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Population genetic study of *Ziziphus jujuba* Mill.: Insight in to wild and cultivated plants genetic structure

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Abstract. *Ziziphus jujuba* (jujube) of buckthorn family (Rhamnaceae) is an important medicinal crop plant cultivated in different provinces of Iran. It has also wild populations in some geographical areas. We carried out population genetic study on 8 populations of cultivated versus wild jujuba by using ISSR molecular markers to produce data on population genetic structure, gene flow, and genetic variability in the studied populations. We also aimed to investigate genetic differentiation between wild and cultivated plants and identify the potential gene pools of this medicinal plant species. The studied populations had a moderate genetic variability and were grouped in two major groups by PCoA plot. AMOVA revealed significant genetic difference among these cultivars. Mantel test showed significant correlation between genetic distance and geographical distance in the studied populations. PCoA analysis showed genetic differentiation between wild and cultivated plants within each province. STRUCTURE analysis identified two potential gene pools for jujube cultivars. Data obtained may be used in genetic conservation and future breeding programs of this medicinal plant species in the country.

Keyword. *Ziziphus jujube*, ISSR, STRUCTURE.

INTRODUCTION

The genus *Ziziphus* Mill. of the buckthorn family (Rhamnaceae), contains about 40 species that are deciduous evergreen trees or shrubs and are distributed in the tropical and subtropical regions of the world (Sing et al. 2007). South and Southeast Asia are considered to be the center of both evolution and distribution of *Ziziphus* species (Sing et al. 2007). These plant species are of medicinal value and are known to be self-incompatible and produce inter-specific hybrids (Asatryan and Tel-Zur 2013, 2014).

Z. jujuba (jujube) is one of the well known species of the genus with great medicinal value. It is mainly distributed in southwestern Asia. 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 are used to alleviate stress and insomnia and as appetite stimulants, digestive aids, antiarrhythmics, and contraceptives (Vahedi et al. 2008).

The fruit is eaten fresh or dried and made into candy; tea, or syrup (Gupta et al. 2004; Jiang et al. 2007). Moreover, some specific saponins, as well as ethyl acetate and water extracts of the fruit and bark, have explored the potential cytotoxicity of jujube. These extracts bring about apoptosis and differential cell cycle arrest, moreover, activity against certain human cancer cell lines has been demonstrated in vitro (Lee et al. 2004; Huang et al. 2007;Vahedi et al. 2008).

Ziziphus jujube is an important plant species to the mankind, due to which its cultivation and conservation gained high importance within recent years. Moreover, as jujube has wide geographical distribution and forms 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 gene 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 diversity in *Z. jujuba* cultivars or wild individuals. For instance, 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 to study cultivar relationships and genetic variability (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).

Population genetic study is an important step for genetic evaluation of medicinally important species as it gives 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 (Sheidai et al. 2013, 2014, 2016). Therefore, the aim of present study was to produce data on genetic diversity, population genetic structure and to compare the cultivars and wild populations of *Ziziphus jujuba* of Iran. We investigated 150 plants of both cultivated as well as wild jujube growing in 23 localities within 8 provinces.

For genetic study we used ISSR molecular markers, as these markers are very useful tool to detect genetic polymorphism, are inexpensive and readily adaptable technique for routine germplasm fingerprinting. They can be used to illustrate genetic relationship between accessions or genotypes and construction of genetic linkage maps (Sheidai et al. 2013, 2014, 2016). The suitability of ISSRs was reported by Alansi et al. (2016), who studied genetic diversity in populations of *Ziziphus spina-christi* (L.) Willd.

MATERIAL AND METHODS

Plant materials

In total 80 plants were studied in 8 provinces (Fig. 1). Ten plants were randomly selected in each population and used for molecular studied.

ISSR assay

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 polyvi-

Figure 1. Distribution map of *Zizphus jujube* populations studied.

nylpyrrolidone (PVP) for binding of polyphenolics during extraction and under mild extraction and precipitation conditions. This promoted high-molecularweight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 0.8% agarose gel.

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 mMTris-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 55 °C, and 1 min at 72 °C. Th e reaction was completed by a fi nal 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 fragment sizes were 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.

Data analyses

The ISSR 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, New gene diversity (He), Shannon information index (I), the number of effective alleles, and percentage of polymorphism (Weising 2005), were determined by using GenAlex 6.4 (Peakall and Smouse, 2006).

For genetic grouping of the studied cultivated and wild plants, 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.

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 (Earl and von Holdt, 2012) was used to perform the Evanno method to identify the proper value of K (Evanno et al., 2005). To study genetic differentiation between wild and cultivated plants, we performed PCoA (Principal coordinate analysis) analysis within each province.

RESULTS

We obtained 31 ISSR bands (Loci) in total (Table 1). The highest number of bands (17 bands) occurred in population 1 (Soth Khorasan), and 2 (Fars) (16 bands), respectively. Some of the populations had private bands with population 4 (Sistan-o-Baloochestan) having the highest number (4 private bands). Few common bands occurred in the studied populations too. These are shared alleles among these populations.

Genetic diversity parameters determined in *Z. jujuba* populations are presented in Table 3. The percentage of genetic polymorphism obtained ranged from 3.25 in population 7 (Golestan) to 51.61 in population 2 Fars). A moderate level of genetic polymorphism (>30%) also occurred in populations 3, and 4 (DNorth-Khorasan, andSistan-o-Baloochestan, respectively). The highest mean value of New gene diversity (He) occurred in populations 1 to 4 (0.10-0.16, Table 2).

Table 1. Details of ISSR bands in *Z. Jujube* populations.

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8
No. Bands	16	17	13	15	12	10	8	13
No. Bands Freq. >= 5%	16	17	13	15	12	10	8	13
No. Private Bands	1	2	0	4	0	1	0	1
No. LComm Bands (<=50%)	6	7	6	5	4	3	3	5

Table 2. Genetic variability parameters determined in *Ziziphus jujube* populations based on ISSR markers (populations numbers are according to Fig. 1).

Pop	N	Na	Ne	I	He	uHe	P%
Pop1	10.000	0.968	1.240	0.223	0.146	0.154	45.16%
Pop2	10.000	1.065	1.262	0.252	0.164	0.172	51.61%
Pop3	10.000	0.742	1.180	0.161	0.107	0.112	32.26%
Pop4	10.000	0.871	1.193	0.177	0.115	0.121	38.71%
Pop5	10.000	0.613	1.141	0.125	0.084	0.088	22.58%
Pop6	10.000	0.484	1.105	0.091	0.061	0.065	16.13%
Pop7	10.000	0.290	1.028	0.021	0.015	0.016	3.23%
Pop8	10.000	0.710	1.167	0.145	0.097	0.102	29.03%

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.

Detailed analysis of ISSR loci revealed that 16 ISSR loci (50% of all ISSR loci), have high Gst value I.e. >0.50 (equivalent of Fst). This indicates that, these loci are different in the studied populations and lead to population genetic differentiation. This ISSR locus had a low value of Nm and therefore, they are not shared by all the populations. On the contrary, 14 ISSR loci had Nm value >1, and low Get value. They are the common alleles shared by the studied populations. The mean Nm value of the studied populations was 0.38, which is very low and indicates lack of extensive gene flow among the studied populations.

The Nei's genetic identity and genetic distance of the studied populations are provided in Table 3. Genetic similarities between 0.70 to 0.96% were observed in the studied populations. The highest genetic identity occurred between populations 1 and 2 (0.96%).

Table 3. Nei genetic identity versus genetic distance in the *Z. Jujube* populations (populations numbers are according to Fig1. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

pop ID	1	2	3	4	5	6	7	8
1	****	0.9609	0.8920	0.8411	0.8574	0.8735	0.8445	0.9128
2	0.0399	****	0.9189	0.8675	0.8526	0.8032	0.7677	0.8477
3	0.1143	0.0846	****	0.9076	0.8505	0.7602	0.7187	0.7955
4	0.1731	0.1422	0.0969	****	0.8741	0.7263	0.6824	0.7415
5	0.1538	0.1595	0.1619	0.1345	****	0.8011	0.7621	0.7748
6	0.1353	0.2192	0.2742	0.3198	0.2218	****	0.9434	0.9539
7	0.1691	0.2644	0.3303	0.3821	0.2717	0.0583	****	0.9548
8	0.0913	0.1652	0.2288	0.2991	0.2552	0.0472	0.0462	****

Genetic differential of Z. Jujube *populations*

Based on Nei genetic distance, PCoA plot was constructed for the studied cultivars and wild populations, separately (Fig. 2). The plot constructed for the cultivars, placed *Z. Jujube* populations in two main groups. Populations 2, 3 and 4 formed the first main group, while populations 1, 6, 7, and 8, comprised the second major group. Some trees in population1 and 5 were intermixed in both groups. This is due to within population genetic variability and the common shared alleys in these two populations.

Similarly, PCoA analysis of the wild populations revealed that these populations differ genetically from each other as they are placed in separate groups (Fig. 3).

Therefore, both cultivated and wild plants of the studied provinces are genetically differentiated from each other. Moreover, AMOVA produced significant genetic difference among *Z. Jujube* populations (PhiPT = 0.57, P = 0.001). AMOVA revealed that 57% of total genetic variability occurred among populations while, 43% of genetic variability was due to within population difference. Paired-sample AMOVA also produced significant difference among the studied populations. These results indicate that although the studied *Z. jujube* cul-

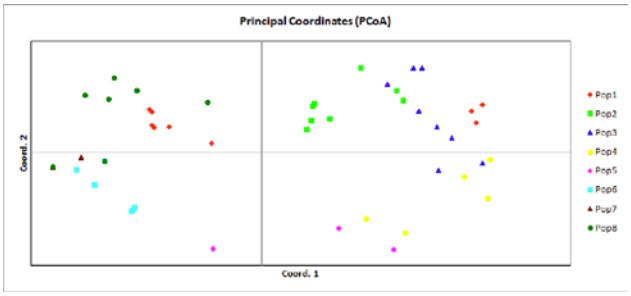


Figure 2. PCoA plot of ISSR data in *Z. Jujube* populations.

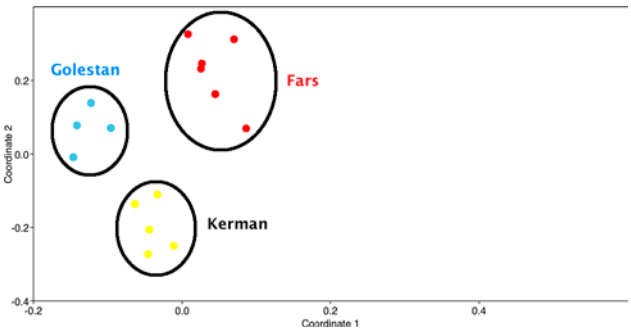


Figure 3. PCoA plot of *Z. Jujube* wild populations based on ISSR data.

tivars and wild populations differ genetically from each other, but also some degree of within population of genetic variability do occur in each population.

Wild versus cultivated Z. Jujuba *plants*

In the other attempt, we investigated the genetic differentiation of wild versus cultivated plants within each locality. In three provinces namely, 1- Fare, 2- Golestan, and 3-Kerman, both cultivated and wild plants were present. The comparison of ISSR bands in these plants revealed almost complete genetic differentiation of wild and cultivated plants in Fars province, while in two other provinces, they were genetically differentiated to some degree (Fig. 4). This indicates that these two types of *Z. jujube*, are not genetically alike and we may have still novel genes in wild plants that can be introduced in to cultivated plants genome. These genetic variability are of high importance in medicinal plant conservation and breeding.

Assocition between genetic diversity and geographical features

Correlation analysis performed did not show significant association between gene diversity with either altitude or latitude in the studied populations (Fig. 5). The same hold true for percentage of genetic polymorphism. This may happen due to cultivation practice and selection made by local gardeners which interfere with local natural adaptation.

However, Mantel test (Fig. 6) between geographical distance (combined distance of longitude and altitude)

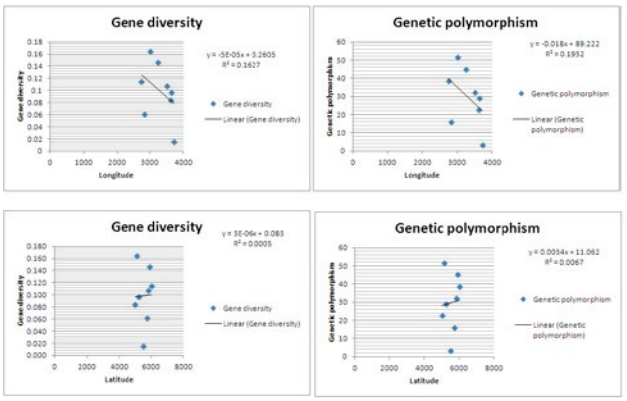


Figure 5. Correlation analysis of genetic diversity and genetic polymorphism with geographical features in *Z. Jujube* populations.

and genetic distance produced significant correlation ($P<0.01$). Therefore, with increase in geographical distance, an increase in genetic difference of the populations occurred. This is called isolation by distance (IBD). This indicates that the combined effect of geographical features as well as genetic background of the studied cultivars bring about significant genetic differentiation among *Z. Jujube* populations.

Genetic structure of Z. Jujube *populations*

The genetic structure of the studied populations and degree of genetic admixture among populations were determined by STRUCTURE analysis. The STRUCTURE plot (Fig. 7) revealed presence of different allele combinations (differently colored segments) in the *Z. Jujube* populations. However, some degree of shared common alleles (similarly colored segments) was observed in populations 1, 2 and 3, and also in populations 6, 7, and 8. Populations 4 and 5 contained distinct allele combinations.

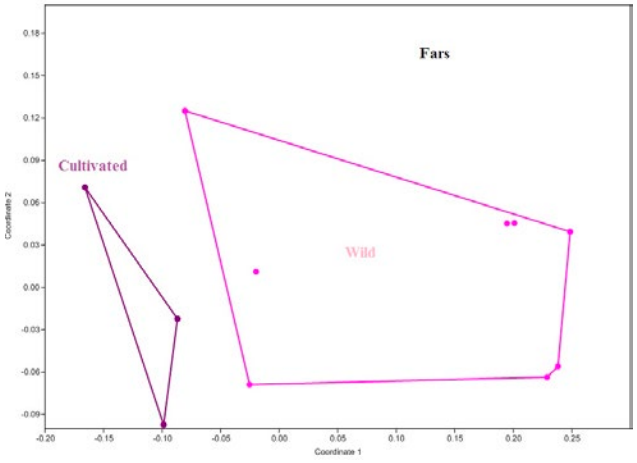
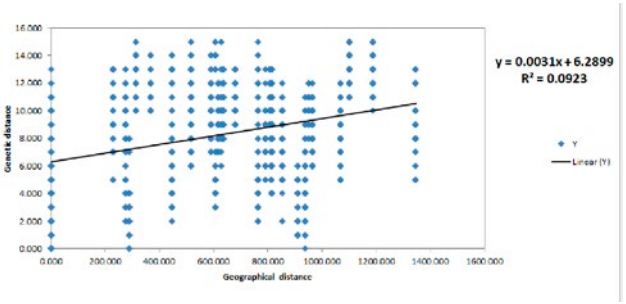


Figure 4. PCoA plot of wild versus cultivated *Z. Jujube* plants within Fars province.



Figuse 6. Mantel test plot between genetic distance and geographical distance of *Z. Jujube* populations.

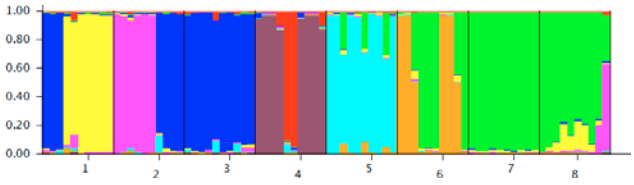


Figure 7. STRUCTURE plot of *Z. Jujube* populations based on k = 8.

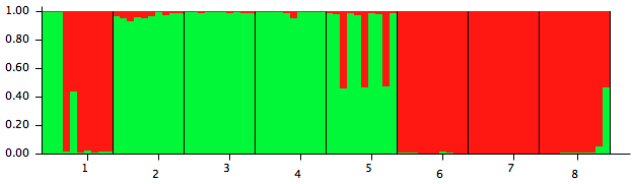


Figure 8. STRUCTURE plot of *Z. Jujube* populations based on k = 2.

Evanno test produced optimal number of genetic group k = 2. Therefore, 13 studied *Ziziphus jujube* populations studied could be grouped in 2 genetic groups. STRUCTURE plot based on k = 2 (Fig. 8), revealed that populations 2-4 comprise the first genetic group, while populations 6-8 comprise the second genetic group. Moreover, populations 1 and 5 stands somewhere in between these two groups. This is in complete agreement with PCoA plot results presented before.

DISCUSSION

In spite of medicinal importance (Vahedi et al. 2008) and wide geographical distribution of *Ziziphus jububa* in our country, we had no detailed information on its genetic variability and structure.The present study revealed the presence of a moderate genetic variability in the cultivated populations. It also showed genetic differentiation between wild versus cultivated plants within each province. Therefore, we can use these plants in a core germ plasm collection of Z. Jujube for conservation and breeding purpose (Sheidai et al. 2013, 2014, 2016).

Alansi et al. (2016), studied genetic diversity in populations of *Ziziphus spina-christi* (L.) Willd. By using ISSR markers and reported the genetic diversity value of 0.26, total genetic diversity Ht = 0.266, and intra-population genetic diversity, Hs = 0.2199.

In present study, AMOVA revealed significant genetic difference among *Z. jujube* cultivars, and also identified a good level of genetic variability within studied population. Moreover, Gst and Nm results revealed that about 50% of ISSR loci was either private on not shared by all populations, and 50% were exchange in populations via gene flow. This may be to some degree related to out-crossing nature of *Z. jujube*.

Zhang et al. (2015) studied genetic variability and differentiation in cultivated jujube and wild jujube by using SSR molecular markers. They reported high levels of genetic diversity (HE=0.659 and HS=0.674) within populations, and moderate differentiation among studied populations (FST=0.091, RST= 0.068, GST=0.271). They also reported a high degree of gene flow (Nm=6.572) and weak correlation between genetic and geographical distances (r 2 =0.026, P>0.05), and suggested that gene flow occurred frequently among populations. AMOVA showed that most of the existing genetic diversity was distributed within populations (88 %), and only 12 % occurred among populations, therefore, the studied populations were not differentiated.

On the other hand, Singh et al. (2017) investigated genetic variation and relationships among cultivars of *Ziziphus mauritiana* (Lamk.) native of India by using start codon targeted (SCoT), ISSR, and ribosomal DNA (rDNA) markers. They reported high level of polymorphism among SCoT (61.6%) and ISSR (61%) markers. SCoT and ISSR dendrograms delineated all the cultivars of Z. *mauritiana* into well-supported distinct clusters. These populations were genetically differentiated as also was indicated with high Get values.

Difference in the results of these studies is probably due to difference in geographical isolation of the studied populations. In present study, the distance between populations is great as they are located in different provinces ranging from south to north of the country with no intermediately plant populations among them (Fig. 1). Genetic differentiation of the studied populations may be attributed to a combination of adaptation to different environmental conditions and limited capacity for long-distance dispersal (Zhang et al. 2015). However, we also noticed good genetic differentiation within each province between wild and cultivated Z. Jujube plants; this is probably due to effects of cultivation practice and artificial selection made by jujube growers in the gardens. Such selection pressure is absent in wild plants.

In conclusion, we have presented data on genetic variability and genetic structure of both *Z. Jujube* cultivars and wild plants in the country. Two main gene pools were identified for jujube cultivars which may be used in future genetic conservation and hybridization programs of this important medicinal plant.

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