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Original scientific paper

GENETIC AND MORPHOLOGICAL VARIABILITY IN *Ziziphus jujuba* Mill.

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Ziziphus jujuba (jujube) one of the well-known species of the *ziziphus* is medicinally important tree species that is also used as food source for its fruits. Conservation and breeding studies are in hand for this horticultural plant and for this reason a thorough population genetic study was performed. We carried out both molecular (Inter-retrotransposon amplified polymorphism: IRAP) and morphological (fruit characteristics) studies in 71 jujube trees collected randomly from 8 geographical regions in Iran. IRAP markers could differentiate the studied populations, as AMOVA revealed significant genetic difference among the studied populations. Networking and STRUCTURE analyses indicated that the jujube populations can be placed in two main genetic groups. Nm analysis revealed limited gene flow among populations. Jujube populations also differed significantly in their fruit characteristics and PCA analysis showed that these populations are diverged in these characters too. Data obtained can be used in future conservation and breeding studies of this important horticultural plant.
Keywords: *Ziziphus jujuba*, IRAP, AMOVA, Fruit morphology

INTRODUCTION

Ziziphus jujuba (jujube), (family *Rhamnaceae*), is one of the well-known species of the *ziziphus* genus for its food value and medicinal importance (VAHEDI *et al.*, 2008). Traditional use of the species dates back to 2,500 years ago, as revealed in the original Chinese materia medica records.

The fruit, seed, and bark of jujube are used to alleviate stress and insomnia and as appetite stimulants, digestive aids, antiarrhythmics, and contraceptives. The fruit is eaten fresh or dried

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and made into candy, tea, syrup (GUPTA *et al.*, 2004; JIANG *et al.*, 2007; VAHEDI *et al.*, 2008). Moreover, some specific saponins, as well as ethyl acetate and water extracts of the fruit and bark, have explored the potential cytotoxicity of jujube. 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).

All these medicinal and food properties have made *Ziziphus jujuba* as an important plant species to the mankind, and therefore, its cultivation and conservation is of immediate and high importance nowadays Moreover, as jujube has wide geographical distribution and formed many local populations, it is important to be studied from population genetic point of view. The species with extensive geographical distribution can be adapted to adverse environmental conditions and harbor different genetic content that may be used in future breeding programs and establishing genetic-rich germ plasm collections (SHEIDAI *et al.*, 2013; 2014; 2016).

Different molecular markers were used to investigate the genetic relationships between different *Z. jujuba* cultivars and/ or wild jujube individuals. These molecular markers are 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 were used (see for example, ZHAO and LIU, 2003; 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; NABAVI *et al.*, 2019).

Population genetic study is an important step for genetic evaluation of medicinaly 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). Therefore the aim of present study was to produce data on population genetic structure of *Ziziphus jujuba* of Iran. We investigated 150 plants of both cultivated as well as wild jujube growing in 32 localities within 8 provinces.

For genetic study we used IRAP molecular markers, as these markers are very useful tool to detect genetic polymorphism, are inexpensive and readily adaptable technique for routine germ plasm fingerprinting and evaluation of genetic relationship between accessions or genotypes and construction of genetic linkage maps (SHEIDAI *et al.*, 2013; 2015).

MATERIALS AND METHODS

Plant materials

In total 71 plants were studied in 8 provinces (Fig. 1). 10 plants were randomly selected in each population and used for molecular and fruit morphological studied.

Fig. 1 Distribution map of *Zizphus jujuba* populations studied: 1(South Khorasan), 2(Fars), 3(Razavi Khorasan), 4(Sistan and Baluchestan), 5(Qazvin), 6(Kerman), 7(Golestan), 8(Mazandaran)

IRAP assay

For molecular studies, the fresh leaves were randomly collected from 71 randomly selected plants in the studied area and were dried in silica gelpowder. The genomic DNA was extracted using CTAB-activatedcharcoal protocol (KRIZMAN *et al*., 2006). The extraction procedure was based on activated charcoal and polyvinylpyrrolidone (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.

Five inter-retrotransposon amplified polymorphism (IRAP) primer pairs including forward primers: LTR 6149, 3'LTR and Nikita and reverse primers: LTR 6150, 5'LRT1, 5'LRT2 were used (KALENDAR *et al*., 1999). PCR reactions were performed in a 25-µL volume containing 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM $MgCl_2$,0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng of genomic DNA, and 3 U of Tag 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. The 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, folowed by ethidium bromide staining. The fragment size was estimated using a 100-bp molecular size ladder

(Fermentas, Germany). The experiment was replicated 3 times and constant ISSR bands were used for further analyses.

Morphological studies

Jujube trees were studied for following fruit morphological characters. Fruit characters studied were: wet weight, dry weight, difference between dry and wet weight, dry / wet weight ratio, length of fruit, width of fruit, difference between dry length and weight.

Data analyses

The IRAP bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The numbers of private versus common alleles were determined. The shared loci among populations were determined by POPGENE ver. 1.3 (2000). Genetic diversity parameters like, the percentage of allelic polymorphism, gene diversity (He), Shannon information index (I), the number of effective alleles, and percentage of polymorphism (WEISING, 2005), were determined in the studied populations by using GenAlex 6.4 (PEAKALL AND SMOUSE, 2006).

For genetic grouping of the studied jujube trees, the Nei genetic distance was determined (WEISING, 2005), and used in clustering as well as ordination methods (PODANI, 2000). Genetic differentiation of the studied populations was determined by AMOVA after 1000 permutations as performed in GenAlex 6.4 (PEAKALL and SMOUSE, 2006). The Mantel test (PODANI, 2000) after 5000 permutation was performed to study the association between genetic distance and geographical distance of the studied populations. DCA (Deternded Correspondence Analysis) was used (PODANI, 2000). Data analyses were performed by PAST ver. 2.17 (HAMMER *et al*., 2012).

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 (1-8) 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 population studied, we used the STRUCTURE Harvester website (EARLAND and VON HOLDT, 2012) was used to perform the Evanno method to identify the proper value of K (EVANNO *et al*., 2005).

For morphological difference among population, ANOVA (Analysis of variance) was performed, while for population divergence, PCA (Principal components analysis) was used (PODANI, 2000). Data analyses were performed by PAST ver. 2.17 (HAMMER *et al*., 2012).

RESULTS

IRAP assay and genetic diversity

We obtained 41 IRAP bands (Loci) in total (Table 1). The highest number of bands (26 bands) occurred in population 3 and 4, followed by population 2 (24 bands). A few private bands occurred in some of the population (Table 1).

Table 1. Details of IRAP bands in Ziziphus jujuba populations (Populations 1-8 are according to Fig. 1)

	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8
No. Bands	17	24	26	26	22	17	18	23
No. Bands Freq. (≥ 5%)	17	24	26	26	22	17	18	23
No. Private Bands	1	2	0	2	0	1	0	0
No. Common Bands (≤ 25%)	0	0	3	3	1	0	1	2
No. Common Bands (≤ 50%)	2	9	11	11	6	3	3	7

DCA (Deternded Correspondence Analysis) revealed that IRAP molecular markers are not closely linked to each other as these loci are distributed in different positions of DCA plot (Fig. 2). This indicates that these markers represent different regions of the genome and are suitable molecular markers for differentiating jujube cultivars.

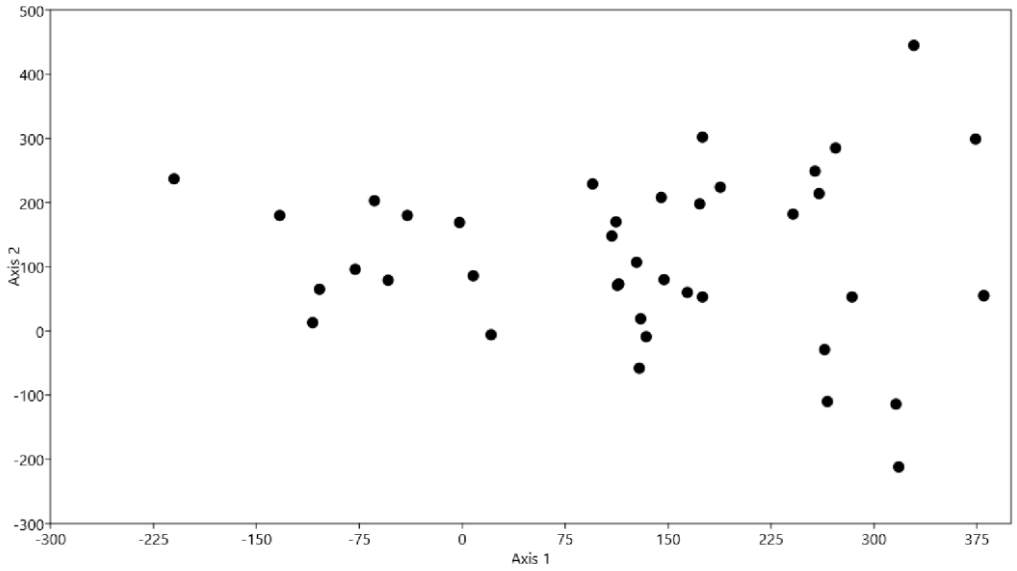

Fig. 2. DCA plot of IRAP loci in *Ziziphus jujuba*

Genetic diversity parameters determined in *Z. jujuba* populations are presented in Table 2. The percentage of genetic polymorphism obtained ranged from 9.76% in population 1 to 48.34% in population 2. A good level of genetic polymorphism (46.34%) also occurred in population 3. The same populations had higher value of gene diversity (He). Discriminating power of IRAP loci in jujube populations is shown in Table 3.

Table 2. Genetic diversity parameters determined in Z. jujuba populations (Pop. 1-8 are shown in Fig. 1)

Pop	N	Na	Ne	I	He	uHe	%P
Pop1	10.000	0.512	1.045	0.046	0.029	0.031	9.76%
Pop2	10.000	1.049	1.229	0.221	0.143	0.151	46.34%
Pop3	10.000	1.122	1.311	0.270	0.182	0.191	48.78%
Pop4	10.000	1.024	1.186	0.175	0.113	0.119	39.02%
Pop5	5.000	0.902	1.231	0.199	0.134	0.149	36.59%
Pop6	10.000	0.610	1.160	0.124	0.086	0.091	19.51%
Pop7	6.000	0.683	1.182	0.148	0.102	0.112	24.39%
Pop8	10.000	0.951	1.254	0.217	0.147	0.155	39.02%

N = No. Of studied plants, Na = No. Of polymorphic alleles, Ne = Effective No. of alleles, He = New gene diversity, uHe = Unbiassed gene diversity, and P% = Percentage of polymorphism.

Table 3. Discriminating power of IRAP loci in jujube populations

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus6	71	0.4636	0.1199	0.7413	0.1745
Locus9	71	0.2569	0.0472	0.8163	0.1126
Locus13	71	0.4332	0.1155	0.7333	0.1819
Locus24	71	0.4950	0.0754	0.8477	0.0898
Locus26	71	0.4997	0.0236	0.9528	0.0248
Locus27	71	0.4969	0.0541	0.8912	0.0610
Locus28	71	0.4750	0.0236	0.9503	0.0261
Mean	71	0.2641	0.1171	0.5565	0.3984

*Nm = estimate of gene flow from Gst or Gcs. E.g., Nm = 0.5(1 - Gst)/Gst

Table 4. Genetic distance versus genetic identity in jujube populations(populations numbers are according to Fig. 1)

Pop ID	1	2	3	4	5	6	7	8
1	****	0.8223	0.7542	0.7328	0.7261	0.8027	0.8453	0.8543
2	0.1956	****	0.8790	0.8012	0.8289	0.8149	0.8064	0.8208
3	0.2821	0.1289	****	0.8692	0.8599	0.7328	0.7591	0.8101
4	0.3109	0.2217	0.1401	****	0.8237	0.7107	0.7025	0.7576
5	0.3200	0.1876	0.1509	0.194	****	0.7979	0.8141	0.79
6	0.2198	0.2046	0.3108	0.3415	0.2258	****	0.9384	0.8947
7	0.1681	0.2152	0.2756	0.3531	0.2057	0.0636	****	0.9480
8	0.1575	0.1974	0.2106	0.2776	0.2357	0.1113	0.0534	****

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

Nei, genetic distance and genetic identity determined among *Ziziphus jujuba* populations (Table 4) revealed that genetic similarity among populations ranged from 0.70 to 0.87. The highest genetic similarity occurred between populations 2 and 3.

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.

Genetic grouping and population affinity

UPGMA clustering of the jujube trees based on IRAP data (Fig. 3), almost placed trees of each population in separate cluster. In few cases some individuals of certain populations were placed inter-mixed, due to common shared alleles they had. This result indicates that IRAP molecular markers can differentiate jujube populations and may be used in jujube germplasm genetic finger printing.

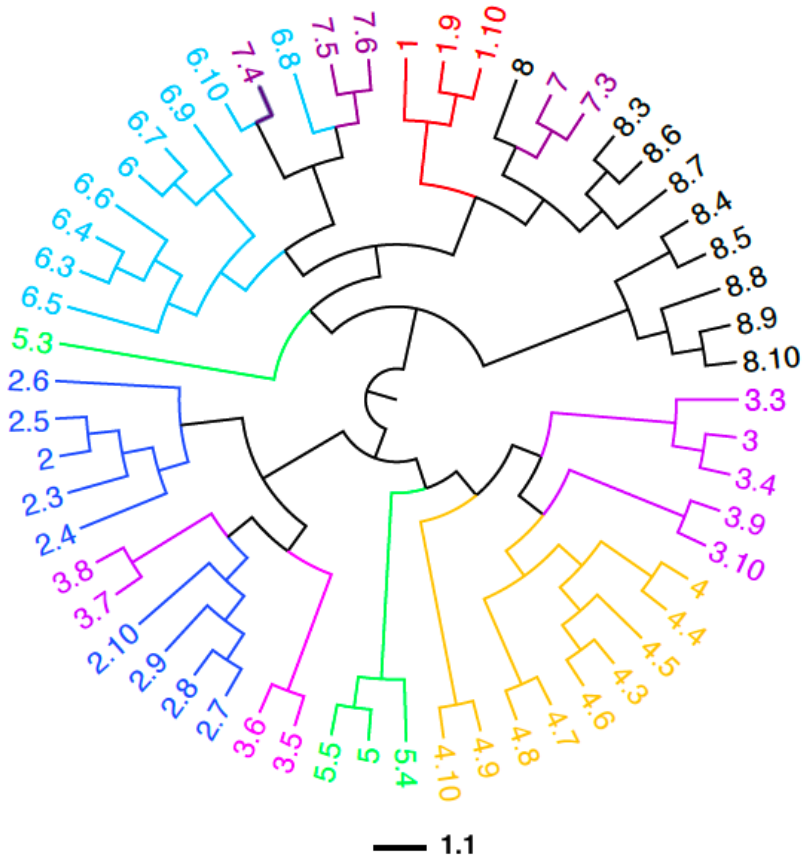

Fig. 3. UPGMA clustering in jujube populations (1-8 are the populations stuied in Fig. 1)

Neighbor-Net network (Fig. 4) revealed both between and within population genetic variability in jujube. Both the length of edges in the network and side bars, indicate genetic difference of the studied jujube trees. The edges and side bars therefore indicate a good level of genetic variability both among jujube populations as well as within each population.

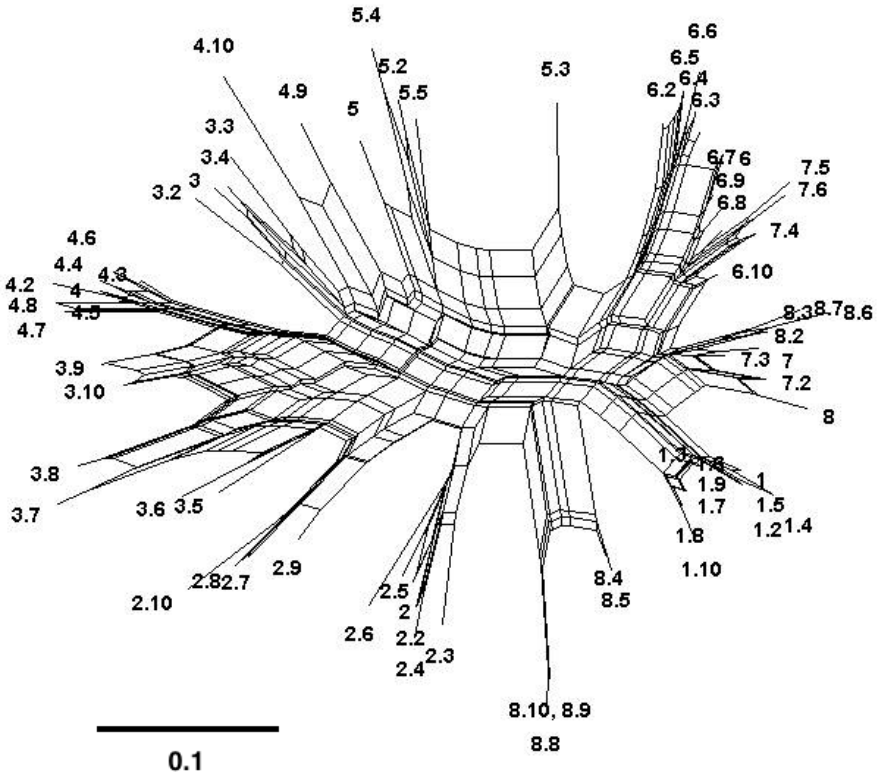

Fig. 4. Neighbor-Net diagram of jujube populations based on IRAP data (population 1-8 are according to Fig. 1)

Genetic affinity of the studied jujube populations was determined by PCoA plot after 1000 times permutation (Fig. 5). It showed that, the studied populations can be placed in two major groups. Populations 6-8 and 1 comprise the first major group. The populations 2-5 form the second major group. This is further supported by Evanno test after STRUCTURE analysis that identified k = 2 as optimum number of genetic groups.

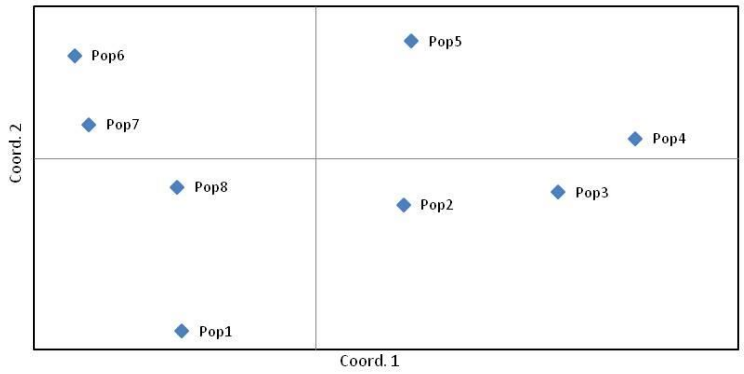

Fig. 5. PCoA plot after 1000 times permutation, showing two main genetic groups in jujube populations (1-8 are the provinces studied in Fig. 1)

Genetic differentiation

AMOVA revealed that these populations differ significantly in their genetic content (PhiPT = 0.54, P = 0.001). AMOVA identified that 54% of total genetic variability occurred among populations while, 46% of genetic variability was due to within population difference. Paired-sample AMOVA also produced significant difference among the studied populations (Table 5).

Table 5. Pair-wise AMOVA between Ziziphus jujuba populations (PhiPT Values below diagonal. Probability values based on 99 permutations are shown above diagonal)

AMOVA	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8
Pop1	0.000	0.0100	0.020	0.010	0.010	0.010	0.010	0.010
Pop2	0.584	0.000	0.010	0.010	0.010	0.010	0.010	0.010
Pop3	0.594	0.310	0.000	0.010	0.010	0.010	0.010	0.010
Pop4	0.755	0.524	0.336	0.000	0.010	0.010	0.010	0.010
Pop5	0.715	0.428	0.343	0.520	0.000	0.010	0.010	0.010
Pop6	0.724	0.560	0.619	0.732	0.591	0.000	0.010	0.010
Pop7	0.632	0.505	0.527	0.676	0.506	0.351	0.000	0.030
Pop8	0.519	0.448	0.453	0.591	0.485	0.467	0.223	0.000

(Populations 1-8 are presented in Fig. 1)

STRUCTURE analysis (Fig. 6), revealed the genetic structure of the studied populations. These populations contained some specific genetic content and allele combinations (differently colored segments). It also revealed some degree of gene flow or ancestral common shared alleles in jujube populations (similarly colored segments).

Fst values obtained in STRUCTURE analysis and based on Bayesian approach is provided at the top of each population (Fig. 6). Both distinctly colored segments in population 1, and its high Fst value (0.8), clearly shows genetic distinctness of this population. This holds almost true for populations 2 and 8 that have high Fst values (0.7). However, they also have some degree of shared common alleles with populations 3, and 7, respectively. Common shared alleles do occur in populations 3 and 4, 6 and 7 too.

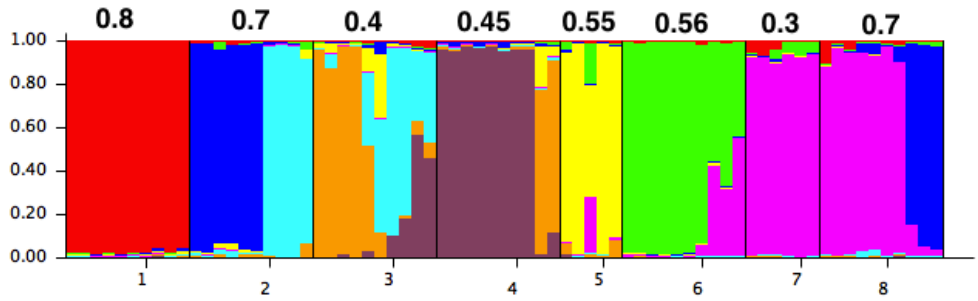

Fig. 6. STRUCTURE plot of *Ziziphus jujuba* populations based on IRAP data.
(Numbers above populations are STRUCTURE Fst values; Number below are according to Fig. 1)

MORPHOLOGICAL DIVERGENCE

The jujube fruits studied in morphological investigations are shown in Fig. 7. Fruit data are presented in Table 6.

Table 6. Fruit characters studied in jujube populations

Province \ Characters	wet weight	dry weight	difference between dry and wet weight	dry / wet weight ratio	length of fruit	width of fruit	difference between dry length and weight
1	2.256	1.687	0.569	1.401	19.29	17.18	2.11
2	1.678	1.304	0.375	1.308	17.95	14.81	3.15
3	1.613	1.338	0.274	1.270	17.77	14.86	2.92
4	1.882	1.649	0.233	1.154	18.23	16.43	1.80
5	1.873	1.559	0.314	1.202	18.88	13.64	5.23
6	1.507	1.281	0.227	1.178	19.20	14.50	4.71
7	1.525	1.022	0.503	1.518	16.28	14.19	2.09
8	1.114	0.762	0.351	1.521	15.23	12.88	2.35

ANOVA revealed significant difference for some of the studied fruit characteristics (P<0.01). Details of these statistical analyses are provided in Table 7. The results indicated that jujube populations are significantly diverged in their fruit characteristics in addition to their genetic divergence.

Table 7. ANOVA results for fruit characteristics studied on jujube populations

ANOVA						
		Sum of Squares	df	Mean Square	F	p-value
wet weight	Between Groups	0.1321	7	0.0188	6.389	*0.001
	Within Groups	0.0472	16	0.0029		
	Total	0.1794	23			
dry weight	Between Groups	0.0686	7	0.0098	7.364	*0.000
	Within Groups	0.0212	16	0.0013		
	Total	0.0899	23			
difference between dry and wet weight	Between Groups	0.0396	7	0.0056	38.97	6.953
	Within Groups	0.0023	16	0.0001		
	Total	0.0419	23			
dry / wet weight ratio	Between Groups	0.0260	7	0.0037	10.6	5.659
	Within Groups	0.0056	16	0.0003		
	Total	0.0316	23			
length of fruit	Between Groups	0.0214	7	0.0030	15.9	4.122
	Within Groups	0.0030	16			
	Total	0.0244	23			
width of fruit	Between Groups	0.2116	7	0.0302	4.342	*0.007
	Within Groups	0.1114	16	0.0069		
	Total	0.3230	23			
difference between dry length and weight	Between Groups	0.0111	7	0.0015	3.959	0.010
	Within Groups	0.0064	16	0.0004		
	Total	0.0176	23			

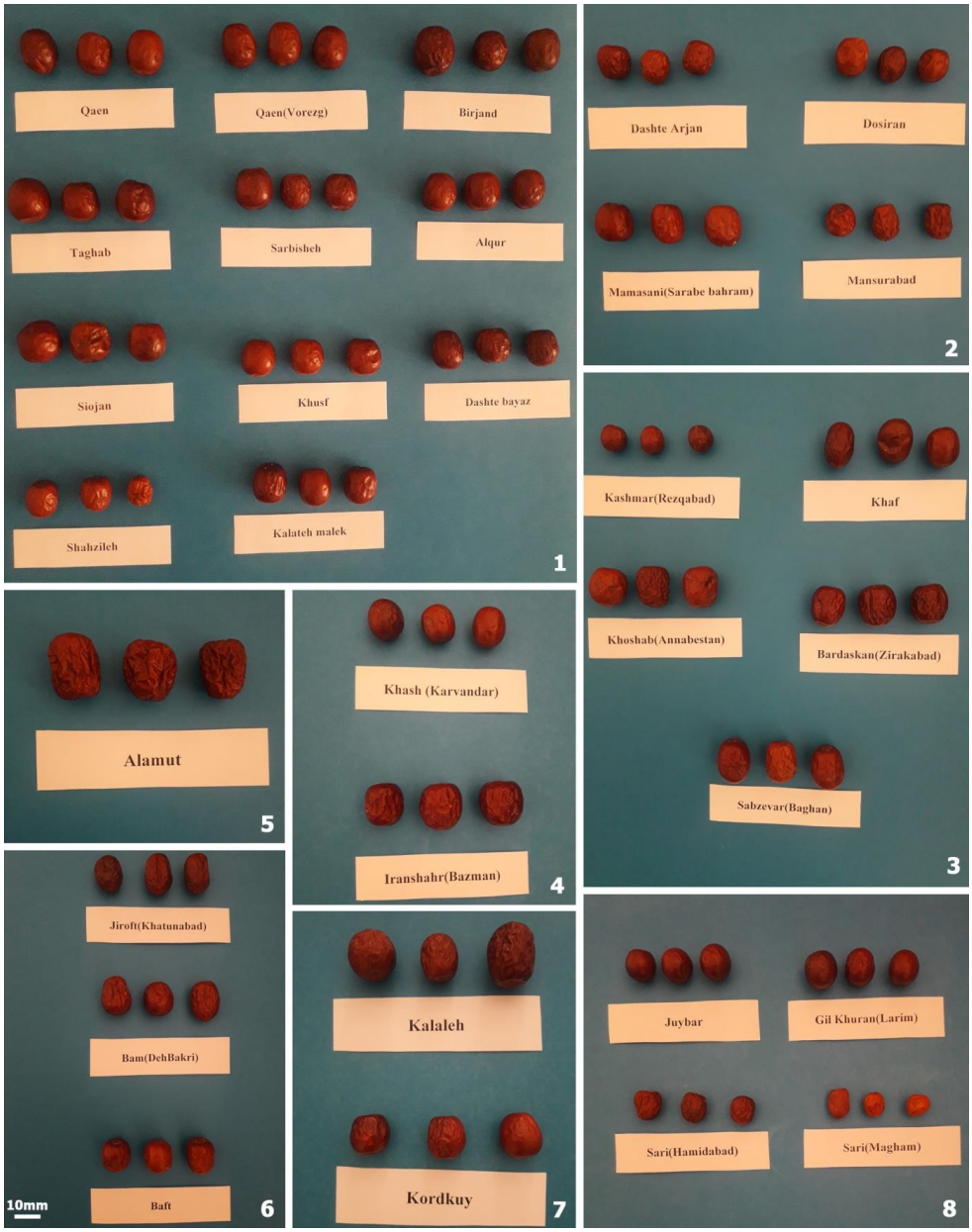

Fig. 7. Jujube fruits in morphological studies (1-8 are the provinces studied in Fig. 1)

Grouping of the populations based on fruit characteristics was done by using PCA analysis. The PCA plot obtained revealed population divergence as trees of each population were almost separated from each other and were placed in different positions within PCA plot (Fig. 8).

Preliminary result of PCA revealed that the first two PCA components comprise about 87% of total variation in jujube populations. PCA loading showed that fruit characteristics dry weight, wet weight and fruit length possesses the highest positive correlation with the first component (r>0.90), while characters difference in the length and width of fruit, and the ratio of wet/dry weight of fruit, are highly correlated (r>0.80) with the second component. Therefore, these are the most variable fruit characteristics that differentiate jujube populations.

The fruit characteristics 1 and 2 differentiate population 4, from the others, while characters 3 differentiate population 5. Fruit characters 4 and 5 differentiate population 6 from the others (Fig. 8).

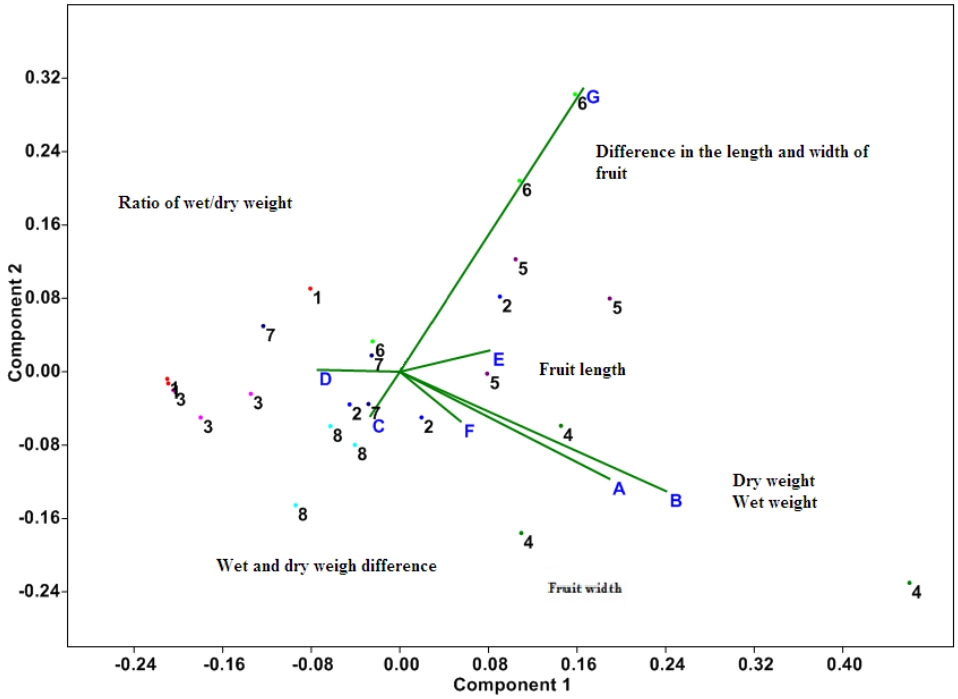

Fig. 8. PCA analysis for fruit characteristics studied on jujube populations

DISCUSSION

Ziziphus jujuba with good medicinal and food importance (VAHEDI *et al.*, 2008) should be studied from genetic and breeding points of view. This tree species grow in different geographical regions of our country and its population genetic study is of immediate importance. Data produced can be utilized in future conservation and breeding programs.

Population genetic study produces insight on the genetic structure, the stratification versus gene flow, as well as genetic divergence of the populations (FREELAND *et al*., 2011).

The present study by applying IRAP molecular markers and different bioinformatics approaches, revealed the presence of genetic diversity both among populations and within each population of jujube.

The Genetic diversity is of fundamental importance in the continuity of aspecies as it is used to bring about the necessary adaptation to the cope with changes in the environment (ÇALISKAN 2012; SHEIDAI *et al*., 2013; 2014). This is particularly essential for *Ziziphus jujuba* as it forms several geographical populations throughout the country.

ALANSI *et al*. (2016), studied genetic diversity in populations of *Ziziphus spina-christi* (L.) Willd. By using ISSR markers and reported mean genetic diversity value of 0.26, and intra-population genetic diversity, Hs = 0.2199. They also reported a high level of gene flow (Nm = 2.37) between populations. It is maybe due to close distance of the studied jujube populations. In our investigation, we collected trees from 8 different provinces which are far from each other and have not meet frequently. This is also supported by significant AMOVA and high Fst values obtained. ALANSI *et al*. (2016) reported that the maximum value of genetic variation was found within populations (90%), whereas a low value of genetic variance was observed among populations. This happened due to extensive gene flow among jujube populations they used in the study. We obtained a higher degree of genetic variability due to among population genetic difference (again due to long distance between populations and possibly due to local adaptations). But we also observed a good level of within population genetic variability. The degree of genetic variability within a species or population 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 TELZUR, 2013; 2014) and therefore, moderate genetic variability in these populations might be related to the out- breeding nature of these trees.

PCoA plot of jujube populations and Evanno test with k = 2, revealed the presence of two distinct genetic groups, which is in agreement with AMOVA and support lack of frequent gene flow among the studied jujube populations. Different mechanisms like isolation drift, founder effects and local selection may act to bring about among population differentiation and therefore, populations differ in phenotypic traits and allele composition (JOLIVET and BERNASCONI, 2007). The present population divergence may be under influence of isolation-by distance across the distribution range of the studied *Ziziphus jujuba* populations.

The dispersal of these populations might be constrained by distance and gene flow is most likely to occur between neighboring populations. As a result, more closely situated populations tend to be more genetically similar to one another (SLATKIN 1993; MEDRANO and HERRERA, 2008).

We also observed that jujube populations differ significantly in their fruit characteristics and PCA showed that these populations are morphological diverged.

In conclusion, the present study revealed that IRAP molecular markers are efficient marker for genetic finger printing and germ plasm evaluation in jujube and that we have a good amount of genetic variability within jujube trees in Iran. These morphological and genetically divergent populations may be used in genetic conservation and future breeding studies in the country.

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GENETIČKA I MORFOLOŠKA VARIJABILNOST KOD *Ziziphus jujuba* Mill.

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Izvod

Ziziphus jujuba (jujuba) je jedna od dobro poznatih vrsta medicinski važne vrste drveta *zizifusis* koja se takođe koristi kao izvor hrane zbog njenih plodova. Studije konzervacije i oplemenjivanja su u toku za ovu hortikulturnu biljku i iz tog razloga je urađeno temeljno istraživanje populacione genetike. Obavili smo molekularna (inter-retrotransposon pojačani polimorfizam: IRAP) i morfološka (karakteristike ploda) na 71 drveću jujube sakupljenom nasumično iz 8 geografskih regiona u Iranu. IRAP markeri mogu razlikovati proučavane populacije, jer je AMOVA otkrila značajnu genetsku razliku između proučavanih populacija. Mrežne i STRUCTURE analize pokazale su da se populacija jujube može svrstati u dve glavne genetske grupe. Nm analiza otkrila je ograničeni protok gena među populacijom. Populacije jujube takođe su se značajno razlikovale po karakteristikama plodova, a to je pokazala i PCA analiza. Dobijeni podaci mogu se koristiti u budućim studijama konzervacije i oplemenjivanja ove važne hortikulturne biljke.

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