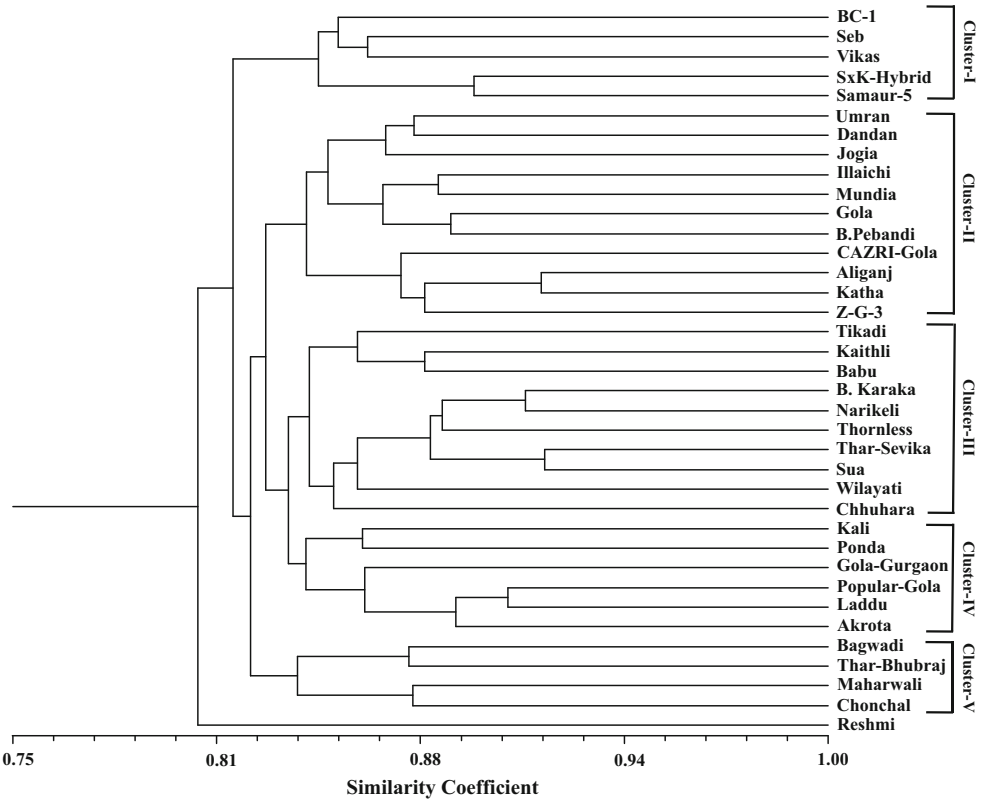

Fig. 2 Dendrogram of 37 cultivars of *Z. mauritiana* based on 17 SCoT informative primers

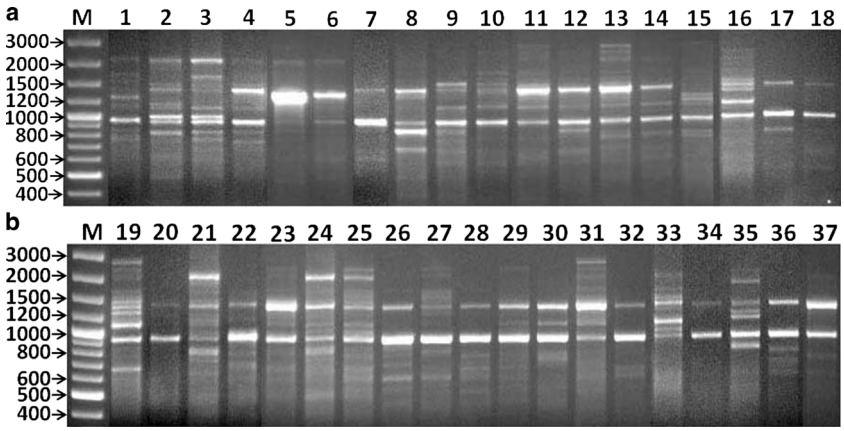

Fig. 3 **a** Profile of 1–18 cultivars of *Z. mauritiana* amplified by ISSR-50 primer. **b** Profile of 19–37 cultivars of *Z. mauritiana* amplified by ISSR-50 primer

Genetic analysis of SCoT markers

Principal coordinate analysis (PCA) of all the seven *Z. mauritiana* populations revealed contribution of the first three eigen factors at 21.74, 20.26, and 16.36%, respectively, explaining a total of 58.36% variability (Fig. 5). The allocation pattern of seven populations across all the principal coordinates indicates their wide distribution except population 5 which had only one cultivar. The mean values of all the seven populations together for Nei's gene diversity (*h*) was 0.2365, and the Shannon information index (*i*) was 0.3456 (Table 3). Results revealed that the genetic diversity of population 1 was the richest. To further explicate the gene differentiation among populations, Nei's unbiased measure of genetic distance ranged from 0.0083 to 0.1812 and the genetic identity ranged from 0.8342 to 0.9931 (Table 4). The maximum genetic distance was recorded between populations 2 and 7 and the least between populations 1 and 3. The distance matrix for all the seven populations permitted a partitioning of the overall variations into two levels: among populations and within populations. The results showed that most of the genetic variations existed within populations. The proportion of variations attributed within populations was 99%, and the remaining variations (1%) occurred among populations (Table 5).

All the seven populations of *Z. mauritiana* representing different geographical areas showed total genetic diversity (H_T) of 0.2295, genetic diversity within each population (Hs) was 0.1451, the coefficient of gene differentiation (Gst) was 0.3680, and the level of gene flow (Nm) was 0.8586 (Table 6).

Genetic analysis of ISSR markers

Seven *Z. mauritiana* populations were subjected to PCA (Fig. 6). The eigen factor analysis indicated that the contributions of the first three factors were 30.26, 20.90, and 17.55%,

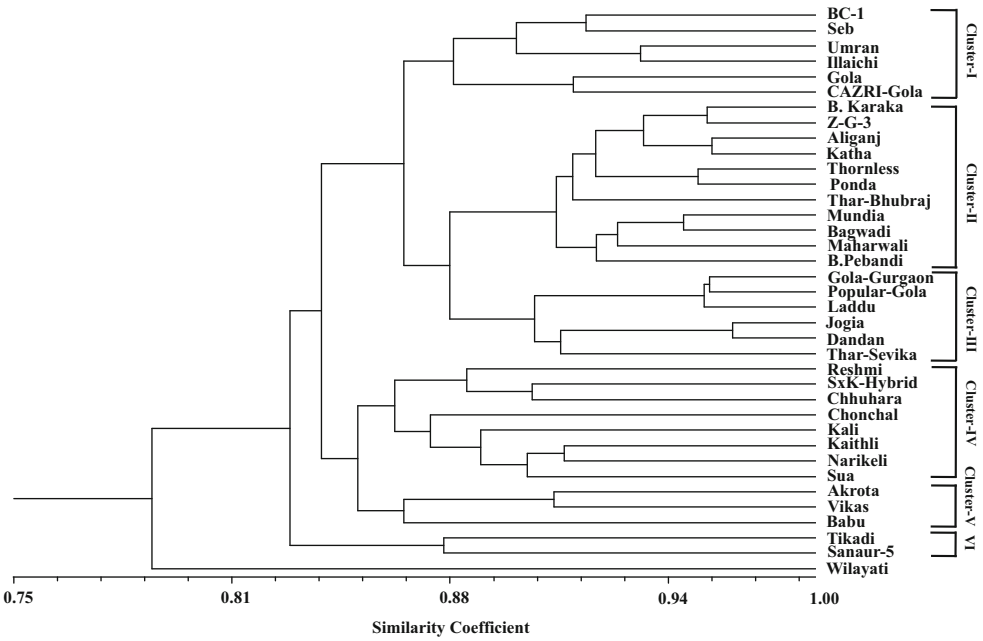

Fig. 4 Dendrogram of 37 cultivars of *Z. mauritiana* based on 14 ISSR informative primers

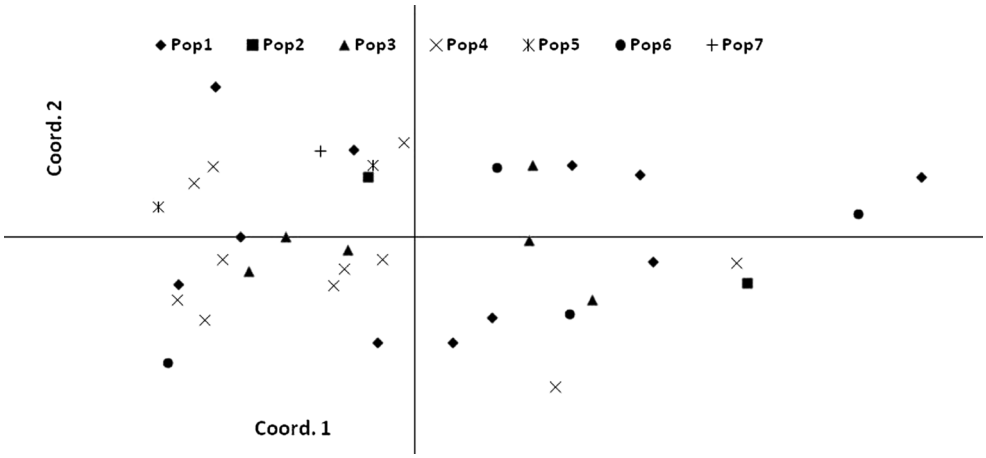

Fig. 5 The principal coordinate analysis (PCA) of seven populations of *Z. mauritiana* using SCoT primers

respectively, explaining a total of 68.71% variability. The distribution pattern of seven populations across principal coordinates indicates their wide distribution except population 5 which had only one cultivar. A summary of mean genetic variation statistics of all the seven populations along with the mean of all loci is presented in Table 3. The mean values of all the seven populations together for Nei's gene diversity (*h*) was 0.2209, and the Shannon information index (*i*) was 0.3280. Results showed that the genetic diversity of *Z. mauritiana* cultivars of population 1 was the richest among all the seven populations. Nei's unbiased measure of genetic distance was employed to further elucidate the gene differentiation among populations (Table 4). Nei's genetic distance ranged from 0.0173 to 0.2189. The largest genetic distance occurred between populations 2 and 7 and the least between populations 1 and 3. The AMOVA of the distance matrix for all the seven populations permitted a partitioning of the overall variations into two levels: among populations and within populations. The results showed that most of the genetic variations existed within populations. The proportion of variations attributed within populations was 95%, and the remainder of variations (5%) occurred among populations. As a result, obvious genetic differentiation existed within the *Z. mauritiana* population (Table 5).

Genetic structure estimates for the seven populations of *Z. mauritiana* representing different geographical areas are presented in Table 6. The total genetic diversity (H_T) was 0.2065, genetic diversity within each population (Hs) was 0.1293, the coefficient of gene differentiation (Gst) was 0.3741, and the level of gene flow (Nm) was 0.8365.

ITS amplification and sequencing

Upon PCR with universal primers ITS-1 and ITS-4, all the 37 cultivars of *Z. mauritiana* generated a single prominent amplified product (approx. 625 bp) of the 5.8S gene region. The amplified product included partial sequence of the 18S gene; complete sequence of ITS-1, 5.8S gene, and ITS-2; and partial sequence of the 28S gene. All the 37 novel gene sequences exhibiting nucleotide variations have been submitted to the NCBI database and assigned with GenBank accession

Table 3 Summary of genetic variation statistics for all loci of SCoT and ISSR markers

Locus (mean)	Sample size	SCoT				ISSR			
		na	*ne*	*h*	*i*	*na*	*ne*	*h*	*i*
Pop 1	11	1.5840	1.3937	0.2265	0.3335	1.5500	1.3811	0.2118	0.3099
Pop 2	2	1.2800	1.1980	0.1160	0.1693	1.2100	1.1485	0.0870	0.1270
Pop 3	6	1.5040	1.3606	0.2015	0.2937	1.4900	1.3763	0.2049	0.2954
Pop 4	11	1.5600	1.3913	0.2211	0.3235	1.5300	1.3578	0.2053	0.3020
Pop 5	2	1.2240	1.1584	0.0928	0.1355	1.1500	1.1061	0.0621	0.0907
Pop 6	4	1.4000	1.2797	0.1576	0.2310	1.3400	1.2398	0.1337	0.1957
Pop 7	1	1.0000	1.0000	0.0000	0.0000	1.0000	1.0000	0.0000	0.0000
Mean of all loci	37	1.6000	1.4190	0.2365	0.3456	1.6300	1.3892	0.2209	0.3280

na observed number of alleles, *ne* effective number of alleles, *h* Nei's gene diversity, Shannon's information index

Table 4 Matrix of unbiased genetic identity and genetic distance according to Nei (1973) among seven populations of *Ziziphus mauritiana* based on 125 ScoT and 100 ISSR markers

Population	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop6	Pop7
SCoT							
Pop 1	****	0.9101	0.9918	0.9931	0.9057	0.9510	0.8655
Pop 2	0.0942	****	0.9185	0.9117	0.8668	0.9013	0.8342
Pop 3	0.0083	0.0850	****	0.9901	0.9034	0.9578	0.8879
Pop 4	0.0069	0.0924	0.0100	****	0.9035	0.9638	0.8760
Pop 5	0.0990	0.1430	0.1016	0.1014	****	0.8686	0.8411
Pop 6	0.0503	0.1039	0.0431	0.0369	0.1409	****	0.8544
Pop 7	0.1445	0.1812	0.1188	0.1324	0.1731	0.1573	****
ISSR							
Pop 1	****	0.9551	0.9829	0.9860	0.9180	0.9624	0.9044
Pop 2	0.0460	****	0.9430	0.9380	0.8478	0.9289	0.8034
Pop 3	0.0173	0.0586	****	0.9698	0.8969	0.9422	0.8925
Pop 4	0.0141	0.0640	0.0307	****	0.9249	0.9486	0.9258
Pop 5	0.0855	0.1651	0.1088	0.0780	****	0.8361	0.8845
Pop 6	0.0383	0.0738	0.0596	0.0527	0.1790	****	0.8676
Pop 7	0.1005	0.2189	0.1137	0.0771	0.1227	0.1421	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal

numbers (Table 7). The conserved 5.8S rDNA portion was recorded with a uniform nucleotide length of 164 bp in all the 37 cultivars. The ITS-2 region was 179 bp long in all the test cultivars except Kaithli (180 bp). Substantial variation was recorded in the ITS-1 region ranging from 255 bp (Tikadi) to 263 bp (Gola-Gurgaon). The total length of the complete 5.8S gene region also varied from 598 to 603 bp.

In the multiple sequence alignment of nucleotide sequences of all the 37 cultivars together, in addition to total length polymorphisms, we detected single nucleotide polymorphisms (SNPs) and insertion/deletions (INDELS) at several positions in ITS-1, 5.8S rRNA gene, and ITS-2 region. The multiple sequence alignment of all the varieties of *Z. mauritiana* generated a phylogram using Clustal X 2.0.22 and NJ plot (Fig. 7). The perusal of the phylogram further delineated *Z. mauritiana* varieties with each other with Illaichi as quite distinct outgroups.

Table 5 Summary of AMOVA of ScoT and ISSR markers

Marker	Source	*df*	SS	MS	Est. var.	%
SCoT	Among pops	6	97.565	16.261	0.218	1%
	Within pops	31	470.303	15.171	15.171	99%
	Total	37	567.868		15.389	100%
ISSR	Among pops	6	77.888	12.981	0.534	5%
	Within pops	31	319.848	10.318	10.318	95%
	Total	37	397.737		10.851	100%

Discussion

Indian jujube germplasm characterization, evaluation, and improvement are fundamentally based on morpho-physiological traits (Gupta et al. 2003; Pareek 2001; Saran et al. 2006; Vashishtha 2001). A high level of morphological variability exists, and similar genotypes are known by different names in different regions of cultivation; it is possibly due to the influence of environment on morphology (Singh et al. 2007), and therefore, morphological appearances are not reliable measures of diversity assessment among *Z. mauritiana* genotypes. Such morphological descriptors are quite homogeneous and often insufficient to distinguish genotypes within morphological groups. The lack of breakthrough has been due to underutilization of genetic variability, superior quality, and high yield potential.

The present study revealed that a high polymorphism could be detected among *Z. mauritiana* cultivars using SCoT and ISSR markers. High level of polymorphism among SCoT (61.6%) and ISSR (61%) primers with higher PIC values ranging from 63.1 to 90.4% of SCoT and 47.3 to 88.8% of ISSR primers are indicative of sufficient genetic diversity

Table 6 Nei's analysis of gene diversity in subdivided populations

Marker	Locus	Sample size	Ht	Hs	Gst	Nm
SCoT	Mean	37	0.2295	0.1451	0.3680	0.8586
ISSR	Mean	37	0.2065	0.1293	0.3741	0.8365

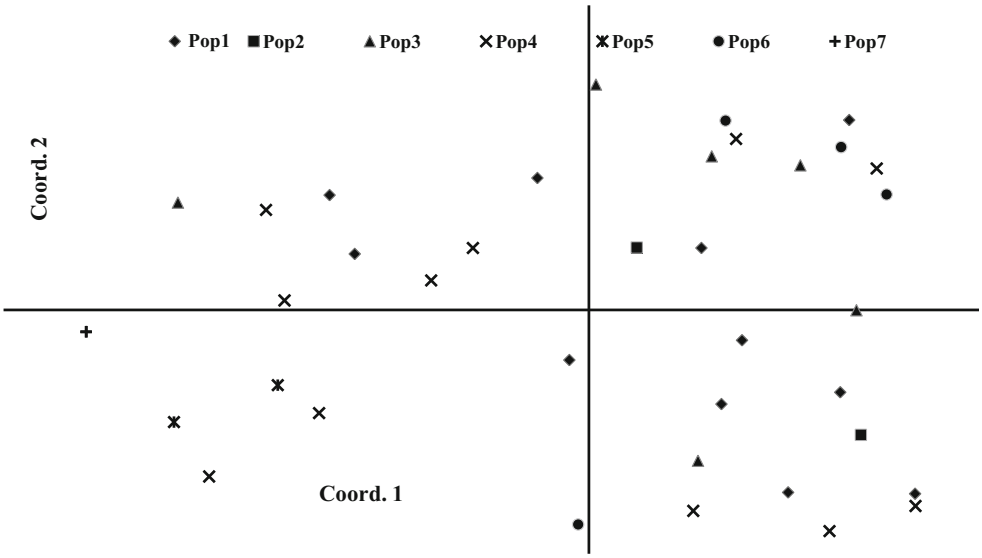

Fig. 6 The principal coordinate analysis (PCA) of seven populations of *Z. mauritiana* using ISSR primers

among 37 cultivars of *Z. mauritiana*. Nevertheless, no single SCoT or ISSR primer could distinguish all Ber cultivars independently. Perusal of the similarity coefficient presented in SCoT and ISSR dendrograms revealed similar values of similarity coefficients ranging from 0.80 to 0.92 and 0.79 to 0.96, respectively, and clearly delineated all the 37 cultivars of *Z. mauritiana* into well-supported distinct clusters which not only reveal appreciable genetic variation and relationships among the test cultivars but also validate the robustness of both the marker systems used. Previous studies on genetic diversity on Indian jujube polymorphism demonstrated similar ranges of genetic similarities using RAPD (Devanshi et al. 2007; Sehrawat et al. 2006; Singh et al. 2014), AFLP (Singh et al. 2006), and ISSR (Singh et al. 2007). Contrary to this, Obeed et al. (2008) using ISSR revealed 60 to 76% genetic similarity among five Ber cultivars from Pakistan. Fu et al. (2007) used ISSR and RAPD primer combinations for identification and classification of *ber* cultivars and reported a very narrow genetic background among Taiwan accessions. Liu et al. (2006) studied the genetic diversity in *Ziziphus jujuba* using RAPD and reported that the percentage of polymorphic loci in Zanhuangdazao population was lower (58%) than that in other Chinese jujube population (66%) and wild jujube population (89%).

It is evident from well-defined clusters of both the dendrograms that SCoT markers delineated *Z. mauritiana* cultivars better than ISSR markers with higher phylogenetic distances. In both the dendrograms, the grouping patterns of cultivars are more or less similar. For instance, grouping of BC-1 and Seb; Umran, Illaichi, Gola, Aliganj, and CAZRI-Gola; Banaras-Karaka and Thornless; Popular-Gola, Laddu, and Gola-Gurgaon; and Bagwadi, Thar-Bhubraj, and Maharwali in the same cluster in both the dendrograms validate reliable grouping and robustness of both the marker systems. The values of mantel test correlation showed a positive correlation between the SCoT and ISSR markers. A moderate correlation coefficient (r) of 0.24 is due to minor variations in the clustering patterns but eventually resulted in about 80% similarities in both the dendrograms indicating good fit for clustering. Pakseresht et al. (2013) performed comparative assessment of ISSR, DAMD, and SCoT markers for evaluation of genetic diversity and conservation of landrace chickpea genotypes and reported that the SCOT and DAMD markers are more effective in fingerprinting of chickpea genotypes. The cophenetic coefficient between the similarity values measured using ISSR and SCoT marker systems was acceptable indicating good fit for clustering.

Significantly higher contributions of the first three factors explaining a total of 58.36 and 68.71 variability using SCoT and ISSR marker systems resulting in wide distribution of *Z. mauritiana* cultivars across principal coordinates validate robustness and applicability of both the marker systems in analyzing the genetic diversity in Indian jujube germplasm. Further, mean genetic variation statistics of all the seven populations together revealed similar and significantly higher values of Nei's gene diversity 0.2365 and 0.2209 together with Shannon information index of 0.3456 and 0.3280 in SCoT and ISSR marker systems, respectively. Higher Gst values signify higher amount of differentiation observed over multiple loci among *Z. mauritiana* populations. On the other hand, higher gene flow demonstrating a very high migration rate between *Z. mauritiana* populations indicates higher rates of transfer of alleles or genes from one population to another. Migration into or out of a population may be responsible for a marked change in allele frequencies which may result in the addition of new genetic variants to the established gene pool or its population (Whitlock and McCauley 1999).

Table 7 Nucleotide base pair lengths of nuclear ribosomal RNA gene of 37 cultivars of *Ziziphus mauritiana*

S.N.	Genotype	Gen accession number	ITS-1 (bp)	5.8S (bp)	ITS-2 (bp)	Total (bp)
1	BC-1	KC155274	259	164	179	602
2	Umran	JQ627033	259	164	179	602
3	Seb	JQ627037	259	164	179	602
4	Illaichi	JQ627030	259	164	179	602
5	Tikadi	JQ627029	255	164	179	598
6	Gola	JQ627032	259	164	179	602
7	Reshmi	JQ627049	257	164	179	600
8	CAZRI-Gola	JQ627050	259	164	179	602
9	Banarasi Karaka	JQ627044	259	164	179	602
10	Aliganj	JQ627042	259	164	179	602
11	Katha	JQ627045	257	164	179	600
12	Z-G-3	JQ627046	257	164	179	600
13	Mundia	JQ627047	256	164	179	599
14	Bagwadi	JQ627048	260	164	179	603
15	Maharwali	JQ627039	257	164	179	600
16	Banarasi Pebandi	JQ627043	257	164	179	600
17	S × K hybrid	KC155275	259	164	179	602
18	Sanaur-5	JQ627036	259	164	179	602
19	Thornless	JQ627038	259	164	179	602
20	Kali	JQ627035	259	164	179	602
21	Jogia	JQ627031	259	164	179	602
22	Kaithli	JQ627041	259	164	180	603
23	Chhuhara	JQ627040	257	164	179	600
24	Dandan	JQ627034	257	164	179	600
25	Gola Gurgaon	KX088495	263	164	179	606
26	Chonchal	KX088496	259	164	179	602
27	Popular Gola	KX088497	259	164	179	602
28	Akrota	KX088498	259	164	179	602
29	Laddu	KX088499	259	164	179	602
30	Thar Bhubraj	KX088500	257	164	179	600
31	Ponda	KX088501	257	164	179	600
32	Wilayati	KX088502	260	164	179	603
33	Thar Sevika	KX088503	257	164	179	600
34	Narikeli	KX088504	260	164	179	603
35	Sua	KX088505	259	164	179	602
36	Vikas	KX088506	259	164	179	602
37	Babu	KX088507	257	164	179	600

Nei's unbiased measure of genetic distance analyses of both the marker systems revealed that the genetic diversity of *Z. mauritiana* varieties of population 1 (Rajasthan) was the richest among all the seven populations. The largest genetic distance occurred between populations 2 (Maharashtra) and 7 (West Bengal) which was due to the largest variable climate and the least between populations 1 (Rajasthan) and 3 (Punjab) which is attributed to a similar genetic background. The AMOVA of the distance matrix for all the seven populations revealed higher genetic variations existed within populations, i.e., 99 and 95%, and among populations (1 and 5%) in SCoT and ISSR molecular analyses, respectively, demonstrating that most of the genetic diversity exists within population rather than among Indian jujube populations. Earlier, researchers have also reported greater genetic variations within populations of various plant species (Raturi et al. 2012; Kakani et al. 2011) including *Z. mauritiana* (Singh et al. 2014).

The appearance of a single prominent amplified product of the 5.8S gene region of approximately 625 bp and the uniform

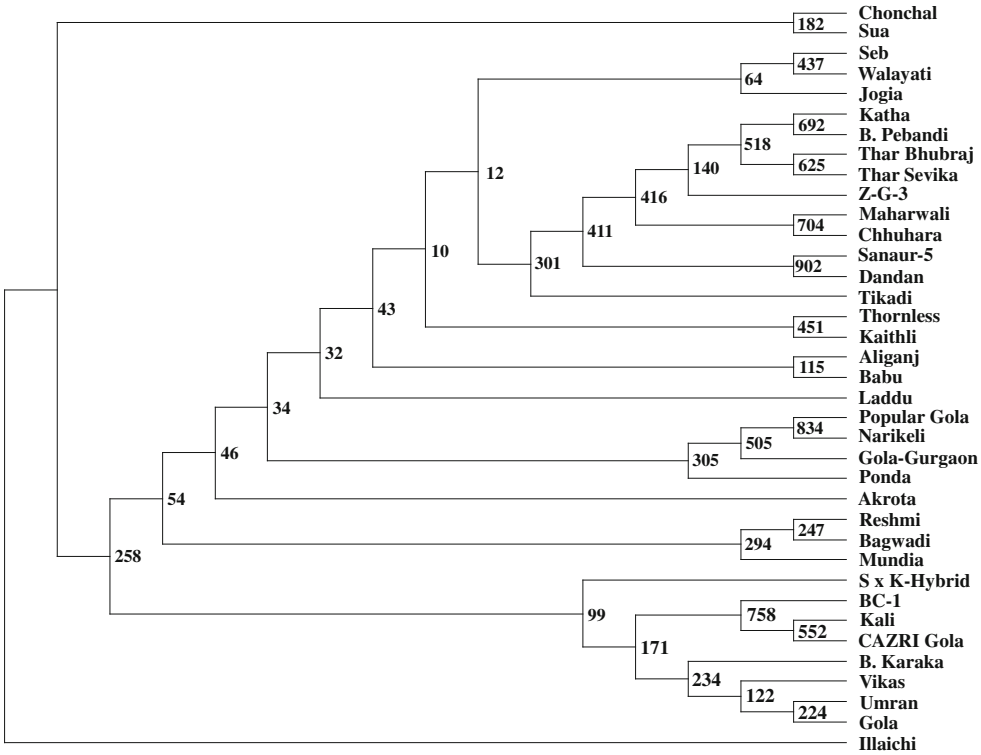

Fig. 7 Phylogram generated using an NJ plot of the multiple sequence aligned rDNA region of 37 cultivars of *Z. mauritiana*

nucleotide length of 164 bp of 5.8S rDNA all the 37 varieties of *Z. mauritiana* demonstrates the conserved nature of rDNA region. Substantial variation in the ITS-1 region ranging from 255 to 263 bp signifies phylogenetic utility specifically in assessing genetic diversity and relationships of the *Z. mauritiana* germplasm. Upon BLAST search, all 37 of *Z. mauritiana* cultivars exhibited 98 to 99% identities with the best aligned GenBank reference sequences. The perusal of the phylogram revealed further delineated *Z. mauritiana* varieties into eight main clusters with Illaichi as quite distinct outgroups. A high degree of nucleotide sequence variation exhibited substantial intraspecific genetic diversity due to variations in the nucleotide sequences by way of SNPs, INDELS, and ITS length polymorphism with significant boot strap values and allowed separation of all the 37 varieties of *Z. mauritiana* in the present study. Bootstrap values are dependable measures of phylogenetic accuracy, and higher values are likely to indicate reliable group (Hillis and Bull 1993).

The perusal of clustering patterns using three molecular marker systems vis-à-vis place of origin exhibited no consistency in grouping of *Z. mauritiana* cultivars as cultivars from the same place of origin were genetically cataloged into different SCoT, ISSR, and ITS phylogram clusters. This indicates that *Z. mauritiana* has wide genetic diversity and is widely distributed across agro-climatic zones. For instance, studies on other plant species *Trigonella foenum-graecum* (Kakani et al. 2011), *Vigna radiata* (Raturi et al. 2012), *Phaseolus vulgaris* (Martins et al. 2006) have also been reported with low correlation between geographic distribution and genetic distances measured using molecular markers. Such clustering of genotypes representing different states ignored the influence of geographical variations within the genetic diversity of 37 *Z. mauritiana* cultivars tested. Such a lack of correlation between geographic and genetic diversity in *Ziziphus* species have also been reported by Devanshi et al. (2007) and Ma et al. (2011).

The genetic variation is of utmost need for designing further breeding strategies in this fruit crop. 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 using molecular markers. This study would help in the consideration of molecular fingerprinting for germplasm conservation, rectification, purification, and identification of genotypes especially using SCoT, ISSR informative markers, and rDNA polymorphism.

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Data archiving statement All the 37 nuclear rDNA sequences have been deposited in NCBI, USA, and are available in public domain. Their GenBank accession numbers are KX688495–KX688507, JQ627029–JQ627050, and KC155274–KC155275. The details of the *Ziziphus mauritiana* cultivars and their sequences are presented in Table 7 of the manuscript.

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