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Molecular Characterization of *Zhumeria majdea* Iranian Germplasms Using ISSR Markers

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Abstract
Investigating genetic diversity in plant species provides a platform for further insight in plant breeding and conservation. Therefore, in the present study genetic diversity of 13 geographically isolated genotypes of *Zhumeria majdea*, as a seriously endangered medicine plant growing exclusively in Hormozgan province (South of Iran) was studied. To do so, the leaf samples of *Z. majdea* were collected from the main growing habitats of this spices including Haji Abad, Geno and Bastak regions. The collected leaf samples were subjected to DNA extraction followed by PCR assay, using Inter-Simple Sequence Repeats (ISSR) markers. Twelve markers produced totally 121 polymorphic bands and revealed a clear-cut among and within *Z. majdea* genotypes. The analysis of molecular variance (AMOVA) showed 86% and 14% variations within and among populations, respectively. Cluster analysis divided genotypes into four main groups. The first and second principle coordinates allocated 28.81% and 15.71% of the variations, respectively. In addition to the innate differences of the individuals, the high intra population variation of *Z. majdea*, can also be explained by differences in the presence or absence of endophytes and differences in the type and genetic pattern of their endophytes.

Keywords: Cluster analysis, genetic variation, ISSR marker, Lamiaceae, *Zhumeria majdea*.

Introduction

Study of genetic diversity and genetic structure of different plant species is necessary to keep them more diverse and reduce the risk of extinction. In addition, this knowledge helps us to reach a logical relationship between the intended traits and the genetic pattern of different populations. Medicinal plants are among the most important plant species that should be given more attention because they are regularly harvested by people for medicinal purposes (Laird and Kate, 2002).

The family Lamiaceae consisted of the popular aromatic plants, growing in many geographical regions of the world.

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Different Lamiaceae species are among the widely used medicine plants for treating human diseases (Alinezhad et al., 2012). *Z. majdae* is one of the most important endangered species in the family Lamiaceae, which needs to receive more attention due to its uncontrolled extraction from natural habitats. This plant was studied by Majdae Zhumer for the first time but its systematic identification was made by Rechinger and Wendelbo (Rechinger and Wendelbo, 1967). *Z. majdae* is locally known as "Mohrekhosh" belongs to the subfamily Neptoideae, order Mentheae and suborder Salviinae (Drew and Sytsma, 2011) and has geographically narrow growing habitats, limited to very sharp slope of the mountains at 520–1450 m altitude in Hormozgan province in the south of Iran (Rechinger and Wendelbo, 1967; Rechinger, 1982).

Zhumeria is unusual within the broader *Salvia* clade in which, in addition to the two fertile stamens, two large staminodes can be easily identified in the corolla, and it is monotypic genus of Lamiaceae (Bokhari and Hedge, 1976). Imani et al. (2015), suggested that *Z. majdae* has a high phenolic content and exhibits significant antioxidant activities and the major part of antioxidant activities in *Z. majdae* comes from its phenolic compounds. In addition, it has been revealed that *Z. majdae* possess flavonoids, diterpenoids and triterpenoids (Izaddoost and Rustaiyan, 1983; Rustaiyan et al., 1995). The cytotoxic, antileishmanial and antiplasmodial activities of 12, 16-dideoxy aegyptinone B from *Z. majdae* has been reported by Moein et al. (2008). There are some reports regarding the anti-inflammatory (Sharififar et al., 2012), antinociceptive, acute toxicity (Hosseinzadeh et al., 2002), antiviral (Ansari-Dogaheh et al., 2013), antimicrobial (Arman et al., 2009) and anticonvulsant (Mandegary et al., 2012) activities of the essential oils of *Z. majdae*. The major organic compounds found in the aerial parts of *Z. majdae*, are linalool (63.4%) and camphor (27.48%) (Imani et al., 2015).

The Inter Simple Sequence Repeat (ISSR) technique is a quick, cost-benefit and high reproducibility PCR-based marker analysis, targeting multiple loci in the genome. ISSRs also require just small amounts of DNA to unveil genetic differentiation and no prior information is required for their application. Many successful examples exist on employing the ISSR technique in disclosing genetic diversity in various plant species (Zietkiewicz et al., 1994; Agarwal et al., 2008) such as *Cunila menthoides* Benth (Agostini et al., 2010), *Mentha cervina* (Rodrigues et al., 2013), *Satureja bachtiarica* Bunge (Khadivi-Khub et al., 2015), *Varronia curassavica* Jacq. (Brito et al., 2016) and *Hyptis pectinata* (L.) Poit (Feitosa-Alcantara et al., 2017). Koohdar et al. (2016), investigated genetic diversity, population structure and morphological variability in the *Lallemantia royleana* (Lamiaceae) and found that geographical populations of *L. royleana* were different both in genetic and morphological perspectives. No documented information is available on the genetic structure of *Z. majdae*, and only available information is related to the studies conducted by Soltanipoor et al. (2017). They studied the karyotypic features of eight populations of *Z. majdae* and found high differences in the position of centromere and total length of chromosomes. Accordingly, they bunched these populations in the different clades, while the morphological analyses did not show any variation among them. In addition, they reported that different populations of *Z. majdae* had a tetraploid pattern ($2n = 4x = 40$).

The present study aimed to investigate genetic diversity and genetic structure in 13 genotypes of *Z. majdae* as representatives of this species in Hormozgan province. It also aimed to determine correlation between genetic and geographic distances of the populations.

Material and methods

Plant material

Leaf samples of thirteen geographically isolated genotypes of *Z. majdae* were collected in March 2017, 5 samples from Haji Abad (55° 55' E, 28° 19' N; 1200 A), 2 samples from Bastak (54º 23' E, 27º 14' N; 400 A) and 6 samples from Geno (56º 9' E, 27º 21' N; 698 A) as three main growing habitats of this plant in Hormozgan province. The average annual rainfall for Haji Abad, Bastak and Geno is 147, 147.8 and 132.35 mm, respectively. Also, the average of temperature for the above mentioned regions is 21.7, 24 and 26.8 °C, respectively.

DNA extraction

Total genomic DNA was extracted from young leaves of *Z. majdae* using the modified CTAB method (Mohammadi et al., 2017). The CTAB extraction buffer (2% CTAB) consisted of 2.0 g cetyl trimethyl ammonium bromide (CTAB), 1.0 g polyvinyl Pyrrolidone (PVP), 10.0 mL Tris-base 1 M (pH 8), 28.0 mL NaCl 5 M, 4.0 mL EDTA 0.5 M (pH 8), 40 mL H_2O, and 100 µL 2-mercaptoethanol was added to each 20 mL of CTAB extraction buffer. For DNA extraction, 0.5 g young leaves from each sample was cryogenically ground in a mortar and pestle after chilling in liquid nitrogen. Then 1 mL pre-heated CTAB extraction buffer at 65 °C was added to the fine homogenized leaf powder. The samples were then incubated at 65 °C for 1 h in a water bath with slow shaking every 10 min. Subsequently, the mixture was centrifuged for 15 min at 13000 rpm and afterward supernatant transferred to a clean microfuge tube and an equal volume of cool chloroform-isoamyl alcohol (24:1) was added to the solution, and the solution was mixed gently by inversion. The mixture was centrifuged for 5 min at 13000 rpm and only the upper aqueous phase (600 µL) transferred to a 1.5 mL clean microfuge tube (the steps of chloroform-isoamyl alcohol addition, spinnig and removing supernatant phase were repeated two times). After adding 400 µL (two thirds sample) of ice cold Isopropanol to the samples, and incubation at -20 °C for 20 min, the mixture was centrifuged for 15 min at 13000 rpm at 4 ºC. Afterward, the supernatant was discarded and the pellet was washed using 70% Ethanol, and allowed the DNA pellet to dry. Finally the DNA pellet was re-suspended in 100 µL sterile water and stored at -20 °C until using in PCR assays. The quality and concentration of DNA samples were analyzed using a UV-visible spectrophotometer, with a Thermo-Fisher Nano-drop 2000. The A260/280 ratio was used to estimate DNA purity. A 1.5% agarose Gel electrophoresis was used for further analysis of DNA purity.

ISSR-PCR

Twelve ISSR primers (Table 1) were used for this investigation (Asadi et al., 2018). The PCR assay was performed in a Thermal Cycler (Applied Biosystems, USA) with a total volume of 25 µL containing, 2 µL primer, 1.5 µL template DNA with 9 µL sterile distilled water (Zeitkiewicz et al., 1994). PCR condition was set at 94 °C for 3 min, following 35 cycles of 94 °C for 1 min, 58 °C for 45 s, 72 °C for 1 min, and followed by a final extension of 5 min at 72 °C. The amplicons were run on a 1.5% agarose gel. The gels were run at 90 volts for 120 min and visualized with ultraviolet light after SYBR Green staining as described by Sambrook and Russell (2001). The nucleic acid marker (100 bp DNA ladder) was used to estimate the amplicon size.

Table 1. Inter Simple Sequence Repeat (ISSR) primers used to study genetic structure in 13 *Zhumeria majdae* genotypes

Primer	sequence	%GC	Annealing temp (°C)	Melt temp (°C)
UBC808	AGAGAGAGAGAGAGAGC	52.9	52.4	52.4
UBC809	AGAGAGAGAGAGAGAGG	47.4	52.9	52.4
UBC810	GAGAGAGAGAGAGAGAT	47.1	50.4	50
UBC811	GAGAGAGAGAGAGAGAC	52.9	52.2	52.4
UBC816	CACACACACACACACAT	47.1	50.4	50
UBC817	CACACACACACACACAA	47.1	52.0	50
UBC823	TGTGTGTGTGTGTGTGC	52.9	54.2	52.4
UBC824	TCTCTCTCTCTCTCTCG	52.9	56.4	52.4
UBC825	ACACACACACACACACT	52.9	52.2	50
UBC841	GAGAGAGAGAGAGAGACC	50	56.2	53.9
UBC844	CTCTCTCTCTCTCTCTAC	50	52.0	53.9
UBC856	ACACACACACACACACCTA	47.4	55.4	55.2

ISSR analysis

By scoring amplified fragments, ISSR products were translated to numerical data as either 1 (present) or 0 (absent) of band in a spread sheet. Combined binary data exported from the scored bands were then used for statistical analyses. The polymorphic information content (PIC) was calculated by using the formula described by Powell et al. (1996). Cluster analysis was done base on genetic similarities using NTSYS software version 2.02 (Rohlf, 1998). Principal coordinates analysis (PCoA) and genetic variation within and among populations were estimated by analysis of molecular variance (AMOVA), using Gene Alex software version 6.5 (Peakall and Smouse, 2012). Nei's gene diversity (H), Shannon's information index (I), Genetic diversity index within populations (HS), Gene flow (Nm) and Genetic differentiation coefficient between populations (GST) were estimated using the Population Genetic Analysis (POPGENE version 1.32) (Yeh et al., 2000).

Results

ISSR polymorphism

Twelve ISSR primers generated 123 bands and revealed 98.37% polymorphism rate. The number of band varied from 5 (primer 825) to 13 (primers 808 and 841) (Table 2). The polymorphic rate was 98.37%. All 12 primers totally produced 123 bands of which 121 bands showed polymorphism and 2 did not show polymorphism.

Table 2. Characteristics of the ISSR makers used to study the genetic structure of *Zhumeria majdae*

Primer	N	H_S	I	H	PIC	P %	G_{ST}	Nm
UBC808	13	0.14	0.33	0.19	0.31	10.74	0.12	4.21
UBC809	11	0.13	0.44	0.28	0.41	9.09	0.47	2.32
UBC810	11	0.24	0.55	0.38	0.49	9.09	0.26	2.43
UBC811	12	0.19	0.33	0.19	0.33	9.91	0.06	16.29
UBC816	9	0.06	0.20	0.10	0.18	7.43	0.08	6.35
UBC817	9	0.24	0.62	0.43	0.45	7.43	0.36	1.04
UBC823	12	0.26	0.45	0.29	0.43	9.91	0.07	15.9
UBC824	7	0.11	0.29	0.15	0.26	5.78	0.16	2.57
UBC825	5	0.05	0.16	0.07	0.14	4.13	0.05	7.85
UBC841	13	0.26	0.44	0.44	0.43	10.74	0.07	26.93
UBC844	9	0.11	0.54	0.37	0.49	6.61	0.52	1.11
UBC856	12	0.07	0.20	0.10	0.21	9.09	0.04	9.25
Total	123	-	-	-	-	121	-	-
Average	10.25	0.15	0.37	0.24	0.40	8.32	0.18	8.02

N: total number of bands; P: Percentage of polymorphic bands; H: Nei's gene diversity; I: Shannon's information index; HS: Genetic diversity index within populations; GST: Genetic differentiation coefficient between populations; Nm: Gene flow.

Population genetic diversity, differentiation (GST), and gene flow (Nm)

The ISSR markers were highly diverse from the point of polymorphism information content (PIC). The estimated PIC varied from 0.14 (primer UBC825) to 0.98 (primer UBC856), with an average of 0.40. The Nei's gene diversity index (H) varied from 0.07 (UBC825) to 0.44 (UBC841), with an average of 0.24. The Shannon's information index (I) ranged from 0.16 (UBC825) to 0.62 (UBC817), with an average of 0.37. The genetic diversity index within populations (HS) varied from 0.05 (UBC825) to 0.26 (UBC823 and UBC841), with an average of 0.15. The genetic differentiation coefficient between populations (GST) ranged between 0.04 (UBC856) and 0.52 (UBC844), with an average of 0.18. The gene flow (Nm) ranged between 1.04 (UBC817) to 26.93 (UBC841), with an average of 8.02. The high intra-genetic variation and polymorphic features indicate that there is high gene exchange among *Z. majdae* genotypes. The highest value of Nei's gene diversity (H) (0.2482) and Shannon's information index (I) (0.3685) were observed in Haji Abad population. However, Bastak population showed the lowest Nei's gene diversity and Shannon's information index (0.0741 and 0.1082, respectively) (Table 3).

The Geno and Bastak populations were the highest in the genetic identity (IN= 0.896) and closest in the genetic distance (0.1093). The Haji Abad and Bastak populations were the lowest in genetic identity (IN= 0.878) and the furthest in genetic distance (D= 0.1305).

Clustering analysis of Z. majdae genotypes

The cluster analysis grouped *Z. majdae* genotypes into four main groups. The first main group consisted of 7 genotypes. The second, third and fourth main groups each consisted of two genotypes. Geno 6 and 2, Haji Abad 1, 2 and 3, and Bastak 1 and 2 were placed in the first group. Geno 1 and 3 were clustered together in the second group. Geno 4 and 5 were bunched in the same group (the third group). The fourth main group consists of Haji Abad 4 and 5 (Fig. 1). In contrast to Bastak population in which all genotypes were clustered in the same group (the first group), the Geno and Haji Abad genotypes were clustered in the different groups. The Geno population was divided into the first, second and third groups each with the same ratio (33.3%). In Haji Abad, 60% of genotypes were clustered together (the first group) and the rest was placed in another group (the fourth group).

AMOVA and principle coordinates (PCoA) both showed that the highest rate of populations variation (86%) is related to the within population variation (Table 4 and Fig. 2). The first and second principle coordinates explained 28.81% and 15.71% of the variations, respectively. The first three coordinates of PCoA explained 58.69% of the variation. The two-dimensional plot of PCoA confirmed results of the cluster analysis (Fig. 2).

Table 3. Genetic variations information revealed by ISSR markers among three populations of *Zhumeria majdae*

	Geno	Bastak	Haji Abad
N	6	2	5
Monomorphic bands	40	101	41
Polymorphic bands	83	22	82
Polymorphism (%)	67.48	17.89	66.67
H	0.1803	0.0741	0.2482
I	0.2883	0.1082	0.3685
Na	1.390	0.472	1.382
Ne	1.279	1.126	1.427
Na/Ne	1.0867	0.4191	0.9684
He	0.180	0.074	0.248
uHe	0.197	0.099	0.276

N: number of genotypes; H: Nei's gene diversity; I: Shannon's information index; Na: Observed number of alleles; Ne: Effective number of alleles; He: expected heterozygosity; uHe: unbiased expected heterozygosity.

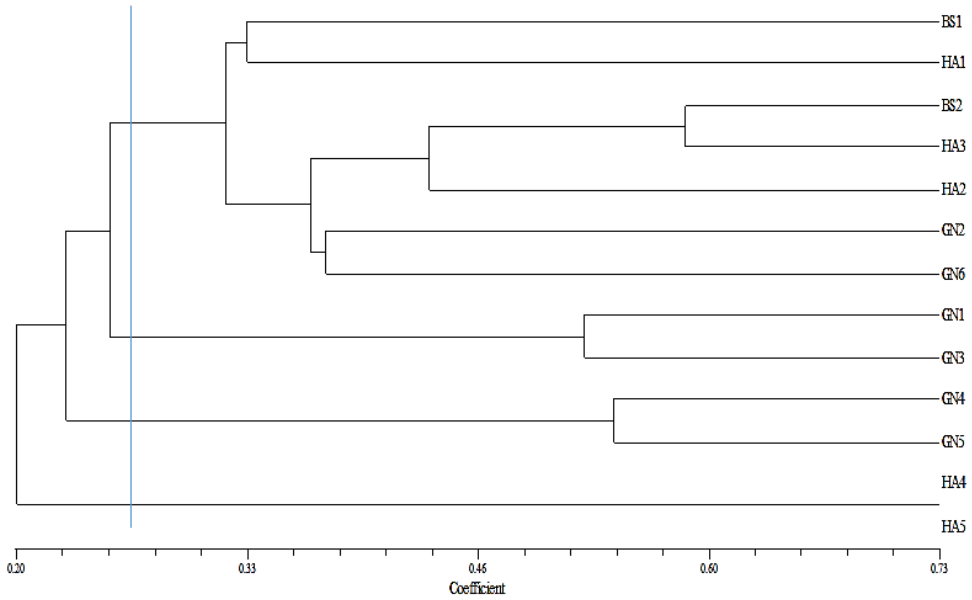

Fig. 1. Dendrogram of 13 genotypes of *Zhumeria majdae* by ISSR analysis based on unweighted pair-group method with arithmetic average (UPGMA). BS (1-2), HA (1-5) and GN (1-6) means; Bastak, Haji Abad and Geno regions and number of genotypes, respectively.

Table 4. Analysis of molecular variance (AMOVA) among and within *Zhumeria majdae* populations.

Source	df	SS	MS	Est. Var	Value %	% of variation	
Among pops	2	60.885	30.442*	2.948	14	14%	0.136*
Within pops	10	186.500	18.650*	18.650	86	86%	
Total	12	247.385	-	21.598	100	100%	

df: degree of freedom, MS: Mean of squares, Est. Var: Estimated variance,* ;significant in p≤0.05.

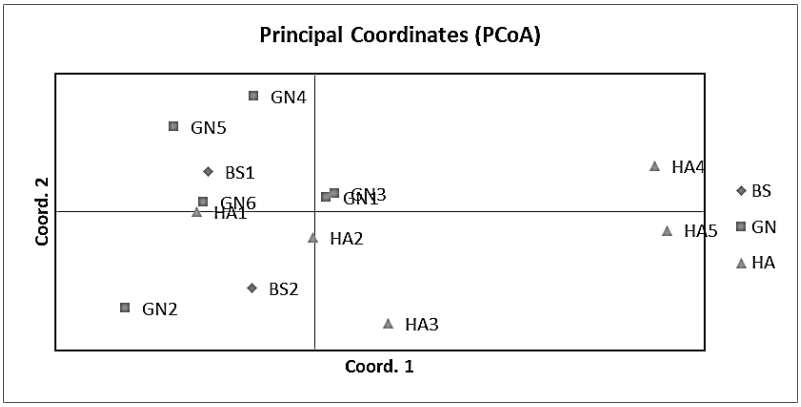

Fig. 2. Two-dimensional scatter diagram of three *Zhumeria majdae* genotypes based on principal coordinate analysis performed by Gen Alex 6.5 software.

Discussion

Although there are several documented studies dealing with ISSR application on the family Lamiaceae, this study can be considered as the first report on the genetic diversity in *Z. majdae*. This plant is an important medicine plant species, growing exclusively in highly limited habitats in Hormozgan province and seriously at risk of extinction. Despite the high importance of *Z.*

majdae, little information is available on the different morphological, biochemical and physiological aspects of this medicinally important plant. Although, many efforts have been made to protect *Z. majdae* from extinction by focusing on its propagation, there is no enough information on the different ecologically aspects of this species and most of the effort failed.

In the present study we unveiled the genetic structure of *Z. majdae* population and provided valuable information for further investigations. These findings can shed light on the different biological and ecological aspects of *Z. majdae*. We gained high polymorphism rate (98.37%) by employing 12 primers, showing the high potential of the primers in finding and targeting the polymorphic loci. This finding is in parallel with outcomes of Rodrigues et al. (2013), who showed high potential of ISSR primers in revealing polymorphism in the different plant species. Although, high polymorphism rate inferred by ISSR markers can stemming from intrinsic variations existing in a plant species, the marker ability to target appropriate loci is also very important issue in qualitative evaluation of a primer (Goodarzi et al., 2015). The highest rate of the polymorphic bands was observed in the Geno population, followed by Haji Abad, indicating high within population variation in these populations. This information serves to increase our knowledge about the proper *Z. majdae* populations which can be involved in the next breeding programs. Accordingly, the Geno and Haji Abad populations, with respect to their high genetic diversity, are of the promising candidates for this purpose. By focusing on these two genetically diverse populations we can move toward saving *Z. majdae* from extinction through determining the suitable habitats for its establishment and providing a germplasm collection for the further investigations.

Gene differentiation and gene flow both are important indices for evaluating the population's genetic structure. The value of calculated GST in the present study was 0.18, indicating superiority of the within population variation than the variation among the populations (86% vs 14%) and a strong gene exchange among *Z. majdae* genotypes. Safaei et al. (2016), using AMOVA test revealed that molecular differences among the studied *Salvia* species is 21% among the studied populations while 79% occurred within species.

This finding was also confirmed by high values of the Nm (8.02). GST>1 is a threshold for this parameter, showing a gene exchange exists among populations. These results are in agreement with Safaei et al. (2016), who showed that GST value in six *Salvia* species was 0.18. Gene flow between the incipient species can homogenize most of the genome, except for loci that experience strong divergent selection pressures or regions that are tightly linked with these loci (Strasburg et al., 2012). In the present study, we found high value of Nm for all primers, indicating high rate of gene exchange among *Z. majdae* genotypes. The seasonal winds may be one of the responsible for this issue, which enables the genotypes to be in association with each other by pollination. The average Nei's gene diversity index (H= 0.24) was very consistent with Wang et al. (2007) that observed H= 0.2509 in evaluation of wild *S. lachnostachys* and also Erbano et al., (2015) with H= 0.2509 in *S. lachnostachys* population. The Soheili is one of the most prevalent winds in southern Iran with southeast to northwest direction, which can facilitate gene exchange among the studied genotypes. Different mechanisms, including isolation by distance, lack of gene flow, local adaptation, and genetic drift followed by strong selection pressure, are responsible for species/population differentiation and genetic divergence (Tero et al., 2003; Freeland et al., 2011; Frichot et al., 2013).

The phylogenetic tree inferred from UPGMA, clustered all *Z. majdae* genotypes into four main groups in which some individuals from the same population were grouped into the different clusters, confirming high rate of within population variation for this species. The high rate of within population variation may come from existing the genetically diverged individuals within each genotype. These diverged individuals may have a close genetic relevance with individuals in another population and this can be a strong reason for lack of correlation between genetic and geographic distances in *Z. majdae*. Genetic dissimilarity among individuals in populations can be explained by either niche partitioning or difference in flora and density of the associating microorganisms.

The endophytes are amongst the most important associating microorganisms inhabiting within plants and can improve the plants adaptability through creating a mutualistic relation with their hosts. They may also play role in protecting the host plants against pests and diseases through producing the secondary metabolites (Strobel et al., 2004). Furthermore, it has been evidenced that in some cases no secondary metabolite is produced in medicine plants in lack of the endophytes, showing their main role in rendering medicinal properties to the hosts. Another reason for the high within population variation can be related to the long-term natural selection pressure which enforces individuals to have an ecological adaptation result in diversification by the occupied niche (Donoghue, 2008). However, further studies are required to determine inconsistency of the genetic and geographical distances. Lack of association between genetic and geographic distances in *Z. majdae* is in agreement with the results of Qiu et al. (2003), Yuan et al. (2007) and Mohammadi et al. (2017), which showed that pomegranate populations can be in association with each other and have gene flow even in geographically long distances.

Conclusion

The present study is the first investigation carried out on the genetic structure of *Z. majdae* as an endangered medicine plant with highly limited habitats. It could provide valuable information on the different genotypes of *Z. majdae* and reveal the genetic diversity status of this species for the upcoming breeding programs. As the quality of a medicine plant like *Z. majdae* is determined by the rate of its secondary metabolites, study of chemical composition in the different populations is strongly recommended to find out any relationship between the level of secondary metabolites and genetic profile of different *Z. majdae* populations.

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