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Population Genetic Studies in *Ziziphus jujuba* Mill.: Multiple Molecular Markers (ISSR, SRAP, ITS, Cp-DNA)

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Abstract. *Ziziphus jujuba* (jujube) is an important horticultural crop with medicinal value. It is under cultivation in many areas of Iran and also grows as wild in several geographical populations throughout the country. We have no information on genetic variability and population structure of this important plant species in our country. Therefore, the aim of the present study was to perform genetic fingerprinting of 13 geographical populations of jujuba for the first time and provide data on population genetic structure, admixture versus genetic fragmentation of this important crop. We used multilocus molecular markers (ISSRs and SRAPs) for genetic fingerprinting and also compared the results with bioinformatics investigation results we did on jujuba cultivars by using nuclear r-DNA and chloroplast inter-genetic cp-DNA sequences. Genetic diversity parameters and AMOVA test as well as Ivanno test support some kind of genetic distinctness of the jujuba populations studied. We found that cp-DNA inter-genic sequences can also discriminate jujuba cultivars as efficient as multilocus molecular markers and therefore, a multiple molecular approaches may be used for genetic fingerprinting of jujuba. The present study revealed good level of genetic diversity among wild/ uncultivated populations of jujuba which can be used in conservation and breeding of this important horticultural crop plant within the country. As this crop has several wild geographical populations throughout the country, we plan to continue our quest to investigate many more populations in nearby future and try to utilize cp-DNA inter-genic sequences along with multilocus molecular markers for genetic discrimination of wild populations.

Keyword. Cp-DNA, ISSR, ITS, SRAP, *Ziziphus jujube*.

INTRODUCTION

The genus *Ziziphus* Mill. belongs to the buckthorn family Rhamnaceae. It is contains about 40 species that are deciduous evergreen trees or shrubs distributed in the tropical and subtropical regions of the world (Sing et al. 2007). The wide geographical and climatic distribution makes it interesting

for genetic diversity investigations and gene pool identification.

South and Southeast Asia is the center of both evolution and distribution of the genus *Ziziphus* (Sing et al. 2007). Tow fossil species are known for *Ziziphus* in Eocene era (US Govt. Printing Office 1982).

Ziziphus species are of medicinal value and are known to be self-incompatible and have synchronous protandrous dichogamy and produce viable inter-specific hybrids (Asatryan and Tel-Zur, 2013). Among *Ziziphus* species, few are well known like: *Z. jujuba* (jujuba), and *Z. spina-christi* (L.)Desf. that grow in south-western Asia, *Z. lotus* in Mediterranean region, ber (*Z. mauritiana*), that is found in western Africa to India and *Z. joazeiro* Mill. that grows in the Caatinga of Brazil (Gupta et al. 2004; Jiang et al. 2007; Vahedi et al. 2008).

Traditional use of jujuba dates back 2,500 years ago in original Chinese material medical records. The fruit, seed, and bark of jujuba are also described in Korean, Indian, and Japanese traditional writings. They are used to alleviate stress and insomnia and as appetite stimulants, digestive aids, anti-arrhythmic, and contraceptives. The sweet smell of the fruit is said to make teenagers fall in love. The fruit is eaten fresh or dried and made into candy; tea, syrup, and wine are also made from the berries (Gupta et al. 2004; Jiang et al. 2007; Vahedi et al. 2008).

The fruit is energy-rich because of the large amount of sugar it contains. It is cultivated and eaten fresh, dry, and in jam. It is also added as a base in meals and in the manufacture of candy. The leaves can be either deciduous or evergreen depending on species, and are aromatic.

The seeds, fruit, and bark of jujuba have been used in traditional medicine for anxiety and insomnia, and as an appetite stimulant or digestive aid. Experiments in animals support the presence of anxiolytic and sedative properties. However, clinical trials are lacking (Gupta et al. 2004; Jiang et al. 2007; Vahedi et al. 2008). Some specific saponins, as well as ethyl acetate and water extracts of the fruit and bark, have explored the potential cytotoxicity of jujuba. Apoptosis and differential cell cycle arrest are suggested to be responsible for the dose-dependent reduction in cell viability. Activity against certain human cancer cell lines has been demonstrated in vitro (Lee et al. 2004; Huang et al. 2007; Vahedi et al. 2008).

Jujuba is one of the important horticultural crops in Iran and about with annual production of 4980 Kg that is about 14.7% of total cold region fruit production (34000 Tones) (Hosseinpour et al. 2016). It has been cultivated in several regions of the country and also is grown wild in several areas throughout Iran.

Different molecular markers have been used for population genetic investigation and phylogenetic studies in *Ziziphus* species. For example, Islam and Simmons (2006) performed an intra-generic classification of 19 *Ziziphus* species by using morphological characteristics and nuclear rDNA internal transcribed spacers, 26S rDNA, and the plastid trnL-F intergenic spacer. Similarly, the genetic relationships between different *Z. jujuba* cultivars and/ or wild jujuba individuals was studied by using random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), sequence-related amplified polymorphisms (SRAP), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR), and chloroplast microsatellite (Cp-SSR) markers (see for example, Peng et al. 2000; Liu et al. 2005; Wang et al. 2007; Singh et al. 2007; Wang et al. 2014; Zhang et al. 2014; Huang et al. 2015).

Population genetic study is an important step for genetic evaluation of medicinally important species as it provides insight on the genetic structure, genetic diversity and gene flow versus genetic fragmentation of these plant species. It also produces data on the number of potential gene pools for conservation and breeding strategies for the studied taxa (Sheidai et al. 2013, 2014, 2016).

The aims of present study are: 1- Produce data on population genetic structure of *Ziziphus jujuba* of Iran for the first time and 2- Investigate the discrimination power of ISSR and SRAP molecular markers in *Ziziphus jujuba* populations and compare them with sequencing data like nuclear r-DNA sequences (ITS = Internal transcribed spacer DNA) and chloroplast gene sequences.

We used ISSR (Inter simple sequence repeats) and SRAP (Sequence related amplified polymorphism) molecular markers, as these markers are very useful tool to detect genetic polymorphism, are inexpensive and readily adaptable technique for routine germplasm fingerprinting and evaluation of genetic relationship between accessions or genotypes and construction of genetic linkage maps (Sheidai et al. 2013, 2014, 2016). Moreover, SRAP markers target the open reading frames (ORFs).

MATERIAL AND METHODS

Plant Materials

In total 130 plants were studied in 13 geographical populations of *Ziziphus jujuba* (Table 1). Ten plants were randomly selected in each population and used for molecular studied (ISSR and SRAP).

Table 1. *Ziziphus jujuba* population in ISSR and SRAP studies.

	Province	Locality	Longitude	Latitude
1	Qom	Kalaghneshin	50. 2536 °	34.4122°
2	Qom	Ghaziolia	50.2850°	34.3222°
3	Qom	Dolatabad	50.3032°	34.1258°
4	Qom	Jafarieh	50.3429°	34.4722°
5	Qom	Khalajestan	50.3844°	34.2852°
6	Markazi	Aveh	50.2523°	34.4732°
7	Markazi	Delijan	50.4102°	33.5926°
8	Markazi	Saveh	50.2124°	35.0117°
9	Esfahan	Kashan niasar	51.0856°	33.5822°
10	Esfahan	Koohpayeh	52.2623°	32.4249°
11	Esfahan	Shahreza	51.5200°	32.0032°
12	Esfahan	Dehaghan	51.3916°	31.5612°
13	Esfahan	Ardestan	52.2238°	33.232.07°

DNA Extraction

For molecular studies, the fresh leaves were randomly collected from 53 randomly selected plants in the studied area and were dried in silica gel powder. The genomic DNA was extracted using CTAB-activated charcoal protocol (Križ man et al. 2006). The extraction procedure was based on activated charcoal and poly vinyl pyrrolidone (PVP) for binding of polyphenolics during extraction and under mild extraction and precipitation conditions. This promoted high-molecular-weight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 0.8% agarose gel.

ISSR Assay

Ten ISSR primers, UBC 807, UBC 810, UBC 811, UBC 834, CAG(GA)7, (CA)7AC, (CA)7AT, (CA)7GT (GA)9A, and (GA)9T, commercialized by the University of British Columbia, were used.

PCR reactions were performed in a 25-µL volume containing 10 mM Tris-HCl buff er at pH 8, 50 mM KCl, 1.5 mM MgCl2 , 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng of genomic DNA, and 3 U of Taq DNA polymerase (Bioron).

Amplification reactions were performed in a Techne thermocycler (Germany) with the following program: 5 min for initial denaturation step at 94 °C, 30 s at 94 °C, 1 min at 52 °C, and 1 min at 72 °C. Th e reaction was completed by a final extension step of 7 min at 72 °C. The amplification products were visualized by running on 2% agarose gel, followed by ethidium bromide staining. The fragments size was estimated by using a 100-bp molecular size ladder (Fermentas, Germany). The experiment was replicated 3 times and constant ISSR bands were used for further analyses.

SRAP Assay

Five sequences related amplified polymorphism (SRAP) primer pairs including forward primers: Me1, Me2, Me3, Me4, Me5 and reverse primers: Em1, Em2, Em3, Em4, Em5 were used (Feng et al. 2014).

PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 1 U of *Taq* DNA polymerase (Bioron, Germany).

The amplifications, reactions were performed in Techne thermocycler (Germany) with the following program: 5Min initial denaturation step 94°C, followed by five cycles of 94°C for 1min, 35°C for 45 sec, and 72°C for 1 min; followed by 35 cycles of 94°C for 1min, 50°C for 45 sec, and ITC for 1 min; followed by 7 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

ITS and cp-DNA Inter-Genic Sequences Analyses

cp-DNA and nuclear-DNA ITS sequences of 11 jujuba cultivars were obtained from NCBI(National Center for Bioinformatic Information) and used to differentiate the studied cultivars. The cultivars accession numbers have been provided in tables 2 and 3.

Data Analyses

The ISSR and SRAP bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The number of private bands versus common bands was determined. Genetic diversity parameters like: The percentage of allelic polymorphism, allele diversity (Weising, 2005), Nei's gene diversity (He), and Shannon information index (I) (Weising, 2005), were determined. We used GenAlex 6.4 for these analyses (Peakall and Smouse 2006).

The Nei genetic distance (Weising 2005) was determined among the studied populations and was used for the grouping of the genotypes. Genetic differentiation of the studied populations was studied by AMOVA with 1000 permutations as performed in GenAlex 6.4 (Peakall and Smouse 2006).

Table 2. The accession numbers of taxa in cp-DNA studies.

No	Species	accession number
1	*Ziziphus jujuba*	HG765030.1
2	*Ziziphus jujuba*	HG765029.1
3	*Ziziphus jujuba*	HG765028.1
4	*Ziziphus jujuba*	GQ435353.1
5	*Ziziphus jujuba*	EU075109.1

Table 3. The accession numbers of taxa in ITS studies.

No	Species	accession number
1	*Ziziphus jujuba*	DQ146578.1
2	*Ziziphus jujuba*	DQ146577.1
3	*Ziziphus jujuba*	DQ146576.1
4	*Ziziphus jujuba*	DQ146575.1
5	*Ziziphus jujuba*	DQ146574.1
6	*Ziziphus jujuba*	DQ146573.1
7	*Ziziphus jujuba*	FJ593183.1
8	*Ziziphus jujuba*	EU075088.1
9	*Ziziphus jujuba*	KF241298.1
10	*Ziziphus jujuba*	KF241297.1
11	*Ziziphus jujuba*	KF186458.1

The Mantel test (Podani 2000) was performed to study the association between genetic distance and geographical distance of the studied populations. We also used Mantel test to investigate the agreement of results between ISSR and SRAP data. PAST ver. 3.14 (Hammer et al. 2001).

Genetic structure of the populations was studied by model-based clustering as performed by STRUCTURE software ver. 2.3 (Pritchard et al. 2000). We used the admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K (1-13) after a burn-in period of 10^5 . Data were scored as dominant markers and analysis followed the method suggested by Falush et al. (2007).

For the optimal value of K in the studied populations we used the STRUCTURE Harvester website (Earl and von Holdt 2012) to perform the Evanno method (Evanno et al. 2005). The choice of the most likely number of clusters (K) was carried out by calculating an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive K values, as described by Evanno et al. (2005).

For ITS and cp-DNA the sequences were aligned by MUSCLE program as implemented in MEGA 7. NJ and Maximum likelihood phylogenetic trees were constructed by MEGA7 software (Tamura et al. 2012). Kimura distance was determined for jujuba cultivars based on ITS and cp-DNA sequences by MEGA ver.7.

RESULTS

ISSR assay

We obtained 40 ISSR bands (Loci) in total (Table 4). The highest Number of bands (27 bands) occurred in population 9 (Neyasar), followed by population 7 (Delijan) (23 bands). Some of the populations had private bands with population 9 having the highest number (6 private bands). Few common bands occurred in the population too. These are shared alleles among these populations.

Genetic diversity parameters determined in *Z. jujuba* populations are presented in Table 5. The percentage of genetic polymorphisma obtained ranged from 7.50 in population 2 (Ghazi-Olya) to 52.50 in population 7 (Delijan). A good level of genetic polymorphism (37.50%) also occurred in three populations 3, 4, and 5 (Doolatabad, Jafariyeh, and Dastjerd, respectively). The same populations had higher value of gene diversity (He).

AMOVA revealed that these populations differ significantly in their genetic content (PhiPT = 0.54, P = 0.001). AMOVA identified that 72% of total genetic variability occurred among populations while, 28% of genetic variability was due to within population difference. Paired-sample AMOVA also produced significant difference among the studied populations.

NJ clustering (Figure 1) revealed that most of the samples in the studied populations are grouped together and are almost separated from the other populations (For example, samples in populations 1, 2, 7, 8, 9, 11, 12, and 13).

Nei, genetic distance and genetic identity determined among *Ziziphus jujuba* populations (Table 6) revealed that genetic similarity among populations ranged from 0.58 between populations 9 and 13, to 0.93 between populations 3 and 5.

Table 4. Details of ISSR bands obtained in the studied populations of *Ziziphus jujuba* (populations numbers are according to Table 1).

Population	1	2	3	4	5	6	7	8	9	10	11	12	13
No. Bands	21	14	20	21	18	16	23	10	27	16	14	15	15
No. Private Bands.	0	1	0	0	0	1	2	0	6	0	0	0	1
No. LComm Bands (<=25%)	1	0	1	1	0	0	1	0	3	1	0	0	1
No. LComm Bands (<=50%)	5	2	4	3	2	2	6	2	6	3	4	3	1

Table 5. Genetic variability parameters determined in *Ziziphus jujube* populations based on ISSR markers (pop ulations numbers are according to Table 1).

Pop	Na	Ne	I	He	uHe	%P
Pop 1	0.800	10157	0.146	0.097	0.116	%27.50
Pop 2	0.425	1.045	0.041	0.027	0.033	%7.50
Pop 3	0.875	1.251	0.209	0.142	0.157	%27.50
Pop 4	0.900	1.283	0.232	0.154	0.176	37.50%
Pop 5	0.825	1.302	0.231	0.161	0.179	%37.50
Pop 6	0.575	1.101	0.092	0.061	0.070	%17.50
Pop 7	1.100	1.352	0.292	0.189	0.220	%52.50
Pop 8	0.425	1.125	0.102	0.070	0.080	%17.50
Pop 9	0.975	1.194	0.169	0.113	0.136	%30
Pop 10	0.550	1.077	0.072	0.047	0.052	%15
Pop 11	0.525	1.131	0.105	0.072	0.080	%17.50
Pop 12	0.600	1.136	0.123	0.082	0.098	%22.50
Pop 13	0.550	1.115	0.097	0.065	0.075	%17.50

N = No. plants, Na = No. alleles, Ne = No. effective alleles, I = Shanon Information Index, He = Nei gene diversity, UHe = Unbiased gene diversity, %P = Percentage of genetic polymorphism

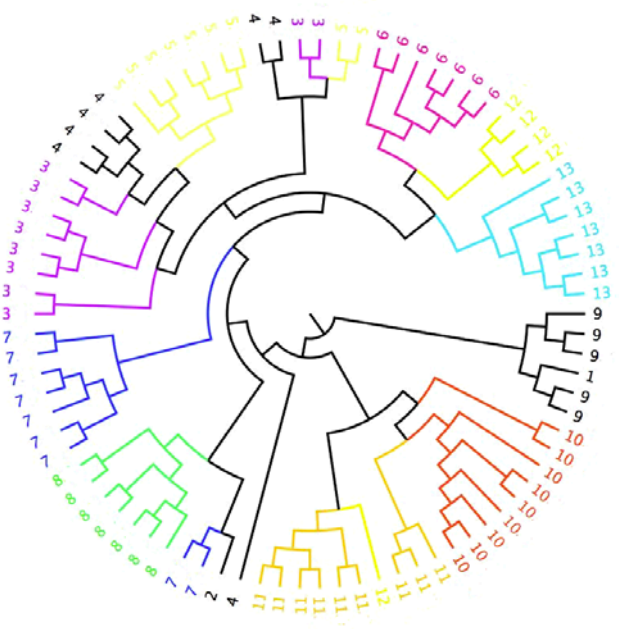

Figure 1. NJ dendrogram of *Ziziphus jujuba* specimens showing genetic differences of the studied populations.

Mantel test between geographical distance and genetic distance produced significant correlation (P<0.01). Therefore, with increase in geographical distance, genetic difference of the populations increased and isolation by distance (IBD) occurred in *Z. jujuba* populations studied.

The genetic structure of the studied populations and degree of gene flow/ or shared common alleles were determined by STRUCTURE analysis. The STRUCTURE plot (Figure 2) revealed presence of different allele combinations (differently coloured segments) in the *Z. jujuba* populations. However, some degree of shared common alleles was observed between populations 3 and 4, and to lesser extent population 5. Similarly, populations 10 and 11 had genetic similarity. The other populations had unique allele combinations (specific coloured segment) as well as some degree of shared alleles.

Evanno test produced optimal number of genetic group k = 8. Therefore, 13 studied Ziziphus jujuba populations studied could be grouped in 8 genetic groups.

SRAP Markers Assay

We obtained 42 SRAP bands (Loci) in total (Table 7). The highest Number of bands (26 bands) occurred in population 13, while the lowest number of SRAP bands occurred in population 4 (14 bands). Populations 1, 4, 8 and 13 had private bands. Few common bands occurred in the population too. These are shared alleles among the studied populations.

Genetic diversity parameters determined based on SRAP molecular markers in *Z. jujuba* populations are presented in Table 8. The percentage of genetic polymorphisma obtained ranged from 7.14 in population 8 to 38.10 in populations 3 and 13. These two populations had higher value of gene diversity (He).

AMOVA revealed that the studied *Ziziphus jujuba* populations differ significantly in their genetic content (PhiPT = 0.65, P = 0.001). AMOVA identified that 66% of total genetic variability occurred among populations while, 34% of genetic variability was due to within population difference. Paired-sample AMOVA also produced significant difference among the studied populations. NJ distance clustering (Figure 3) revealed that most of the samples in the studied populations are grouped together and are almost separated from the other populations (For example, samples in populations 1, 9, 12 and 13). This indicates that SRAP molecular markers can efficiently differentiate jujube populations and may be used in germplasm diversity evaluation.

PCoA plot of the studied populations (Figure 4) obtained after 99 permutations, almost separated the studied populations in two major groups (with populations 1 and 9 somewhere in the middle).

The populations 2-7 formed the first group, while populations 8, 10-13, comprised the second group. Therefore, *Zizphus jujuba* populations can be genetically discriminated by ISSR markers.

Table 6. Nei genetic distance and genetic identity (populations numbers are according to Table1).

Pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13
1	****	0.7857	0.7781	0.7202	0.7947	0.7194	0.8011	0.7576	0.8117	0.7012	0.7697	0.7462	0.7660
2	0.2412	****	0.7929	0.7385	0.8074	0.6631	0.7905	0.7260	0.6737	0.7130	0.6715	0.6947	0.7035
3	0.2509	0.2321	****	0.9339	0.9511	0.8575	0.9064	0.8043	0.7280	0.7418	0.7362	0.8516	0.8704
4	0.3282	0.3031	0.0684	****	0.9309	0.8262	0.8844	0.7837	0.6696	0.7370	0.6891	0.8023	0.7803
5	0.2298	0.2139	0.0502	0.0716	****	0.8507	0.9397	0.8183	0.6888	0.7431	0.7417	0.8282	0.8274
6	0.3294	0.4109	0.1537	0.1909	0.1617	****	0.8230	0.7832	0.5932	0.7619	0.7230	0.8882	0.8224
7	0.2218	0.2351	0.0982	0.1228	0.0622	0.1948	****	0.8516	0.6906	0.7853	0.8219	0.8283	0.8054
8	0.2776	0.3202	0.2178	0.2437	0.2006	0.2443	0.1606	****	0.6428	0.7891	0.7644	0.7943	0.7376
9	0.2086	0.3950	0.3175	0.4011	0.3728	0.5222	0.3703	0.4419	****	0.5813	0.6721	0.6473	0.7684
10	0.3549	0.3383	0.2986	0.3052	0.2970	0.2719	0.2416	0.2369	0.5425	****	0.8698	0.7940	0.7124
11	0.2617	0.3983	0.3062	0.3724	0.2988	0.3244	0.1961	0.2686	0.3973	0.1395	****	0.8327	0.6940
12	0.2928	0.3643	0.1606	0.2203	0.1886	0.1186	0.1883	0.2303	0.4350	0.2307	0.1831	****	0.8511
13	0.2666	0.3517	0.1388	0.2481	0.1895	0.1956	0.2164	0.3043	0.2634	0.3392	0.3653	0.1613	****

Table 7. Details of SRAP bands obtained in the studied populations of *Ziziphus jujuba* (populations numbers are according to Table 1).

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8	Pop9	Pop10	Pop11	Pop12	Pop13
No. Bands	21	21	20	14	18	17	21	16	17	20	19	20	26
No. Bands Freq. >= 5%	21	21	20	14	18	17	21	16	17	20	19	20	26
No. Private Bands	1	0	0	1	0	0	0	1	1	0	0	0	2
No. LComm Bands (<=25%)	3	0	0	0	2	1	1	0	2	1	1	1	1
No. LComm Bands (<=50%)	8	7	7	4	5	5	8	1	5	6	7	8	11

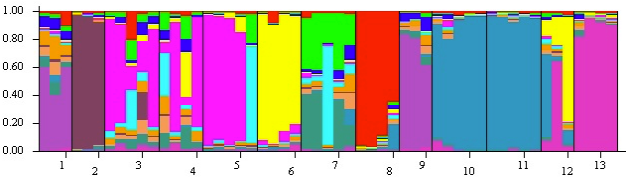

Figure 2. STRUCTURE plot of *Ziziphus jujuba* populations studied (populations numbers are according to Table1).

Table 8. Genetic distance among jujube cultivars based on cp-DNA PSBA sequences (populations numbers are according to Table 2).

	1	2	3	4
2	0			
3	0	0		
4	0.58	0.58	0.58	
5	0.58	0.58	0.58	0

STRUCTURE plot of SRAP molecular markers (Figure 5) revealed more detailed information on the genetic affinity of the studied populations. It also revealed the presence of specific allele combinations (differently coloured segments) versus available common shared alleles (similarly coloured segments) in these populations. For example, close affinity between populations 1 and 9 that were identified by PCoA plot seems to be due to some low degree of shared common alleles between these populations. The same is true for the other studied populations.

Evanno test produced delta k = 2 as the optimal genetic groups. Therefore, the studied jujuba populations can be differentiated in two broader and distinct genetic groups. The populations 1-7 form the first group, while populations 8-13 comprise the second group.

Mantel test performed between ISSR and SRAP data produced significant correlation (P = 0005). Therefore, both types of molecular markers efficiently differentiate jujuba populations and also show similar genetic grouping.

Similarly, Mantel test produced significant correlation (P = 0.001) between the studied molecular markers with geographical distance of the populations. Therefore, with increase in geographical distance among jujube populations, the genetic difference of these populations also increases. This indicates the occurrence of IBD (Isolation by distance) in the studied jujuba populations.

Table 9. Genetic distance among jujube cultivars based on nuclear DNA (ITS sequences) (populations numbers are according to Table 2).

	1	2	3	4	5	6	7	8	9	10
2	0									
3	0	0								
4	0	0.003	0.003							
5	0	0.003	0.003	0						
6	0	0.003	0.003	0	0					
7	0	0.003	0.003	0	0	0				
8	0	0.007	0.007	0.003	0.003	0.003	0.003			
9	0	0.007	0.007	0.003	0.0037	0.003	0.003	0		
10	0	0.007	0.007	0.003	0.0037	0.003	0.003	0	0	
11	0	0.007	0.007	0.0037	0.0037	0.003	0.003	0	0	0

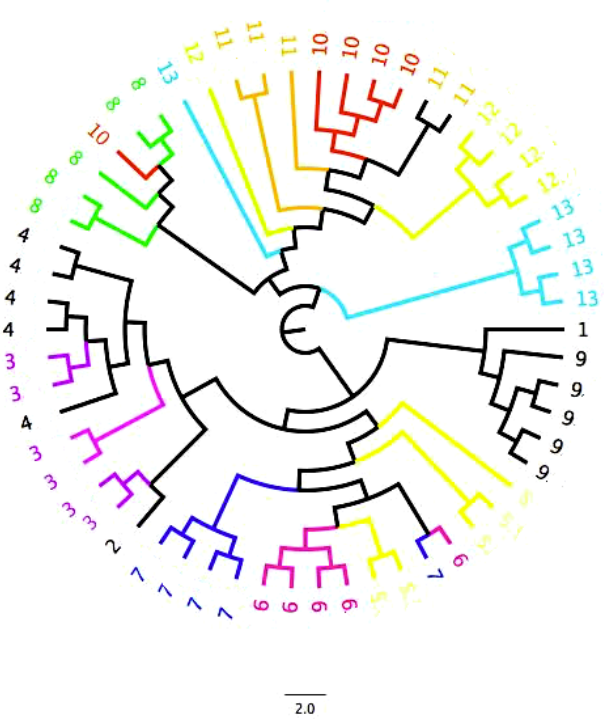

Figure 3. NJ dendrogram of the studied *Ziziphus jujube* populations based on SRAP molecular markers. (Populations 1-13 are according to Table 1).

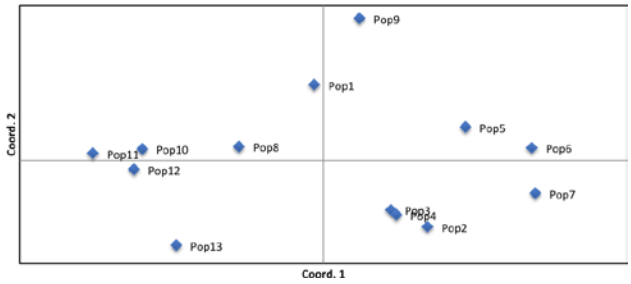

Figure 4. PCoA plot of *Ziziphus jujube* populations based on SRAP molecular markers. (Populations 1-13 are according to Table 1).

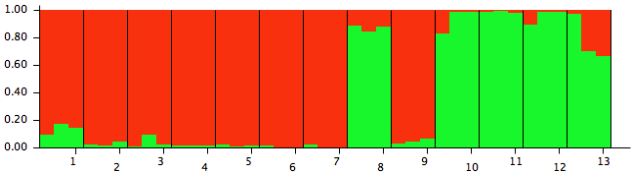

Figure 5. Top: STRUCTURE plot of *Ziziphus jujuba* populations based on SRAP data. Bottom: STRUCTURE plot based on k = 2 (Populations 1-13 are according to Table 1).

ITS and cp- DNA Sequences

Nuclear r-DNA (ITS) and chloroplast inter-genic region of trnH-psbA sequence data were obtained for few jujuba cultivars. Phylogenetic tree based on these sequences (Figures 6 and 7) differentiated the studied cultivars in three clusters with high bootstrap values. Therefore, we can also apply these sequence-based molecular markers in future studies to investigate jujuba cultivar discrimination, the methods that have not been utilized in genetic finger printing of this important horticultural plant species.

Pair-wise genetic distances in the studied jujube cultivars are provided in Tables 9 and 10. In case of trnH-psbA, we obtained the mean genetic distance of 0.58 which is comparable to the genetic distance obtained for ISSR and SRAP molecular markers. However, in case of ITS sequences, we obtained much lower genetic distance value (0.003-0.007). This is probably due to much more conservative nature of ITS sequences compared to that of cp-DNA inter-genic sequences. Therefore, we may suggest using cp-DNA inter-genetic sequences for future

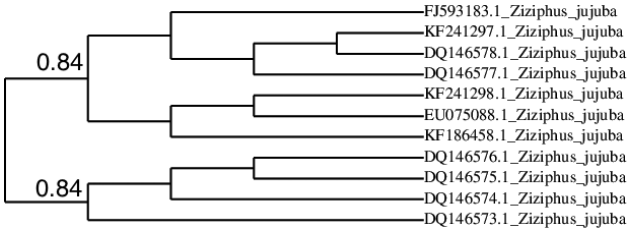

Figure 6. Maximum parsimony phylogenetic tree of jujube cultivars based on ITS sequences (Numbers above branches are bootstrap value).

Table 9. Genetic distance among jujube cultivars based on cp-DNA PSBA sequences (populations numbers are according to Table 2).

	1	2	3	4
2	0			
3	0	0		
4	0.58	0.58	0.58	
5	0.58	0.58	0.58	0

genetic finger printing of jujube cultivars and populations, but also keeping in mind that using multilocus molecular markers (ISSRs and SRAPs) are more cost-benefit approaches.

DISCUSSION

Population genetic study provides valuable information on genetic structure of plants, the stratification versus gene flow among the species populations, genetic divergence of the populations, etc. (Sheidai et al. 2014). These information have different applications, and from pure understanding of biology of the species to conservation of endangered species, choosing of proper parents

Figure 7. Maximum parsimony phylogenetic tree of jujube cultivars based on trnH-psbA sequences (Numbers above branches are bootstrap value).

for hybridization and breeding and phylogeography and mechanism of invasion (Freeland et al. 2011). *Ziziphus jububa* is of wide spread in our country and it has several medicinal applications (Vahedi et al. 2008), however we had no information on its genetic structure. The present study revealed interesting data about its genetic variability, and genetic stratification of this medicinally important species in the country.

Assessment of the genetic variation within collections of *Ziziphus jujuba* genetic resources is crucial for the effective conservation and utilization of these resources in breeding programs, and could be dramatically enhanced by using molecular genotyping tools. The present study revealed that multilocus molecular markers like ISSRs and SRAPs are powerful technique for the assessment of genetic variability among *Ziziphus jujuba* collections. Moreover, we can also use cp-DNA inter-genic sequences for genetic finger printing and discriminating jujuba cultivars and populations. For

Table 10. Genetic distance among jujube cultivars based on nuclear DNA (ITS sequences) (populations numbers are according to Table 2).

	1	2	3	4	5	6	7	8	9	10
2	0									
3	0	0								
4	0	0.003	0.003							
5	0	0.003	0.003	0						
6	0	0.003	0.003	0	0					
7	0	0.003	0.003	0	0	0				
8	0	0.007	0.007	0.003	0.003	0.003	0.003			
9	0	0.007	0.007	0.003	0.0037	0.003	0.003	0		
10	0	0.007	0.007	0.003	0.0037	0.003	0.003	0	0	
11	0	0.007	0.007	0.0037	0.0037	0.003	0.003	0	0	0

grouping of the cultivars we can also utilizenuclear r-DNS sequences.

We obtained about 40 bands for either of ISSR and SRAP molecular markers and almost good level of genetic variability within each population (ranging from 17 to 35%). These markers have good discriminating power to differentiated jujuba populations. Cp-DNA inter-genic sequences also revealed high degree of genetic difference among jujuba cultivars (0.58).

Saleh et al. (2016), studied genetic diversity in populations of *Ziziphus spina-christi* (L.) Willd. By using 11 ISSR markers and reported the occurrence of 105 scorable loci, of which 93.4% were found to be polymorphic. They obtained genetic diversity value of 0.26, and total genetic diversity Ht = 0.266, as well as intra-population genetic diversity, Hs = 0.22.

These values are in good agreement with genetic variability obtained here by both multilocus molecular markers (ISSRs and SRAPs) as well as cp-DNA sequences.

The genetic variability within the studied populations is of fundamental importance in the continuity of a species as it is used to bring about the necessary adaptation to the cope with changes in the environment (Sheidai et al. 2013, 2014). This is particularly expected in *Ziziphus jujuba* as it forms several geographical populations throughout the country.

Degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/ cross breeding brings about higher level of genetic variability in the studied taxon (Freeland et al. 2011). *Ziziphus jujuba* is a self-incompatible species (Asatryan and Tel-Zur 2013) and therefore, moderate genetic variability in these populations may be related to the open pollination nature of this species.

AMOVA revealed significant genetic difference among the studied populations of jujube, while Ivanno test identified 8 genetic groups within these populations. Moreover, Mantel test showed positive significant correlation between genetic distance and geographical distance. All these data support some kind of genetic distinctness of the jujuba populations studied. Different mechanisms like isolation, drift, founder effects and local selection may act to bring about among population differentiation (Jolivet and Bernasconi 2007; Sheidai et al. 2014).

In conclusion, the present study revealed good level of genetic diversity among wild/ uncultivated populations of jujube which can be used in conservation and breeding of this important horticultural crop plant within the country. As this crop has several wild geographical populations throughout the country, we plan to continue our quest to investigate many more populations in nearby future and try to utilize cp-DNA inter-genic sequences along with multilocus molecular markers for genetic discrimination of wild populations.

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