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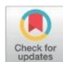

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Original Article

Genetic Diversity Among Economically Important *Zataria multiflora* Accessions Through ISSR Markers: The Main Step for Breeding and Exploitation Programs

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Abstract

Introduction: *Zataria multiflora* is an important medicinal plant of the Lamiaceae family in Central and Southern Iran. This plant is at the risk of extinction as a result of wasteful harvesting due to growing demand and high economic value.

Materials and Methods: In this study, the genetic diversity of 25 different accessions of *Z. multiflora,* collected from provinces including Hormozgan, Fars, Sistan. and Balouchestan, Bushehr, Yazd, Kerman and Isfahan were examined using Inter Simple Sequence Repeat (ISSR) markers. To extract the DNA, five samples taken from the leaves of each accession were transformed into bulks; then, their concentrations were homogenized and 15 primers were used for the remainder of the experiment.

Results: The primers produced 83 polymorphic strands altogether, with an average polymorphism percentage of 77.30%; meanwhile, the highest polymorphism percentage (100%) was achieved via primers including 816D, 824H, 836P, and 844S. The lowest polymorphism percentage was obtained from 811C and 834N primers. The results obtained from the Jaccard similarity coefficient in the NTYSIS software showed that the genetic similarity of *Z. multiflora* accessions varies between 0.32-0.82. The lowest similarities were observed in accessions taken from Fanuj and Ashar, Mehriz, and NasrAbad, and two accessions of Mehriz and Khafr. However, the highest similarities were seen among accessions of NalShah GhandAab and Kerman. In principal component analysis, three of the first components explained 40.44% of changes in the entire data. Following a cluster analysis based on the UPGMA algorithm, accessions were classified into six groups.

Conclusions: It can be concluded that the ISSR markers are suitable for examining the genetic diversity of *Z. multiflora* accessions.

Keywords: Shirazi Thyme, Population, Domestication, Cluster Analysis, PCR, Molecular Marker

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Introduction

Iran is a prominent, high-ranking country in terms of richness in medicinal and aromatic plants. The majority of these plants which are consumed in the form of tea include common sage, savory, thyme, mint, and marjoram, which all belong to the Lamiaceae family.[1] Here, *Zataria multiflora* Boiss., a.k.a. Shirazi Thyme is one of the important species of mint grown in the central and southern regions of Iran; which naturally grows in Pakistan and Afghanistan as well.[2] In terms of appearance, it is in the form of a multi-year shrub with dark green leaves and white flowers, and a height of up to 80 cm.[3] The medicinal parts of this plant are its leaves and flowers.[2] The *Z. multiflora* species involve a vast spectrum of biological traits including painkiller, antimicrobial, anti-spasm, and anti-inflammatory effects.[4-6] Numerous studies focusing on the essence of *Z. multiflora* for its dominant active substance have reported thymol, linalool and carvacrol as the three main compounds.[7-10] The essence of *Z. multiflora* also has antioxidant effects by reducing oxidative stress.[11-13] *Z. multiflora* is used for treating respiratory system disorders, digestion system disorders, fever, premature labor pain, tear, bone and joint pain, headache, diarrhea, vomiting and the common cold.[2]

Z. multiflora is one of the most important and most consumed medicinal plants of Iran's southern provinces. They are highly sold in the markets and pharmacology stores of these regions and have been traditionally exported to the Persian Gulf countries. Factors including its extensive applications and significant role in financial aspects of rural households, its traditional export, and the rise in its price during the past three years have led to improper harvesting of the plant.[14] Consequently, it is necessary to take certain steps with respect to the cultivation and domestication of this species. Considering the substantial role of genetic diversity in proceeding with modification plans, the first step entails the assessment of genetic diversity and potentials of different populations of a given species. Such assessment of

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diversity helps to better identify the latent genetic power and enables accurate planning in line with making a suitable choice.[15] Genetic improvement in any beings depends on the presence, nature, and extent of genetic diversity that is accessible for manipulation. Not only the modification plans for plants in the present and future requires access to such diversity, it also depends on the conservation and management of the biological diversity.[16,17]

In conventional methods of the past, genetic diversity examination was carried out based on morphological and phenological features. However, these methods were problematic as they were time-consuming, they were influenced by environmental changes, and the markers were not highly accurate as a result of bilateral effects between environment and genotypes, and phenotypes of the plants. Among the various methods for estimating genetic diversity across plant species and their dependent populations, molecular markers are a strong tool for assessing genetic diversity in plant genotypes and plant modification.[18] Molecular markers can also be used as a powerful tool to identify individuals with close genetic affinity from various modification sources.[19] It is also possible to identify and separate the genes responsible for different stages of secondary metabolite cycles of medicinal plants using molecular markers.[20] Today, methods based on Polymerase Chain Reaction (PCR) are widely used to examine genetic diversity in various plants. Having been reported as an efficient method, the Inter Simple Sequence Repeat (ISSR) marker is also PCR-based and widely used by medicinal plant researchers for analyzing genetic diversity.[21-27] A study was conducted to examine genetic diversity of 15 ecotypes of *Z. multiflora* collected from Kerman, Sistan and Balouchestan, Boushehr, Fars, and Khuzestan provinces using RAPD and ISSR molecular makers presented the selected 15 regions in three distinct groups and the created cluster was consistent with the geographical conditions.[28] Primers produced 207 polymorphic strands in total with polymorphism percentage of 86.9%. Results obtained from Dice similarity coefficient in NTYSIS suggested that the genetic similarity in *Z. multiflora* varies between 0.43-0.84. Ultimately, findings demonstrated that the RAPD and ISSR markers are suitable for examining genetic diversity among the ecotypes of this species.[28] In another study, Hadian et al., (2011) used AFLP markers and chemical polymorphism of 18 accessions from *Z. multiflora* to examine its genetic diversity. Four chemical types were identified in the essence of the examined 18 accessions through principal component analysis. The application of AFLP markers divided the 18 accessions into five groups. Results showed that there are no particular relationships between the location of sample collection, chemical polymorphism and AFLP molecular assessment. The difference between genetic and chemical markers depends on the different share of genetic and environmental control over the production and occurrence of chemical types.[7]

Despite the importance of *Z. multiflora* in Iran and the necessity of conserving and maintaining its germplasm, there has been no comprehensive examination conducted on this species within its distribution range across Central and Southern Iran to examine its germplasm. It is also necessary to study the genetic relations for modification, domestication, and production of numbers proportionate to the needs of the related industries. Subsequently, the present study examined the genetic diversity among 25 accessions of *Z. multiflora* in Iran using ISSR molecular markers to assess the present genetic diversity and report the relations among them.

Materials and Methods

Plant Material

To conduct the experiment, first the natural *Z. multiflora* habitats were indicated using the present primary resources including Flora Iranica,[29] examination of scientific references, expert reports, interviews with professionals at the centers for natural resources and agricultural research and education and direct observations. Following the identification of these regions and direct observation of different accessions, the phenological data on various regions were collected and a proper time for sampling was determined. Ultimately, on March 2018 and the first half of spring in the same year, 25 natural habitats for *Z. multiflora* were identified in provinces including Fars (Khafr, Juyom, Fasa, Abadeh, Khonj, Tashk, Darab and Pasargad), Hormozgan (Roodkhaneh, Lavar Sheikh, Faryab, Tang-e Zagh, Bashagard and Bandar Khamir), Sistan and Balouchestan (Ashar and Fanuj), Boushehr (Boushehr), Yazd (Bafgh, Yazd, Mehriz, and Behabad), Kerman (Kerman and Jiroft) and Isfahan (Nasr Abad and NalShah GhandAab) (Table 1; Figure 1). Sampling was carried out from each habitat on five separate spots, during the growing season before the plant's reproductive phase. Following sample collection, the leaves taken from each habitat were mixed and then transferred to the medicinal plant technology lab at Hormozgan University for the subsequent stages of the experiment.

Genomic DNA Extraction

In this stage, 1 g of leaf sample from different *Z. multiflora* ecotypes were powdered inside a glass pounder in the presence of liquid nitrogen; DNA extraction was then carried out using Doyle & Doyle method, albeit with a slight difference.[30] Following DNA extraction, its quantity and quality were assessed using the Agarose gel electrophoresis method. Band quality of DNA in each sample was indicated via 1% Agarose gel electrophoresis. The existence of DNA, its concentration and quality were assessed based on the gel image. Accordingly, the DNA concentration of each sample was estimated by comparing the width of resulting bands

Table 1. Localities of *Z. multiflora* Sample Accessions Studied

No.	Collection places	Province	Longitude (E)	Latitude (N)	Altitude (m)
1	NalshahGhandab	Isfahan	52 37´	´31 74	2156
2	Nasrabad	Isfahan	52 05´	´32 27	1652
3	Yazd	Yazd	54 35´	´31 88	1235
4	Mehriz	Yazd	54 44´	´31 58	1487
5	Bafgh	Yazd	55 40´	´31 60	992
6	Behabad	Yazd	56 01´	´31 87	1432
7	Abadeh	Fars	52 64´	´31 16	2056
8	Pasargad	Fars	53 18´	´30 19	1867
9	Tashk	Fars	53 72´	29 80´	1591
10	Fasa	Fars	53 64´	´28 94	1380
11	Khafr	Fars	´53 20	´28 97	1296
12	Darab	Fars	54 55´	´28 75	1159
13	Juyom	Fars	53 98´	´28 25	856
14	Khonj	Fars	53 43´	´27 88	668
15	Bushehr	Bushehr	50 83´	´28 96	162
16	Kerman	Kerman	57 07´	´30 28	1769
17	Jiroft	Kerman	57 48´	28 33´	603
18	Faryab	Hormozgan	57 27´	´27 82	492
19	Lavar Sheikh	Hormozgan	´54 69	27 11´	894
20	Bandar Khamir	Hormozgan	55 58´	´26 95	154
21	Tang-e-zagh	Hormozgan	55 97´	´27 84	894
22	Roodkhaneh	Hormozgan	57 22´	´27 74	485
23	Bashagard	Hormozgan	57 89´	´26 45	735
24	Fanuj	Sistan & Baluchestan	59 38´	26 35´	725
25	Ashar	Sistan & Baluchestan	61 66´	26 68´	1044

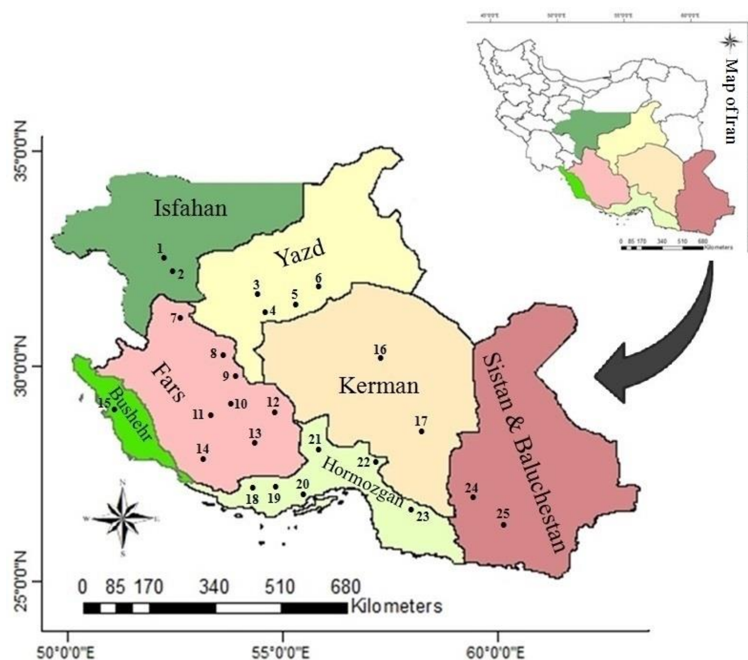

Figure 1. Geographic Locations of Collection Sites for the Studied Wild *Z. multiflora* Germplasm.

with those of standard DNA bands. After indicating DNA concentration in each sample, a DNA consumption solution was prepared with the concentration of 10 nm/μL and was used in the experiments.

Polymerase Chain Reaction (PCR) Conditions and PCR Product Electrophoresis

In this study, 15 ISSR markers obtained from Pishgam Biotech Co. were used. To indicate the most suitable connection temperature for each Marker, a thermal gradient test was conducted via a thermo-cycler device (manufactured by Bio-Rad Company; Model: T100 Thermal Cycler) using one DNA (Table 2). PCR was carried out by preparing a 15 μL mixture containing 7.5 μL of PCR kit (obtained from Pishgam Biotech Co.), 2 μL of primer, 2.5 μL of DNA with a concentration of 10 nm/μL, and 3 μL of double-distilled water. PCR was carried out using the thermo-cycler device under the following conditions: initial denaturation of genomic DNA at 95 °C for 5 minutes, 35 cycles with each cycle involving 50 seconds of 96 °C for denaturation, 50 seconds for the attachment of

primer to the denatured DNA, 2.5 minutes of 72 °C temperature for expansion of new DNA strand, and 10 minutes of 72 °C temperature for final expansion. Following the completion of PCR, 7 µL of each solution was poured into 1.8% Agarose gel sinks. Green gel was used for coloring, 1 kb marker size (Pishgam Biotech Co.) was used for estimating the length of PCR product, and TBE buffer was used for electrophoresis. Electrophoresis was carried out for 120 minutes at constant 85 volts. KIACCD Gel Documentation System- CCD-5 device was employed to photograph the gel.

Table 2. Primer Used for the ISSR Analysis in Investigating Genetic Diversity of *Z. multiflora* Sample Accessions

No.	Primer	Primer sequence	Annealing temperature (°C)
1	808Z	5′-AGAGAGAGAGAGAGAGC-3′	56.5
2	809A	5′-AGAGAGAGAGAGAGAGG-3′	60
3	811C	5′-GAGAGAGAGAGAGAGAC-3′	52.4
4	810B	5′-GAGAGAGAGAGAGAGAT-3′	59.6
5	816D	5′-CACACACACACACACAT-3′	56.8
6	817E	5′-CACACACACACACACAA-3′	55.2
7	823F	5′-TGTGTGTGTGTGTGTGC-3′	60.4
8	824H	5′-TCTCTCTCTCTCTCTCG-3′	51.7
9	825M	5′-ACACACACACACACACT-3′	58.3
10	834N	5′-AGAGAGAGAGAGAGAGTT-3′	54.6
11	836P	5′-AGAGAGAGAGAGAGAGTA-3′	49.6
12	841T	5′-GAGAGAGAGAGAGAGATC-3′	54.3
13	844S	5′-CTCTCTCTCTCTCTCTAC-3′	52.8
14	844R	5′-CTCTCTCTCTCTCTCTGC-3′	50.1
15	856W	5′-ACACACACACACACACCTA-3′	55.1

Statistical Calculations

In order to conduct statistical analysis on data in line with examining polymorphisms among genotypes, values one and zero were assigned to the presence and absence of each band, respectively. After 0/1 matrix was produced, data obtained from softwares including NTSYS-pc ver. 2.02[31] and GeneAlex ver. 6.4[32] were placed under statistical analyses. Genetic diversity for all allelic locations was calculated using Nei analysis.[33] In this assessment, the number of observed alleles, the number of effective alleles and Shannon information index for ecotypes and each primer were calculated.[34]

Results and Discussion

In this study, the genetic diversity of 25 different *Z. multiflora* accessions was examined using 15 ISSR primers; overall, 107 bands were produced with high resolution, out of which, 24 and 83 bands were monomorphic and polymorphic, respectively. On average, 7.13 bands were obtained per primer, with 5.53 polymorphic bands (Table 3). The lowest number of duplicated bands (4) were obtained from 844R and 844S primers while the highest number of bands (11) were achieved from 808Z primer. The highest polymorphism percentage (100%) was achieved via primers including 816D, 824H, 836P, and 844S; the lowest polymorphism percentage (50%) was obtained from 811C and 834N primers. Overall, the polymorphism mean percentage in all primers was 77.30% (Table 3). The range of produced bands varied between 500-2500 base pairs. Primers with anchor bases at the end of 3´ act exclusively and produce a band pattern with lower numbers, yet higher resolution.[35] It is reported that primers with additional bases (AG), (GA), (CT), (TC), (AC), and (CA) reveal higher polymorphisms with additional base (AT).[36-38] In a study to examine genetic diversity in 15 ecotypes of *Z. multiflora* using ISSR and RAPD molecular markers, it was shown that 238 bands were obtained from the 20 primers employed. Subsequently, 207 polymorphic bands with polymorphism percentage of 86.9% were produced with 31 monomorphic bands.[28] In another inquiry, Hadian et al., (2011) investigated genetic diversity in 18 accessions of *Z. multiflora* using AFLP Markers. Results demonstrated that out of the 560 observed bands, 323 bands (58.8%) were polymorphic.[7] Another study assessed genetic diversity in 59 different cultivated clones of *Thymus daenensis* via ISSR markers. Findings showed that out of the 12 applied primers, 126 bands could be observed and rated out of which, 103 (81.5%) and 32 bands were polymorphic and monomorphic, respectively.[39] Finally, based on the obtained results, the highest extent of polymorphism among 25 ecotypes of *Z. multiflora* was obtained via 808Z, 823F, and 816D out of the 15 primers employed. The high polymorphism percentage can be attributed to how this plant pollinates (cross-pollination).[7] Results obtained from assessing molecular diversity with respect to the ISSR primers used in this study showed that the highest number of observed alleles in the examination of 25 *Z. multiflora* accessions were related to 816D, 824H, and 844S while the lowest numbers belonged to 811C and 834N primers. Additionally, the highest number of effective alleles on assessing various *Z. multiflora* accessions was observed in 836P and 841T, while the lowest number belonged to 810B primer. Based on Shannon's index and the obtained heterozygosity in the assessment of 25 *Z. multiflora* accessions, the highest and lowest values respectively belonged to 836P and 834N primers. Given the examination of allelic frequency obtained from 15 used primers for various *Z. multiflora* accessions under examination, it was shown that the highest

and lowest extent of allelic frequency were obtained from 811C and 824H primers, respectively (Table 4). In general, results suggest that the high extent of both genetic diversity indices and Shannon's index for primers including 816D, 824H, 844S, 808Z and 836P shows the efficiency of these primers in differentiating between 25 *Z. multiflora* accessions; thus, this primer can be used in assessing genetic diversity of various accessions and its relation to quantitative traits. Considering how the primers were used in this study and how they showed acceptable polymorphisms, they can be used in future studies on this plant by combining them with retrotransposon markers, as REMAP markers.

Table 3. Number of Monomorphic and Polymorphic Bands, and Observed Polymorphism Percentage and by Used ISSR Primers in Different Accessions of *Z. multiflora*

No.	Primer	Total observed bands	Number of monomorphic bands	Number of polymorphic bands	Polymorphism percentage
1	808Z	11	1	10	90.91
2	809A	8	2	6	75
3	811C	8	4	4	50
4	810B	9	3	6	66.66
5	816D	8	0	8	100
6	817E	7	3	4	57.14
7	823F	10	1	9	90
8	824H	6	0	6	100
9	825M	7	2	5	71.42
10	834N	6	3	3	50
11	836P	7	0	7	100
12	841T	6	2	4	66.66
13	844S	4	0	4	100
14	844R	4	1	3	75
15	856W	6	2	4	66.66
	Mean	7.13	1.6	5.53	77.30
	Total	107	24	83	-

Table 4. Number of Observed Alleles (Na), Number of Effective Alleles (Ne), Shannon's Information Index (I), Heterozygosity (He) and Allele Frequency (Allele Freq.) by Used ISSR Primers in Different Accessions of *Z. multiflora*

No.	Primer	Na	Ne	I	He	Allele Freq.
1	808Z	1.91	1.46	0.44	0.28	0.43
2	809A	1.75	1.48	0.42	0.28	0.61
3	811C	1.50	1.30	0.28	0.19	0.72
4	810B	1.67	1.17	0.23	0.13	0.47
5	816D	2.00	1.38	0.44	0.27	0.30
6	817E	1.57	1.36	0.32	0.22	0.69
7	823F	1.80	1.29	0.32	0.20	0.42
8	824H	2.00	1.27	0.35	0.20	0.22
9	825M	1.71	1.36	0.36	0.23	0.55
10	834N	1.50	1.18	0.22	0.13	0.66
11	836P	1.86	1.49	0.45	0.30	0.50
12	841T	1.67	1.49	0.39	0.27	0.72
13	844S	2.00	1.35	0.39	0.24	0.27
14	844R	1.75	1.36	0.37	0.24	0.52
15	856W	1.67	1.33	0.33	0.21	0.58

Results obtained from Jaccard similarity matrix in NTSYS software showed that genetic similarity in *Z. multiflora* accessions under examination varied between 0.32-0.82. Given the high similarity domain, it can be inferred that these markers have successfully been able to reveal genetic diversity among the examined *Z. multiflora* accessions. The least significant similarity or the farthest genetic distance were observed among samples from habitats including Fanuj and Ashar (Both in Sistan and Balouchestan province), Mehriz (Yazd provive) and NasrAbad (Isfahan province), and two accessions of Mehriz (Yazd province) and Khafr (Fars province) with a value of 0.32. The highest genetic similarity or the closest genetic distance were observed between accessions from NalShah GhandAab (Isfahan province) and Kerman (Kerman province) with a value of 0.82 (Table 5). In the study conducted on genetic diversity among 15 *Z. multiflora* ecotypes using RAPD and ISSR markers, it was shown that the genetic similarity between these ecotypes varied between 0.439-0.846. In this inquiry, the lowest similarity or the farthest genetic distance were reported among populations from Iranshahr (Sistan and Balouchestan province) and Jiroft (Kerman province), while the highest similarity or the closest genetic distance was observed among populations from Andimeshk and Izeh (Both in Khuzestan province). Additionally, the results of this study showed that given the high genetic diversity, the examined ecotypes can be a suitable genetic resource for *Z. multiflora* modification programs.[28] In another research carried out on 18 *Z. multiflora* accessions, a mean similarity of 0.61 was obtained between the accessions.[7] In the present study on 25 *Z. multiflora* accessions, a high genetic diversity was found which could stem from cross-pollination in this plant.[2] Cross-pollinating plants can produce desirable diversity via molecular markers. One important criterion on

Table 5. Similarity Matrix for Studying *Z. multiflora* Ecotypes Based on Jaccard's Coefficient

No.	Accession	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	Jiroft	1.00																								
2	Darab	0.61	1.00																							
3	Fasa	0.61	0.67	1.00																						
4	Behabad	0.53	0.53	0.59	1.00																					
5	Juyom	0.56	0.64	0.64	0.53	1.00																				
6	TangZagh	0.52	0.60	0.54	0.60	0.55	1.00																			
7	B. Khamir	0.47	0.47	0.54	0.50	0.51	0.60	1.00																		
8	Bafgh	0.61	0.57	0.49	0.51	0.52	0.58	0.56	1.00																	
9	Khonj	0.48	0.42	0.46	0.48	0.50	0.49	0.63	0.58	1.00																
10	L. Sheikh	0.56	0.52	0.52	0.56	0.53	0.55	0.64	0.61	0.63	1.00															
11	Kerman	0.56	0.45	0.49	0.42	0.46	0.43	0.54	0.64	0.78	0.63	1.00														
12	Ashar	0.56	0.52	0.52	0.44	0.49	0.50	0.63	0.66	0.62	0.57	0.66	1.00													
13	Nasrabad	0.50	0.58	0.53	0.53	0.58	0.56	0.43	0.50	0.38	0.45	0.41	0.47	1.00												
14	Khafr	0.45	0.53	0.50	0.48	0.53	0.51	0.41	0.46	0.36	0.40	0.36	0.47	0.72	1.00											
15	Bushehr	0.50	0.61	0.59	0.47	0.55	0.56	0.48	0.46	0.43	0.44	0.44	0.48	0.72	0.62	1.00										
16	Abadeh	0.49	0.47	0.53	0.49	0.52	0.52	0.54	0.52	0.61	0.53	0.58	0.44	0.60	0.55	0.64	1.00									
17	Bashagard	0.45	0.51	0.49	0.45	0.52	0.56	0.62	0.50	0.58	0.59	0.53	0.51	0.53	0.50	0.58	0.66	1.00								
18	Pasargad	0.50	0.46	0.48	0.44	0.54	0.49	0.56	0.57	0.73	0.59	0.69	0.53	0.43	0.36	0.45	0.55	0.55	1.00							
19	Yazd	0.55	0.47	0.49	0.59	0.56	0.50	0.45	0.50	0.47	0.53	0.48	0.43	0.53	0.55	0.48	0.50	0.48	0.52	1.00						
20	N.Ghandab	0.50	0.38	0.42	0.41	0.43	0.44	0.60	0.67	0.79	0.60	0.82	0.62	0.38	0.34	0.39	0.58	0.50	0.73	0.43	1.00					
21	Fanuj	0.44	0.54	0.47	0.53	0.54	0.61	0.41	0.44	0.45	0.45	0.42	0.32	0.62	0.53	0.63	0.59	0.57	0.50	0.58	0.39	1.00				
22	Mehriz	0.45	0.37	0.40	0.45	0.42	0.43	0.63	0.51	0.80	0.63	0.75	0.59	0.32	0.32	0.38	0.51	0.55	0.70	0.49	0.75	0.44	1.00			
23	Tashk	0.48	0.58	0.55	0.48	0.54	0.52	0.48	0.45	0.44	0.48	0.41	0.48	0.58	0.56	0.52	0.53	0.53	0.46	0.49	0.40	0.47	0.35	1.00		
24	Faryab	0.40	0.44	0.42	0.46	0.45	0.53	0.62	0.52	0.61	0.61	0.58	0.53	0.47	0.47	0.47	0.52	0.60	0.54	0.41	0.61	0.45	0.60	0.46	1.00	
25	Roodkhaneh	0.48	0.52	0.54	0.36	0.56	0.49	0.55	0.45	0.46	0.53	0.50	0.48	0.52	0.46	0.61	0.54	0.62	0.54	0.46	0.46	0.48	0.48	0.54	0.56	1.00

Table 6. Grouping of *Z. multiflora* Accessions Using of Principal Components Analysis

No.	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
1	Tang-e zagh	Fasa	Jiroft	Bafgh	Bandar Khamir	Faryab	Abadeh	Bushehr	Nasrabad
2	Tashk	Darab		Lavar Sheikh	Khonj		Bashagard	Fanuj	Khafr
3	Yazd	Behabad		Ashar	Kerman		Roodkhaneh		
4		Juyom			Pasargad				
5					NalshahGhandab				
6					Mehriz				

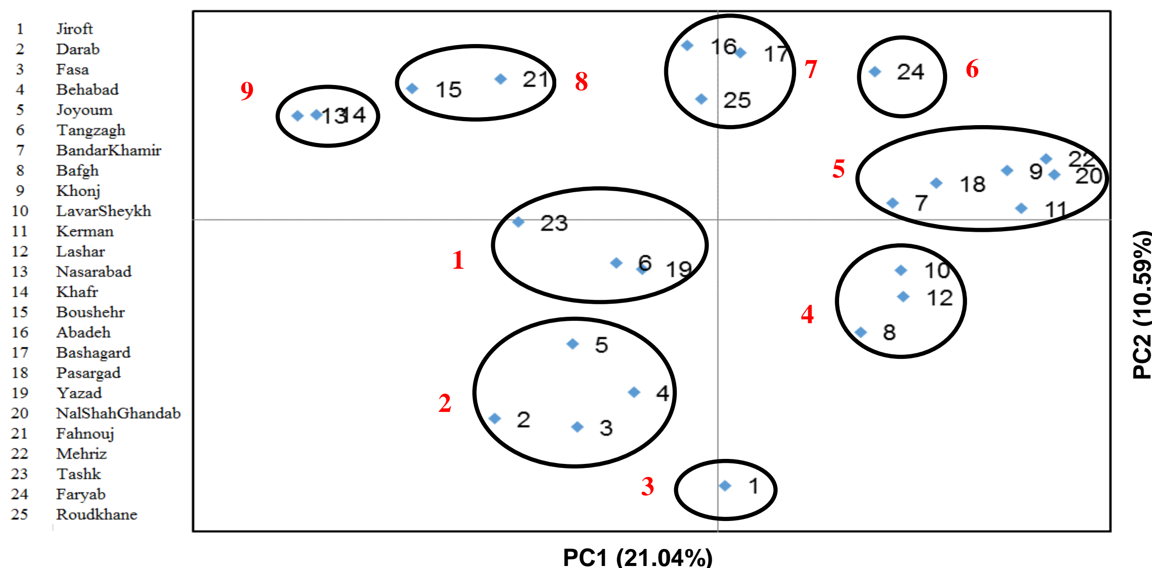

Figure 2. Scatter Plot for *Z. multiflora* Ecotypes Based on Two First Axes from Principal Coordinate Analysis

choosing parents for crossing and using heterosis phenomenon in cross-pollinating plants is genetic distance; the farther the distance between parents genetically, the higher the diversity and heterosis in outcomes.[40] In this study, accessions from Fanuj and Ashar, Mehriz and NasrAbad and two accessions of Mehriz and Khafr involved the lowest similarity or in other words, the farthest genetic distance, with a value of 0.32.

Principal component analysis is typically conducted prior to cluster analysis in order to clarify the relative importance of variables that play a role in classification of clusters.[41] Based on the results of principal component analysis in this study, data with values of over 1 were placed in three factors which, overall, could explain 40.44% of the total changes. Among these factors, the first factor explained 21.04% of variance; next were the second and third factors with 10.59% and 8.80%, respectively. In molecular data, the lower the percentage of variance allocated to the main factors, the better the distribution; this shows that molecular markers have examined more extensive areas of genome, therefore targeting and investigating more information from various areas of genome that contain genes which control different traits.[42]

Consequently, it can be concluded that the ISSR markers have had a suitable efficiency in this study and have been able to cover a broad range of genome. Drawing different *Z. multiflora* ecotypes based on the first and second consonant components in the form of 2D chart (biplot) showed different ecotypes in nine distinct groups (Figure 2). The highest number of accessions (six) was related to group 5 and the lowest (one) were placed in groups 3 and 6. Other accessions belonged to the other six groups (Table 6).

The dendrogram obtained from cluster analysis using the UPGMA method based on Jaccard similarity coefficient is presented in figure 3. Considering the results of Cophenetic coefficient for various coefficients and methods of classification, Jaccard coefficient and the UPGMA method had the highest Cophenetic coefficient value; also the accessions were classified accordingly. Accessions were divided into six subgroups via sectioning the dendrogram at 0.57 genetic distance; this line of fitness was, to a high extent, consistent with principal component analysis results (Figure 3).

The first subgroup included four accessions of Jiroft, Darab, Fasa, and Juyom with the highest similarity between the two accessions of Darab and Fasa and the lowest between Jirfot and Juyom. The second subgroup only included Tashk accession and accessions including Tang-e Zagh, Fanuj, NasrAbad, Khafr and Boushehr were in the third subgroup in which the highest and lowest similarities were observed in accession pairs of NasrAbad and Khafr, and Tang-e Zagh and Boushehr, respectively. The three accessions of Abadeh,

Bashagard and Roodkhaneh where classified in the fourth subgroup. The fifth subgroup contained Behabad and Yazd accessions, both located in Yazd province. Finally, the sixth subgroup included accessions of Bandar Khamir, Lavar Sheikh, Faryaab, Bafgh, Ashar, Khonj, Mehriz, Kerman, NalShah GhandAb, and Pasargad. In this subgroup, the highest similarity belonged to Kerman and NasrAbad accessions while the lowest similarity was between Pasargad and Bandar Khamir accessions. The lowest and highest numbers of accessions were observed in the second (1) and sixth (10) clusters. In a study conducted on 15 *Z. multiflora* accessions using the ISSR marker, cluster analysis was carried out via the UPGMA method and ecotypes were classified into three distinct groups at 0.72 similarity coefficient distance.[28] In another study, Hadian et al. (2011) conducted cluster analysis resulted from molecular examination of 18 *Z. multiflora* accessions via the AFLP marker at 0.60 genetic distance; ultimately, they classified the 18 accessions into five distinct groups.[7] According to observations, *Z. multiflora* accessions in each subgroup involved higher genetic closeness compared to accessions of other subgroups. Therefore, in case of need for hybridization, phenomena such as heterosis or transgressive segregation can be used for better productivity, given the present accessions in different groups. Typically, to offer genetic diversity or transfer desirable traits from one parent to the other or in other words, improving and modifying a specific plant in breeding operations, the cross between two or several different parents is used. Therefore, more cross between individuals with lower similarity yields more diverse traits. Moreover, to make use of the heterosis phenomenon, individuals with more genetic distances should be used. Cluster analysis enables the classification of individuals based on different traits in a manner that those with higher similarities are placed at close groups while individuals with lower similarity are placed within distant groups. Accordingly, suitable individuals can be selected for crossing or other modifications in line with the intended purposes.[43] Given these results, Jiroft and Pasargad accessions can be used in case the objective of modification programs involves using ecotypes with maximum genetic distance for crossing.

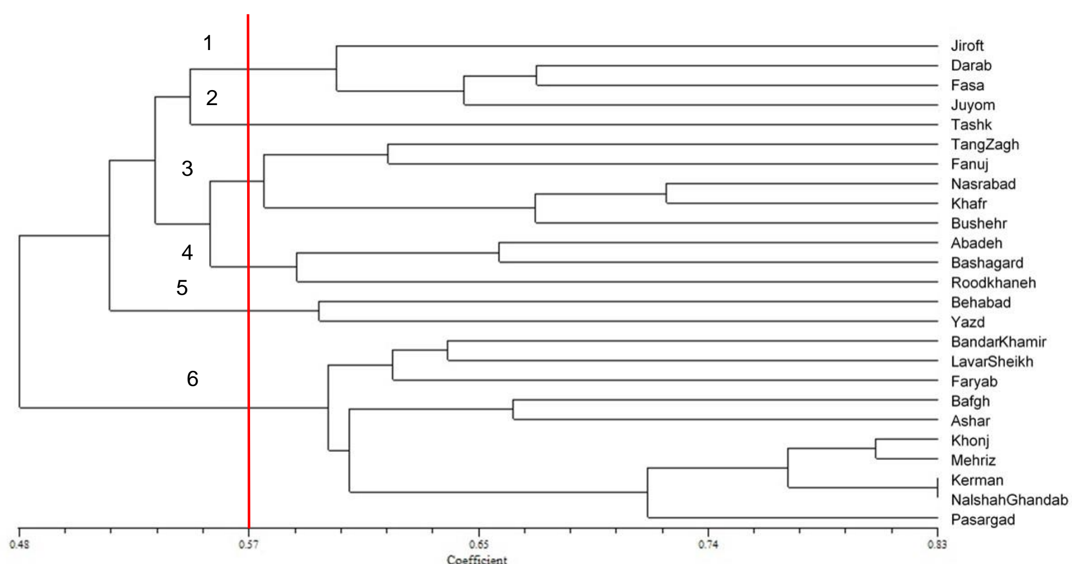

Figure 3. Dendrogram of Cluster Analysis for *Z. multiflora* Accessions Using ISSR Marker Based Jaccard Coefficient by UPGMA

Conclusion

Z. multiflora is a cross-pollinating (allogamy) plant; subsequently, its different accessions are genetically heterogeneous. The assessment of genetic diversity in germplasm of wild medicinal plants such as *Z. multiflora* with a high chance of beneficial gene occurrences is considered as one of the most necessary prerequisites to planning in line with sustainable conservation and use of these species. Furthermore, the conservation of genetic pools results in the maintenance of genetic diversity, leading to increased resistance against pests and diseases and ultimately, the survival of the species. Because the study of genetic relationships is necessary for plant breeding as well as the conservation of genetic resources, this study demonstrated that despite the endangerment of different accessions of *Z. multiflora* due to unscientific and excessive exploitation and droughts of the past few years, it showed a considerable genetic diversity among the examined accessions. As a result, suitable medication methods should be adopted in line with conserving and domesticating the species as well as for modification planning. Furthermore, the findings of the study also demonstrated that ISSR markers are a suitable and fast instrument to examine *Z. multiflora* kinship relations and genome analysis.

Considering how modification and domestication plans require high genetic diversity among ecotypes, the present diversity among the 25 examined *Z. multiflora* accessions can be substantially helpful in proceeding with modification plans.

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Authors' Contributions

SM, AY, and TSH designed the study. MB collected samples from natural habitats. SM and TSH performed the experiments (Genomic DNA extraction). AY prepared the samples, carried out the experiments and wrote the manuscript with input from all authors.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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