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USE OF ISSR HYBRID MARKERS TO ASSESS THE GENETIC DIVERSITY IN WILD MEDICINAL *ZIZIPHUS NUMMULARIA* (BURN.F.) WIGHT & ARN. COLLECTED FROM DIFFERENT REGIONS OF IRAN

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ABSTRACT

Ziziphus nummularia (Burn.f.) Wight & Arn. Is a valuable medicinal tree that belongs to the Rhamnaceae family with a wide distribution in the southern areas of Iran? In this study, 20 populations of *Z. nummularia* were collected from the southern areas of Iran to evaluate their characteristics as well as genetic diversity. Twelve ISSR primers produced polymorphic 101 bands with about 100% polymorphism. The highest polymorphism percentage was seen in population K16 (51.49) and the lowest was related to population F12 (0.16). The highest and lowest heterozygosity were observed in populations K16 (0.203) and F12 (0.074), respectively. Genetic parameters such as Shannon index, Nei's genetic diversity index, and the number of effective alleles for each primer indicated a genetic diversity of 42% within the populations. In addition, a variation of 58% was observed among the populations. The highest and lowest percentages of the Shannon index were observed among populations K16 and F12, respectively, confirmed by the analysis of molecular variance (AMOVA). The cluster analysis of molecular data (UPGMA) and morphological traits allowed well separation of populations and species, where populations were isolated in clusters.

Keywords **:** *Ziziphus nummularia*, ISSR markers, diversity,

Introduction

Ziziphus nummularia (Burn. f.) Wight & Arn. Belongs to the Rhamnaceae family. It is spread in tropical regions and southwestern regions of North Africa, Afghanistan, North India, South China, Malaysia, Iran, Syria, Sri Lanka, and part of the Mediterranean (Akhtar *et al*., 2017). This plant grows in sandy and rocky soils of arid and semi-arid areas (Bailey 1947, Singh *et al*., 2000, Dinarvand and Zarinkamar, 2006), and optimum temperature for its growth is 25-35 °C (Pandey, 2010). This species is evergreen, but some of its leaves fall during hot seasons. Family Rhamnaceae has been widely used in traditional medicine to treat diseases (Ullah *et al*., 2013, Goyal *et al*. 2013). The leaf and fruit of *Z. nummularia* are used in treating coughing, sputum, headache, and skin diseases, as well as in weight loss, digestion, and laxative, confusion, burning sensation, thirst, and vomiting, with clear uses in the treatment of tuberculosis and blood diseases. Additionally, it has been examined as an antidote for poisoning and abdominal pain during pregnancy. Seeds are also useful in eye diseases (Meena *et al*., 2003). The medicinal potential of this plant is due to presence of some phytochemicals and metabolites such as ascorbic acid, thiamine, riboflavin, and pectin (Farooq *et al*., 2005). Molecular markers based on polymerase chain reaction (PCR) have been used to examine morphological and genetic characteristics (Akhtar *et al*., 2017). Nowadays, genetic variation detection and identification methods have gained new dimensions using molecular markers. Molecular markers such as RAPD, SSR, ISSR, AFLP, and so on have been widely used as desirable markers for genetic variation at various levels, such that identification of species has been performed with confidence and ease through molecular markers in recent decades (Fang *et al*., 1997). Singh *et al*. (2009) studied genetic variation among 48 different genotypes of *Z. nummularia* from different geographical regions of India using RAPD and ISSR markers. The study suggested the superiority of ISSR compared to RAPD markers for the evaluation of genetic diversity in *Z. mauritiana.* Obead *et al*. (2008) examined the genetic diversity of five varieties of *Z. mauritiana* in Saudi Arabia using ISSR molecular marker. Their results revealed that ISSR method had a unique capability in differentiating cultivars. In different populations of *Z. Nummularia,* there is a significant variation in the inputs of different regions, as obtained by analyzing five qualitative and 25 quantitative morphological traits of leaves, fruits, and seeds. ISSR and morphological markers can be successfully used to identify the genetic diversity of plants in different geographic areas (Akhtar *et al*., 2017). Concerning the quantitative and qualitative characteristics of the Iranian *Z. jujuba*

populations, Gao *et al*. (2011) reported that molecular markers t be a useful tool for evaluating morphological and genetic characteristics and for more accurate identification of *Ziziphus* ecotypes. With these markers, one can well define the variety of ecotypes and their correspondence with geographic patterns. Information obtained from molecular studies can be regarded as complementary to those gained from conventional morphological studies. ISSR markers have been used as a means of identifying genotypes as well as studying plant constructs such as corn (Pejic *et al*., 1998; Kantety *et al*., 1995), potatoes (Prevost and Williams, 1999), orange (Fang *et al*., 1997), and wheat (Nagaoka and Ogihara, 1997). The aim of this study, therefore, was to compare and determine the relationship between genetic characteristics of *Z. nummularia*, and to identify and Introduce superior populations of *Z. nummularia* species in different regions of Iran.

Materials and Methods

Plant material

Parts of 20 *Z. nummularia* genotypes (including fruit, leaf, and samples of shoots) were collected from different geographical regions of Iran including Khuzestan, Fars and Kohgiluyeh and Boyer Ahmad provinces during summer and autumn 2017 (Table 1). The shoots were prepared for herbarium sheets after determination of taxonomic position based on Flora Iranica at the Institute of Medicinal Plants Herbarium (IMPH) of Iran.

Statistical analysis

Statistical analyses for genetic variation were the similarity matrix between the cultivars based on the population-bonding pattern using Jacard similarity coefficients. Then, cluster analysis was performed on all amplified bands (Rohlf 1998, Kumar *et al*., 2003) with UPGMA and neighbor joining (NJ) methods to group the samples using NTSYS-pc 2.02e software. The cophenetic correlation between the dendrograms and their matrices was calculated by Mantel test using NTSYS-pc software. Finally, the dendrogram with the highest correlation coefficient with the corresponding matrix was used as the strongest shrub to analyze the relationships between populations and species (Kumar *et al*., 2003). High genetic parameters were examined at the population level. The genetic variation within and between the studied populations was examined by AMOVA analysis using GenAlex 6.4 software.

Genetic evaluation

DNA was extracted from 0.2 gr of leaf tissue using the modified CTAB (cetyl trimethylammonium) bromide method (Khan *et al*. 2007). The material was ground to a fine powder with a pestle and mortar using liquid nitrogen. The fine powder of each sample was transferred in a 2-mL Eppendorf tube and suspended in 700 mL of extraction buffer with 3% CTAB (w/v), 100 mmol/L of Tris-HCl (pH 8), 2 mol/L of NaCl, 25 mmol/L of ethylenediaminetetraacetic acid (EDTA, pH 8), 3% b-mercaptoethanol (v/v), and 3% polyvinylpyrrolidone (w/v). The suspension was mixed for 5 min and incubated in a water bath at 65 ºC for 30 min. The mixture was then removed from the water bath and cooled, followed by adding an equal volume of chloroform: isoamyl alcohol (24:1). The suspension was mixed for 10 min, and centrifuged at 10,000 rpm at room temperature for 10 min. The supernatant was collected, transferred to another 1.5 mL tube, and precipitated with 2/3 volume ice-cold isopropanol at 20 ºC for 1 h. DNA was pelleted by centrifugation at 10,000 rpm at 4 ºC for 10 min. The pellet was then washed with 70% alcohol, dried at room temperature, and suspended in Tris-EDTA (TE) buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and pH 8). The isolated DNA was assayed by a Nanodrop 8000 spectrophotometer (Thermo Scientific), and then used for PCR amplification. Genomic DNA was amplified with ISSR primers (Table 2) using the PCR bead (GE Healthcare, UK). Each reaction was performed in 25 mL reaction volume containing 20 mL of sterile deionized water, 4 mL of DNA (50 ng), and 1 mL of 10 pmol/L ISSR primer. After mixing the PCR components, the reaction was carried out in an Applied Biosystems Thermal Cycler (Singapore) using the following conditions: initial denaturation at 94 ºC for 4 min, followed by 40 cycles of denaturation for 1 min, annealing step at 45 ºC for 1 min, extension at 72 ºC for 1 min, and final extension at 72 ºC for 7 min. The amplified fragments were electrophoresed on 1.5% agarose gel (Hoefer, Richmond CA, USA). Finally, the gel was documented using a Syngene BIO IMAGING system (Ingenius L, UK) (Ghasemzadeh *et al*. 2018).

Results

12 ISSR markers produced 101 polymorphi bands. The length of fragments in the band ranged from 150 to 3000 bps all fragments in 150 to bps. Also, 12 primers including UBC807, UBC810, UBC811, UBC823, UBC834, UBC849 (simple primers, UBC807/811, UBC807/823, UBC807/849, UBC811/823, UBC811/849, and UBC823/849 (combined primers) (Table 2) produced 101 polymorph fragments. The length of fragments ranged from 150 to 3000 bps. UBC 180 and 708/118, respectively, produced maximum and minimum number of polymorphic bands (2 fragments). The shortest amplified DNA fragment was produced by UBC 810 (150bps) mean, while the longest amplified fragment was produced by UBC 807 (3000 bps), both of which were combined primers. Similarity matrix was developed based on all traits (including ISSR markers) according to Jacard similarity coefficient.

Similarity matrix was obtained using Jacard similarity coefficient based on the data obtained from the ISSR markers. The shrubs were drawn using different clustering methods including Neighbor Joining, UPGMA, and WARD. The dendrogram of the UPGMA analysis was based on ISSR data, where the populations were divided into four main groups. The first cluster consisted of five sub-clusters involving populations 1, 2, 4, 6, 7, 8, and 9. The second cluster consisted of 3, 5, 10, and 12 populations. Then, two sub-clusters were observed in the third cluster, where populations 11, 13, 14, 17, 18, 19, and 20 were clustered in this group, and populations 15 and 16 were observed in the fourth cluster (Fig. 1).

The genotypes in cluster analysis based on ISSR data had good cluster analysis confirming geographical distribution. All genotypes occupying the first and second clusters were distributed in 49 to 51 N latitude. The genotypes occupying the third and fourth cluster were distributed from 50 to 51 N area. Intra-population diversity was not detected based on AMOVA (Table 3). The results revealed that the whole diversity estimated based on ISSR was highly significant indicating inter-population diversity.

The highest percentage (51.49) of polymorphism belonged to population 16 while the lowest (0.16) was related to population 12. The highest number (1.109) of effective alleles belonged to population 12 and the minimum (0.079)

was found for population 7. The highest heterozygosity (0.203) was observed in population 16 while population 12 showed minimum level (0.074). In terms of Shannon index, population 16 had the highest (0.298) while population 12 showed the lowest (0.107) index. Using the first and second components (PCA), the charting chart was compiled for each population separately. In these graphs, the populations were divided into four distinct groups. There were populations 3, 5, 10, 12, 13, 14, 15, and 16 in the first group, populations 4, 6, 7, 8, and 9 in the second group, populations 11, 17, 18, 19, and 20 in the third group and populations 1 and 2 in the fourth group. Grouping with the drought algorithm obtained from the UPGMA analysis was acceptable except in some populations such as one and two. This appears to be due to geographical proximity and climatic similarities such as longitude and altitude. The two populations were more distant than the other ones. In the charting and clustering diagrams, however, the populations are slightly apart. The results of the cluster analysis confirmatory regimen are based on UPGMA method (Fig. 2).

According to the results, the high polymorphism percentage in the selected loci indicates the ISSR molecular marker.

Discussion

Higher Shannon index was estimated in ISSR based polymorphism with the application of combined primers. Accordingly, combined primers can be recommended for genetic biodiversity studies because of their high resolution. In addition, a significant difference was detected between *Z. nummularia* populations based on ISSR markers according to AMOVA results (Table 7). Therefore, the genetic effects were better highlighted after separating the environmental effects from the ground diversity. Khakdaman (2006) expressed the gene flow and cross-pollination system as the reason for genetic biodiversity among populations. Lei *et al*. (2004) studied genetic variation among 117 *Ziziphus* genotypes and reported a significant genetic variance. Therefore, breeding programs can be planned to improve the value of traits. Shahhosseini *et al*. (2012) reported a significant genetic variance among *Z. nummularia* derived from different regions of Iran. They declared that genetic diversity among populations under study was highly affected by geographical conditions such as geographical coordinates and altitude, confirming the results obtained in the present paper. More than 72% of the total variation among the population under study could be described by the first three principal components (Fig. 2). In addition to Akhtar *et al.* (2017), the mentioned researchers suggested climatic changes and mutations as the influential factors. The cause of the genetic distance between Indian populations. In the PCA, 58 percent of intera-population genetic variation was estimated based on ISSR markers. It is noteworthy that inter-population diversity was lower than within population variation. The geographical distance between the studied populations was very high (more than 100 and up to 500 km), which can limit gene flow among the populations. Resulted to low within population variation considering high among population diversity pollens cannot immigrate in this very far distance therefore there is a low genetic diversity within populations. Environmental and climatic situation is a key factor in the development of diversity. Cheang *et al.* (2007) contended that gradual water temperature changes in the surface of high seas could affect morphological changes.

Furthermore, 72% of total variation was described by the first three principal components in our experiment, which is in line with that of Akhtar *et al*. (2017). They reported climate and mutation as the two main factors affecting the variation among *Z. nummularia* ecotypes in India contributing to placement of genotypes in different groups in the PCA. Intra-population variation was estimated as 58% based on ISSR markers. The narrow gene flow caused by long geographical distance between populations (more than 100 km) could be considered as a reason for high among-population variations. Long distances lead to less gene flow among populations. Some geographical factors can also influence diversity among populations. Cheang *et al*. (2008) found gradual thermal changes in the surface of seas as a factor affecting morphological traits in *Z. nummularia* populations. Cluster analysis based on SM (simple matching coefficient) via UPGMA method showed a very high cophenetic coefficient of correlation (CCC = 97%) in ISSR markers. The results demonstrate the efficiency of UPGMA method in clustering the populations. Recognition of a population is not possible unless all traits of the population are studied. Our findings showed significant differences between populations . This variation may be attributed to the basic diversity among populations and selection by the beneficiaries who selected content genotypes over years. Therefore, further phytochemical and molecular studies are recommended to understand the relationship and proximity of ecotypes, which will help researchers to select superior ecotypes of *Z. nummularia*. The results of our study revealed the high importance of genetic and phytochemical traits in differentiation of populations compared with qualitative characteristics. The quantitative characteristics, such as phytochemical traits, are affected by the environment, which is considered as a factor causing intra-population genetic variations. In order to introduce the best population, it is necessary to consider all its determinant components. For more accurate identification, supplementary phytochemical and molecular research is required to determine the relationship with qualitative identification of these materials and the relationship between the ecotypes to improve the selection of the best cultivars. According to our results, fluctuations in the temperature and altitude, as well as in the geographic coordinates of the populations have led to the variation among the cultivars. Morphological, phytotoxic, and genetic studies on the populations studied here suggest that the populations can be segregated through genetic and phytochemical traits. Note that they cannot differentiate population's as much as genetic and phytochemical traits. Qualitative traits showed differentiation variety only at the species level, which, coupled with some qualities, could separate populations from each other. The environment generally affects genetic and phytochemical traits, and the existence of diversity in these traits among the populations shows environmental and ecological changes in the area, causing intra-population variations in these areas. The populations of any species also confirms the intra-population diversity of all populations in genetic traits. The current study indicated that molecular markers could be used to determine the genetic diversity and genetic relationship of the populations, which could be used in breeding programs. It is suggested that priority should be given to the importance of the medicinal and nutritional value of these species and to their significance for soil stabilization and desertification. Furthermore, it is also suggested to conduct similar studies

by examining other species of this genus using morphological and molecular traits through other molecular markers and other genomic sections, thereby examining the relationship between ecological characteristics of the regions and morphological plus molecular markers to determine superior populations.

Table 1: Information on collection area of Z. *nuummularia* populations.

No	Population no.	Region originated	Latitude (N)	Longitude (E)	Altitude (m)	Average maximum temperature (˚C)	Average minimum temperature (˚C)
1	KH1	Khuzestan - Behbahan	30º 59" 68'	50º 35" 21'	328	35.2	17
2	KH2	Khouzestan - Ramhormoz	31º 25" 55'	49º 63" 36'	181	30.1	20.3
3	KH3	Khuzestan - Haftgol	31º 45 49'	49º 56" 55'	315	34	17.2
4	KH4	Khuzestan - Baghmalek	31º 52" 36'	49º 50" 12'	630	28	15
5	KH5	Khuzestan – Ghaleh tool Baghmalek	31º 65" 68'	49º 92" 19'	701	29	15.3
6	KH6	Khuzestan - Izeh Turkab	31º 69" 18'	49º 75" 05'	786	28	14
7	KH7	Khuzestan - Izeh Jaghband	31º 70" 94'	49º 81" 28'	791	29	13.5
8	KH8	Khuzestan - Izeh Koolfarh	31º 52" 01'	49º 52" 00'	790	29.3	13.4
9	F9	Fars - Qaemea	29º 84" 14'	51º 60" 67'	883	23	11
10	F10	Fars - Khomezar Noorabad	30º 02" 41'	51º 56" 10'	908	25.8	11.8
11	F11	Fars-Fahliyan Norabad	30º 15" 61'	51º 53" 12'	901	25	10
12	F12	Fars-Mosiry Rostam	30º 15" 56'	51º 53" 14'	920	22	10
13	F13	Fars - Tel pir Babaamidan	30º 26" 83'	51º 49" 10'	948	23	8
14	F14	Fars - Dehno Babaamidan	30º 34" 04'	51º 30" 98'	935	23	10
15	F15	Fars - Coupon	30º 33" 12'	51º 27" 84'	917	29.1	15.4
16	K16	Kohgiluyeh and Boyerahmad - Basht	30º 32" 93'	51º 17" 99'	781	25	11
17	K17	Kohgiluyeh and Boyerahmad-Gachsaran	30º 35" 12'	50º 80" 68'	719	26.5	12.4
18	K18	Kohgiluyeh and Boyer Ahmad-lick	30º 90" 22'	50º 09" 67'	673	25	9.1
19	K19	Kohgiluyeh and Boyer Ahmad-Dehdasht	30º 52" 32'	50º 45" 21'	803	26	8
20	K20	Kohgiluyeh and Boyer Ahmad – haft cheshmeh Gachsaran	30º 04" 71'	50º 61" 43'	735	27.5	13.8

Table 2 : Specifications of the primers used in the ISSR reaction

No	Primer code	Sequence 5'-3'
1	UBC807	(AG) 8T
2	UBC810	**(**GA)8T
3	UBC811	(GA) 8C
4	UBC823	(TC) 8C
5	UBC834	(AG)8YT
6	UBC849	(GT)8YA
7	UBC807/811	
8	UBC807/823	
9	UBC807/849	
10	UBC811/823	
11	UBC811/849	
12	UBC823/849	

Table 3: Results obtained from the molecular variance analysis (AMOVA) based on ISSR data

Source	df	SS	MS	Est. Var.	%
Among Pops	19	668.333	35.175	7.997	42%
Within Pops	40	447.333	11.183	11.183	58%
Total	59	1115.667		19.181	100%
Stat	Value	P(rand >= data)			
PhiPT	0.417	0.010			

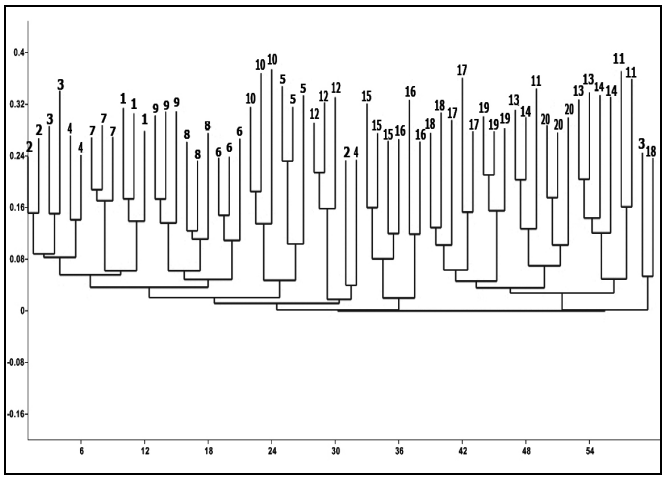

Fig. 1: UPGMA dendrogram derived from cluster analysis based on Jacard similarity coefficient based on ISSR data

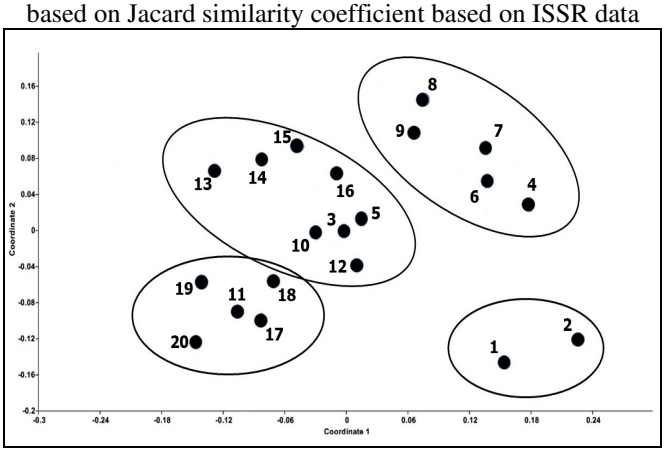

Fig. 2: PCA biplot analysis of *Z. nummularia* populations based traits based on ISSR data

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