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Genetic diversity and population structure of Iranian *Ephedra major*

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Abstract

As a highly valuable source of ephedrine, *Ephedra major* is well-adapted to various habitats in Iran. Over the last few decades, human's activity, in particular, over exploitation of its populations to extract medicinally valuable constitutes, have endangered this species. In this investigation, the level of genetic diversity and genetic structure in 16 natural populations of *E. major* for the very first time have been assessed utilizing molecular markers, inter-simple sequence repeat (ISSR). A high level of genetic diversity was revealed among the population where Dehbar region (Khorasan Razavi province, Iran) exhibited the highest quantity of genetic parameters (P = 67.96%, H = 0.365 236, I = 0.365). Intra-population genetic diversity (71%) was significantly higher than inter-population genetic diversity (29%) and only 1% genetic diversity among-regions. The unweighted pair group method with arithmetic mean (UPGMA) and Bayesian clustering methods indicated the presence of a relatively strong population structure and to some extent in accordance with the geographical origin of populations. However, the success of UPGMA in grouping populations in congruence with their geographical location was more tangible. From the outcomes of this study, it could be asserted that, ISSR markers are effective in detecting the pattern of genetic diversity in *E. major* populations. On the other hand, by having reliable knowledge of the status of genetic diversity and structure of populations of this species, the first steps can be taken to protect populations with low genetic diversity, and also pave the way for breeding programs to finding and selection of superior populations.

Keywords: *Ephedraceae*, clustering, conservation, genetic distance, ISSR

مطالعه تنوع ژنتیکی و ساختار جمعیتی *Ephedra major* در ایران*

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خلاصه

Host *Ephedra major* (*Ephedraceae*)، به عنوان منبع بسیار ارزنده ماده افدرین، با زیستگاه‌های مختلف در ایران سازگار شده است. طی چند دهه اخیر فعالیت‌های انسانی، به ویژه بهره‌برداری بیش از حد از جمعیت‌های گونه مذکور جهت استخراج مواد دارویی ارزشمند، این گونه را در معرض خطر قرار داده است. در این تحقیق، سطح تنوع ژنتیکی و ساختار ژنتیکی در ۱۶ جمعیت *E. major* برای نخستین بار با استفاده از نشانگرهای مولکولی inter-simple sequence repeat (ISSR) ارزیابی شد. سطح بالایی از تنوع ژنتیکی در میان جمعیت مشاهده شد که منطقه دهبار واقع در استان خراسان رضوی بیش‌ترین تنوع در پارامترهای ژنتیکی را نشان داد (P = 67.96%, H = 0.365 236, I = 0.365). تنوع ژنتیکی درون جمعیت (۷۱٪) به طور قابل توجهی بالاتر از تنوع ژنتیکی بین جمعیت (۲۹٪) بود و تنها ۱ درصد تنوع ژنتیکی بین مناطق وجود داشت. روش‌های متوسط فاصله بین گروه‌ها (UPGMA) و همچنین روش‌های خوشه‌بندی بایسین (Bayesian) بیانگر وجود ساختار نسبتا قوی جمعیت است تا حدودی منطبق با منش جغرافیایی جمعیت می‌باشد. با این حال، موفقیت UPGMA در گروه‌بندی جمعیت متناسب با موقعیت جغرافیایی آن‌ها محسوس‌تر بوده و با توجه به نتایج این مطالعه، می‌توان ادعا کرد، نشانگرهای ISSR در تشخیص الگوی تنوع ژنتیکی در جمعیت‌های *E. major* مؤثر هستند. از سوی دیگر، با داشتن دانش قابل اعتماد از وضعیت تنوع ژنتیکی و ساختار جمعیتی این گونه، می‌توان گام‌های نخستین را در راستای حفاظت از جمعیت‌های با تنوع ژنتیکی پایین استوار نمود تا راه برای اجرای برنامه‌های اصلاحی در انتخاب جمیعت‌های برتر هموار گردد.

واژه‌های کلیدی: افدرا، حفاظت، خوشه‌بندی، فاصله ژنتیکی، ISSR

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Introduction

Intensive human-driven alternation and destruction in natural habitats (i.e., over 30% of the land surface on earth) have notably endangered a number of plant species as critical food and medicinal resources (Lienert 2004, Bakhshipour *et al*. 2019). Habitat disruption imposes significant threats on plant species through shrinking population size and contributing to geographical isolation which given their association with the extinction risk of plant species acquiring a profound understanding on genetic variability and population structure of plant species is an indispensable prerequisite to establishing comprehensive programs to conserve plant biodiversity since information on population genetic holds a key in conserving populations in many approaches, for instance, the intensified decline in genetic diversity of small and isolated populations could be the result of inbreeding and genetic drift (Méndez *et al*. 2014, Ellegren & Galtier 2016, Lee *et al*. 2018, Mafakheri *et al*. 2020). The literature is enriched with information on genetic diversity and population structure of important medicinal plants (Mafakheri *et al*. 2020). However, little attention has been paid to *Ephedra* L. with approximately 65–75 species commonly widespread in Asia, Central and North America, and Europe. Iran is known to hold a great diversity of *Ephedra* with 10 species (Riedl 1967) and in some references reported eight species from Iran (Assadi 1998), among them *Ephedra major* Host, a dioecious evergreen plant belonging to the family *Ephedraceae* from an evolutionary viewpoint, considered as an advanced gymnosperm with a number of angiosperm characteristics (Owens *et al*. 1990). This species is profusely branched woody stems and herbaceous branchlets with shrub growth habit and height from 20 to 150 cm (Vidaković 1991).

Classification of *Ephedra* has long been a matter of debate, as individuals have few morphological characteristics to assist classification. In particular, the distinction between *E. procera* C.A. Mey. And *E. major* is uncertain due to morphological similarities (Assadi 1998). Based on some gross morphological and geological examinations, *E. procera* falls has been reduced as *E. major* subsp. *procera* (Fisch. & C.A. Mey.) Bornm. (Rydin *et al.* 2010). Therefore, during the present study, an effort was made to illustrate any grouping at the rank below species. Being dioecious and lacking well-developed leaves has made it very difficult to efficiently evaluate the populations of *E. major* using morphological characters. Therefore, using molecular markers as a tool for clarifying possible relationships between its populations, seems to be the best solution.

Ephedra extract is sympathomimetic have a 5000-year application as an effective remedy for respiratory issues as asthma, colds, influenza, typhoid fever, and cough in traditional Chinese medicals (Lee & Lee 2009, Parsaeimehr *et al*. 2010) besides recently many reports asserted the presence of potent antioxidant and antibacterial potentials (Jaradat *et al*. 2015) as well as its positive influence on improving athletic performance (Luther 2012) which these phytochemical capabilities to a large degree are owing to ephedrine and associated alkaloids such as pseudoephedrine, methyl ephedrine, methyl pseudoephedrine, norephedrine, and norp-seudoephedrine (Rustaiyan *et al.* 2011, Gul *et al*. 2017).

To evaluate genetic diversity in plant species, there are numerous approaches that each one comes with its own advantageous and drawbacks, but PCR-based molecular markers have long proven to be a reliable method and are available in great diversity, albeit, opting markers of interest affected by various factors and considerably depend on the goals of the study. Here, in this research, Inter Simple Sequence Repeats (ISSR) markers were employed to estimate the genetic variation and population structure in

E. major wildly grow in west, north-western, central Alborz, and north-eastern regions of Iran.

In spite of being dominant markers with some drawbacks, ISSRs still a potent and user-friendly biotechnological tool with notable abundancy throughout genomes that can be applied to determine polymorphism, and differentiate populations reliably (Costa *et al*. 2016).

Holding such merits, molecular markers have been frequently applied alone or as complementary with other markers (Meena *et al*. 2016, Mafakheri *et al*. 2020) to assess the genetic variation within and between plant population and species. Previously nuclear and plastid markers have been utilized for phylogenetic investigation in *Ephedra* (Huang *et al*. 2005, Qin *et al*. 2013, Ickert-Bond & Renner 2016), and only a couple studies have undertaken more readily available markers for studying the relationship between species or populations of *Ephedra* such as RAPD (Rong-li 2003, Takeuchi *et al*. 2003, Hanyu *et al*. 2006, Ghafoor *et al*. 2007, Ehtesham-Gharaee *et al*. 2017), ISSR (Zhu *et al*. 2013) or combination of RAPD and ISSR (Saeed *et al*. 2015) and most lately directed amplification of mini-satellite DNA (DAMD) and ISSR (Meena *et al*. 2016, Meena *et al*. 2019). Given the fact that genetic diversity determines the potential fitness of a population and, ultimately, its long-term persistence, because genes encode phenotypic information. The risk of plant species extinction has often been linked with low genetic diversity, and several researchers have documented reduced fitness in populations with low genetic diversity. For example, low heterozygosity has been associated with low seedling survival and reduced population growth. Thus understanding the genetic diversity status of plant populations can help in locating the decline in genetic diversity of populations and practice conservation approaches before the extinction of the population (Costa *et al*. 2016, Mafakheri *et al*. 2020). Extensive studies are required to comprehend the status of plant populations, particularly those that have received the economical and medicinal attention of humans, such as *E. major*. Other species of *Ephedra*, including *E. gerardiana* Wall. ex Stapf, and *E. foliata* Boiss. that have medicinal applications as *E. major* have been reported to be endangered already (Meena *et al*. 2016, Meena *et al*. 2019). Despite the medicinal use of *E. major* in Iran and the negative impacts of human exploitation, no study has been so far conducted to evaluate the genetic status of its populations. That is why here we attempted to: a) estimate the genetic variability of 16 populations of *E. major* in Iran using ISSR markers, b) evaluate intra-population and inter-population variation, and c) discuss possible underlying factors affecting the distribution pattern and means that, this information can contribute in conserving of *E. major* populations.

Materials and Methods

- Plant materials

The specimens collected from 16 populations of *E. major* covered areas from north-western, north, and north-eastern Iran (Table 1) by relying mainly on Iran. Flora Iranica (Riedl 1967) and Flora of Iran (Assadi 1998). From each population, six individuals as samples were taken by considering a 20 m distance between individuals. Collectively, 94 individuals were obtained from 16 populations. Voucher samples have been deposited in the Islamic Azad University Herbarium (IAUH), Tehran, Iran (Table 1).

Table 1. Localities along with related data of collected plant material examined in this study

Locality	Country	Latitude	Longitude	Date	Altitude (m)	Collector	Herbarium No.
Mazandaran prov.: 20 km from Chalous to Marzanabad	Iran	36°31′	50° 21′	30.09.2016	316.17	Ghanbari	IAUH0000 15316
Khorassan (N) prov.: Bojnord, 13 km from Hesar-hoseini road to Rakhtian	Iran	37°21'	57° 12'	01.08.2016	1814	Eskandari	IAUH0000 15318
Zanjan prov.: Sarcham road	Iran	37°8'	47° 47'	12.09.2016	1400	Eskandari	IAUH0000 15317
Kordestan prov.: 15 km from Ghorveh to Songhor	Iran	35°6'	47° 57'	12.10.2016	1829	Ghanbari	IAUH0000 15319
Gilan prov.: 10 km from Lowshan to Amarloo	Iran	36° 46'	49° 44′	15.11.2016	1800	Eskandari	IAUH0000 15320
Ardabil prov.: Hir, 28 km from Hir to Ardebil	Iran	37°37'	48° 32'	10.11.2016	1400	Eskandari	IAUH0000 15321
Golestan prov.: Azadshahr, 10 km from Azadshahr to Shahroud	Iran	36° 56'	55° 22′	07.08.2016	1400	Eskandari	IAUH0000 15322
Markazi prov.: Saveh road, 85 km from Nowbaran to Saveh	Iran	35° 07'	49° 33'	13.10.2016	1755.65	Ghanbari	IAUH0000 15323
Ardebil prov.: Khalkhal	Iran	37° 40'	48° 29'	08.11.2016	1700	Eskandari	IAUH0000 15324
Hamedan: Abbas Abad	Iran	34° 46'	48° 27'	01.09.2016	2189	Ghanbari	IAUH0000 15325
Khorasan Razavi prov.: 12 km from Torghebeh to Dehbar	Iran	36° 5'	59° 29′	13.08.2016	1578	Ghanbari	IAUH0000 15326
Alborz prov.: Chalous road, 15 km from Shahrestanak to Gachsar	Iran	36°02′	51° 30′	14.07.2016	2015.49	Ghanbari	IAUH0000 15327
W. Azarbaijan prov.: Takab	Iran	36°35′	47° 11′	16.07.2016	1850	Eskandari	IAUH0000 15328
Alborz prov.: Baraghan, 10 km from Baraghan to Karaj	Iran	35°055′	57° 47′	07.07.2016	1625	Ghanbari	IAUH0000 15329
Tehran prov.: Soleghan, 10 km from Soleghan to Tehran	Iran	35°47'	51° 16′	16.07.2016	1430	Ghanbari/ Eskandari	IAUH0000 15330
Tehran prov.: Damavand, 5 km from Absard to Khosravan	Iran	35˚ 39′	52˚ 13′	18.05.2017	2273	Ghanbari	IAUH0000 15331

- DNA extraction and ISSR assay

To extract of genomic materials from silica gel dried leaf, the CTAB method was employed (Doyle & Doyle 1987). Utilizing agarose gel (1%) the quality of the DNA was assessed. Of the molecular markers, 12 ISSR primers were tested which eight generated polymorphic bands (CAA)5, (AGA GAG)2AGAGT, (ACA CAC)2ACACT, (CAC ACA)2GC, (GACA)4, (AGA GAG)2AGAGT, (ACA CAC)2ACACYT, and (CAC ACA)2CACARG (Biolegio, Netherland) were used for amplification of each individual (Agarwal *et al*. 2015). PCR amplification procedure was followed as detailed in Morales *et al*. (2011). Briefly, 13 µl PCR reaction mixture composed of 6.5 µl master mix, 0.5 µl DMSO, 4.75 µl water, 0.5 µl primer, and 0.75 µl of genomic DNA. The PCR (LabCycler Basic thermocycler, Sensoquest, Göttingen, Germany) program was that after initial denaturation at 94 °C for 4 min, each cycle consisted 1 min of denaturation at 94 °C, 1 min of annealing at 54 °C, 2 min extension at 72 °C along with 7 min final extension at the end of 35 cycles. Further, the success in the reaction was examined by a 1% agarose gel. A molecular ladder (100 bp) was utilized to estimate the size of fragments (Fermentas, Germany).

- ISSR data analyses

The data obtained from ISSR primers aligned with the aim of GeneMarker Ver. 1.95 (GeneMarker, SoftGenetics, State College, Pennsylvania). Peaks (fragments) in length from 50 to 500 bp were manually scored as 1 (present) or 0 (absent). To confirm the presence or absence of peaks, each sample was checked considering signal intensity higher than 200. To make sure on peak accuracy, assay was repeated in a triplet.

- Genetic diversity and population structure

Utilizing GenAlex 6.4 (Peakall & Smouse 2006) parameters related to the genetic diversity of each population was evaluated including Polymorphic bands (PB), Polymorphism (%P), Nei's gene diversity (He), Shannon information index (I), the number of effective alleles (Ne), and UHe = unbiased Nei gene diversity (Weising *et al*. 2005, Ferreira *et al*. 2013). To assess the significant genetic difference amongst populations and their geographical locations an AMOVA (analysis of molecular variance) test was carried out (with 1000 permutations) (Podani 2000). The cluster analysis of plant samples performed with the unweighted pair group method with arithmetic mean (UPGMA) and Principal Coordinates Analysis (PCoA) using PAST Ver. 3.18 (Hammer *et al*. 2001). Exploiting the Bayesian-based model STRUCTURE (Ver. 2.3.4) analysis (Pritchard *et al*. 2000), the genetic structure of *E. major* was investigated.

Results and Discussion

- Genetic diversity

The ISSR markers used in this study generated a high number of repeatable bands provided a proper opportunity to investigate the genetic diversity of *E. major* populations. The indices of biodiversity observed in 16 *E. major* populations indicated a relatively high genetic biodiversity (Fig. 1). The number of private bands (unique alleles) for populations was mainly zero, except for three cases; Dehbar, Tekab, and Absard were 1, 2, and 1, respectively. The highest number of common bands (≤ 50%) greatly varied amongst populations where populations from Dehbar (71), Chalous road (67), and Soleghan (66) were found to have the highest values, respectively.

Samples which collected from populations of *E. major* in the northwest (Ardebil, Azadshar, and Khalkhal) and center of Iran (Nobaran and Hamedan) tend to show the lowest number of common bands (≤ 50%). Given the number of common bands (≤ 25%), to some extent, a pattern observed similar to ≤ 50%, in which populations of Chalous (18) and Dehbar (14) in addition to Marzanabad (14) and Qurveh (13) exhibited maximum values. Other critical indices for genetic diversity, heterozygosity (He) found to be significantly diverse among populations, the highest He was observed in Dehbar, Qurveh, and Bojnord with 0.236, 0.210, and 0.210, respectively (Fig. 1 & Table 2).

Several other parameters of genetic diversity were assessed in populations (Table 2) which PB in populations Dehbar and Bojnord found to be the highest (167 and 143, respectively), while %P in populations of Dehbar and Soleghan were higher than the rest of the populations. Again, Dehbar and Bojnord with 1.413 and 1.308 exhibited the maximum number of different alleles (Na), and Shannon information index (0.356 and 0.318, respectively). Further, populations from Dehbar and Qurveh indicated the highest number of effective alleles (Ne; 1.397 and 1.364, respectively), and unbiased Nei gene diversity (HUe; 0.258 and 0.234, respectively).

More assessments using AMOVA revealed notable molecular differences amongst populations, equal to 28%, of the existing genetic diversity among investigated populations of *E. major*, whereas the percentage of within-population diversity was significantly higher with 71%. Also, the remaining 1% belonged to diversity among regions (Fig. 2).

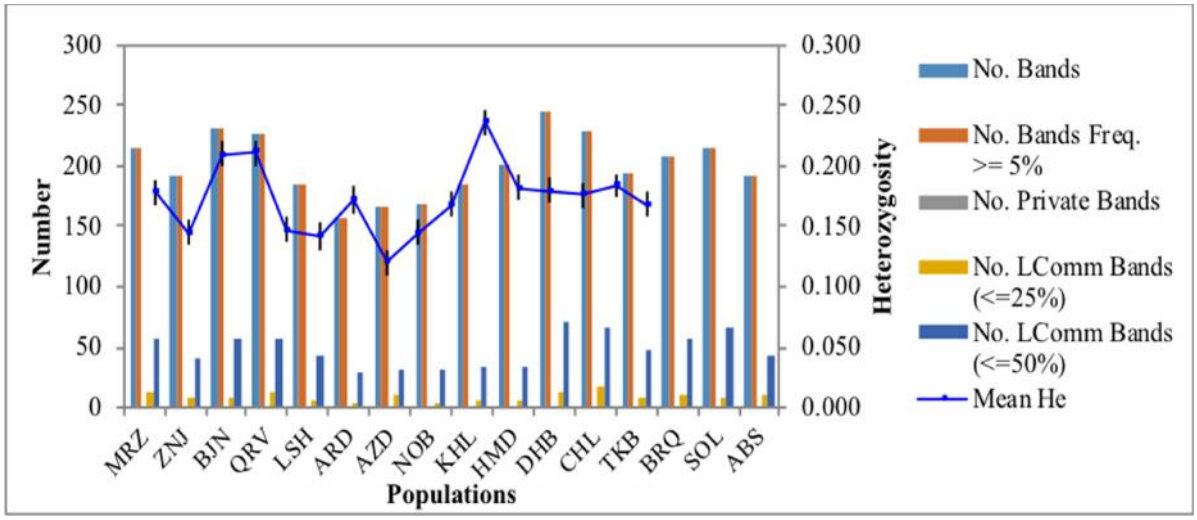

Fig. 1. Band patterns across populations: No. LComm Bands ≤ 25%) = No. LComm bands (Freq ≥ 5%) found in 25% or fewer populations, No. LComm bands (≤ 50%) = No. LComm bands (Freq. ≥ 5%) found in 50% or fewer populations. Pop.: population, MRZ: Marzanabad, ZNJ: Zanjan, BJN: Bojnord, QRV: Qorveh, LSH: Lowshan, ARD: Ardebil, AZD: Azadshahr, NOB: Nowbaran, KHL: Khalkhal, HMD: Hamedan, DHB: Dehbar, CHL: Chalous road, TKB: Takab, BRQ: Baraghan, SOL: Soleghan, ABS: Absard.

Table 2. Biodiversity indices of 16 natural populations of *Ephedra major*

Pop.	N	PB	%P	Na	Ne	I	He	UHe
MRZ	6	120	55.39%	1.201	1.290	0.273	0.178	0.194
ZAJ	6	143	44.31%	1.015	1.243	0.221	0.145	0.158
BJN	6	85	61.38%	1.308	1.351	0.318	0.210	0.229
QRV	6	129	58.08%	1.257	1.364	0.313	0.210	0.234
LSH	6	79	42.22%	0.973	1.248	0.221	0.147	0.160
ARD	6	59	38.02%	0.847	1.247	0.210	0.142	0.157
AZD	6	75	46.11%	0.961	1.303	0.253	0.172	0.187
NOB	6	55	33.83%	0.841	1.208	0.179	0.120	0.131
KHL	6	78	42.22%	0.976	1.246	0.218	0.145	0.158
HMD	6	89	44.91%	1.054	1.294	0.249	0.168	0.183
DHB	6	168	67.96%	1.413	1.397	0.356	0.236	0.258
CHL	6	127	54.19%	1.228	1.301	0.276	0.182	0.198
TKB	6	102	52.69%	1.108	1.302	0.271	0.180	0.196
BRQ	6	109	52.40%	1.147	1.300	0.266	0.176	0.192
SOL	6	139	63.47%	1.275	1.290	0.288	0.184	0.200
ABS	6	96	50.00%	1.075	1.283	0.255	0.168	0.183
Mean	-	103.3	50.45	1.10	1.29	0.26	0.172	0.188

Abbreviations: N = number of samples, PB = polymorphic bands %P = polymorphism Na = number of different alleles Ne = number of effective alleles, I = Shannon information index, He = Nei gene diversity, UHe = unbiased Nei gene diversity. Pop.: Population, MRZ: Marzanabad, ZNJ: Zanjan; BJN: Bojnord; QRV: Qorveh, LSH: Lowshan, ARD: Ardebil, AZD: Azadshahr; NOB: Nowbaran, KHL: Khalkhal, HMD: Hamedan, DHB: Dehbar, CHL: Chalous road, TKB: Takab, BRQ: Baraghan, SOL: Soleghan, ABS: Absard.

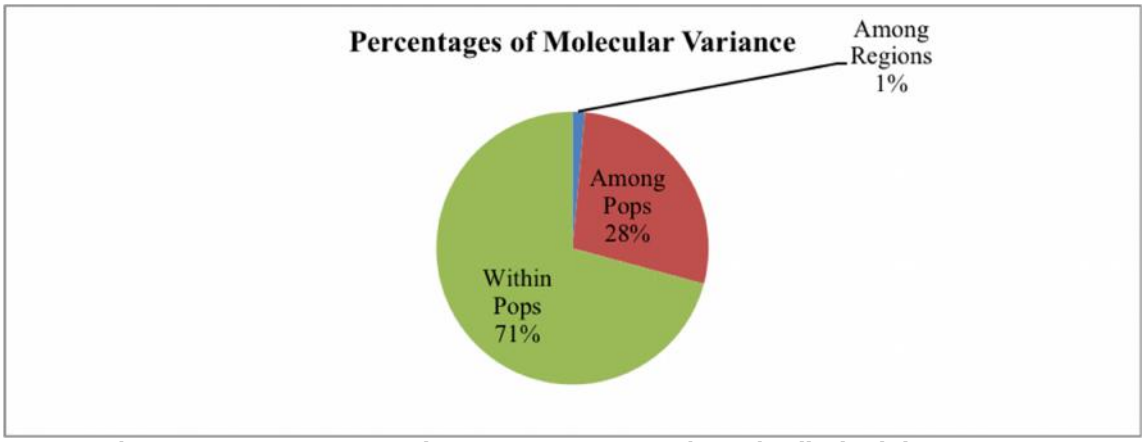

Fig. 2. A pie chart presenting AMOVA results. Genetic variance was hierarchically divided into two parts: 1. Among populations (28% of total genetic variance), 2. Within population (71% of total genetic variance), and among regions (1% of total genetic variance).

- Population structure

The UPGMA based clustering method of populations (Fig. 3) manifested a relatively well-structured paradigm of genetic distances. The 16 studied populations of *E. major* were divided into two main clusters: Cluster I, populations 1 to 13, and cluster II composed of populations from Bojnord and Dehbar. The former cluster encompassed two large sub-clusters where in the first sub-cluster successful clustered for example, Hamedan and Qorveh or Lowshan and Chalous populations together. Also, populations from Markazi and Nobaran, and those from northwest of Iran (Takab, Zanjan, and Ardebil) were clustered together. Individuals of each population are mainly grouped together, and clustering of populations corresponding to their geographical location. However, discrepancy among individuals was observed in which individuals from Khalkhal and Borghan populations did group together mainly but not according to their geographical origins at the population level. Overall, the populations, to a

significant degree, grouped close to their geographical locations, as detailed above. Further analysis with PCoA to ensure the accuracy of the grouping of the population in UPGMA revealed the absence of a grouping pattern in PCoA. Populations mainly clustered in one group (Fig. 4).

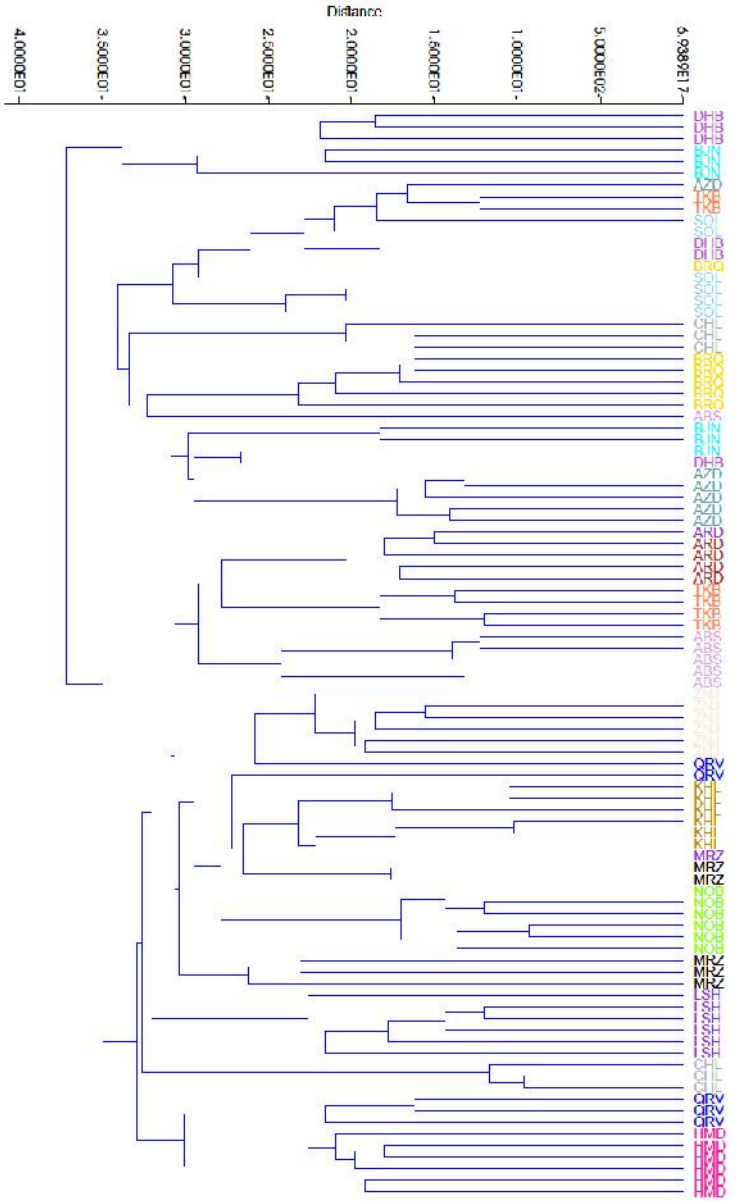

Fig. 3. Clustering of individuals of 16 *Ephedra major* populations based on UPGMA method using ISSR data. Pop.: population, MRZ: Marzanabad, ZNJ: Zanjan; BJN: Bojnord; QRV: Qorveh, LSH: Lowshan, ARD: Ardebil, AZD: Azadshahr, NOB: Nowbaran, KHL: Khalkhal, HMD: Hamedan, DHB: Dehbar, CHL: Chalous road, TKB: Takab, BRQ: Baraghan, SOL: Soleghan, ABS: Absard.

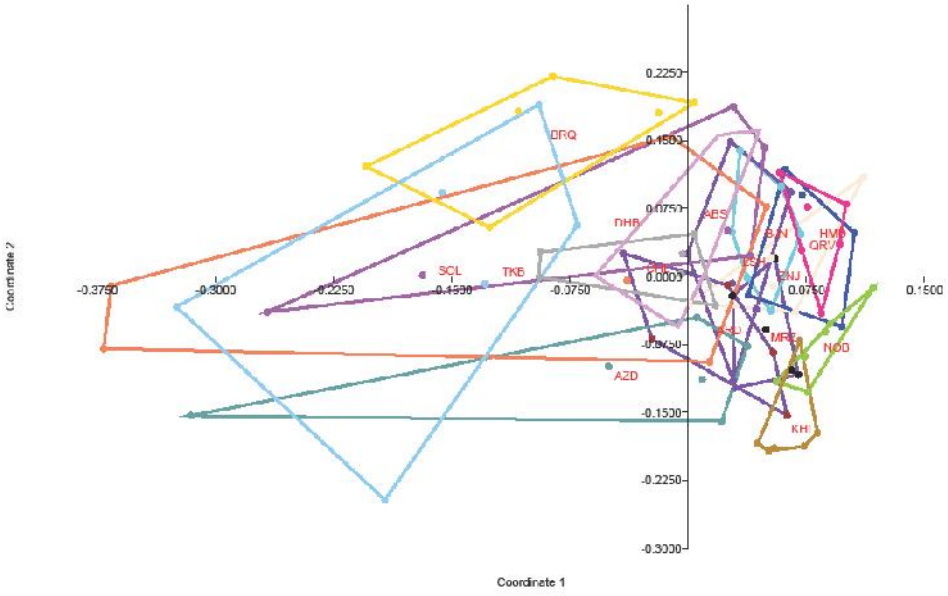

Fig. 4. Principal coordinate analysis (PCoA) of *Ephedra major* populations.

The analysis of Nei's genetic distance across 16 *E. major* populations are shown in Table 3. The genetic distances were the shortest between Soleghan and Takab (0.073) and between Absard and Marzanabad (0.083), while the highest genetic distance was between Nowbaran, Khalkhal, and Hamedan with Baraghan (0.207, 0.210, and 0.221, respectively). The genetic relationship between populations was mainly not close to the geographical location of the populations.

Table 3. Pairwise population matrix of Nei's genetic distances of 16 *Ephedra major* populations

MRZ	ZNJ	BJN	QRV	LSH	ARD	AZD	NOB	KHL	HMD	DHB	CHL	TKB	BRQ	SOL	ABS	
0.000																MRZ
0.100	0.000															ZNJ
0.104	0.122	0.000														BJN
0.098	0.099	0.108	0.000													QRV
0.130	0.165	0.150	0.130	0.000												LSH
0.103	0.151	0.140	0.126	0.144	0.000											ARD
0.097	0.134	0.108	0.132	0.161	0.130	0.000										AZD
0.132	0.149	0.183	0.137	0.187	0.156	0.182	0.000									NOB
0.095	0.148	0.141	0.130	0.170	0.132	0.130	0.138	0.000								KHL
0.134	0.139	0.144	0.098	0.178	0.159	0.177	0.183	0.153	0.000							HMD
0.119	0.139	0.103	0.121	0.140	0.126	0.108	0.163	0.154	0.164	0.000						DHB
0.111	0.128	0.147	0.116	0.172	0.146	0.114	0.186	0.153	0.154	0.126	0.000					CHL
0.103	0.145	0.122	0.141	0.160	0.091	0.106	0.186	0.150	0.178	0.102	0.146	0.000				TKB
0.155	0.166	0.148	0.158	0.181	0.156	0.144	0.207	0.210	0.221	0.128	0.158	0.128	0.000			BRQ
0.114	0.148	0.123	0.139	0.151	0.113	0.096	0.188	0.156	0.191	0.087	0.151	0.073	0.128	0.000		SOL
0.083	0.114	0.113	0.127	0.161	0.106	0.111	0.166	0.150	0.149	0.108	0.126	0.100	0.114	0.108	0.000	ABS

Pop.: population, MRZ: Marzanabad, ZNJ: Zanjan, BJN: Bojnord, QRV: Qorveh, LSH: Lowshan, ARD: Ardebil, AZD: Azadshahr, NOB: Nowbaran, KHL: Khalkhal, HMD: Hamedan, DHB: Dehbar, CHL: Chalous road, TKB: Takab, BRQ: Baraghan, SOL: Soleghan, ABS: Absard.

STRUCTURE analysis based on the Bayesian model utilized to investigate the genetic structure of 16 *E. major* populations. Evanno test indicated an optimum of K = 2, the highest clustering likelihood of molecular data (Table 4, Figs 5 and 6). Cluster I composed of 62 individuals of assumed populations from Hamedan, Qurveh, Zanjan, Khalkhal, Ardabil, Marzanabad, Loshwan, Azadshahr, Bojnord, and Nobaran; and cluster II encompassed 32 individuals covering six remaining populations including Takab, Dehbar, Soleghan, Chalous, Absard, and Baraghan. This result reflects the existence of two genetic population's type with a high degree of admixing of individuals in *E. major* populations collected from 16 various locations in Iran. The UPGMA and Bayesian analyses in this investigation revealed the presence of a relatively potent geographical affiliation of natural populations of *E. major*.

Table 4. Value of the best K in the genetic structure of 16 populations of *Ephedra major* obtained using Bayesian model

K	Reps	Mean LnP (K)	St-Dev. [L(K)]	L"/std.d	Ln'(K)	Ln" (K)	Delta K
1	20	-9858.505	0.1932	-	-	-	-
2	**20**	**-9183.335**	**2.6731**	**-166.3957**	**675.174**	**-444.795**	**166.3957**
3	20	-8952.960	6.2947	-6.93517	230.374	-43.655	6.93517
4	20	-8766.240	12.7416	-1.174105	186.721	-14.959	1.174105
5	20	-8594.480	2.6128	-65.73683	171.762	-171.76	65.73683

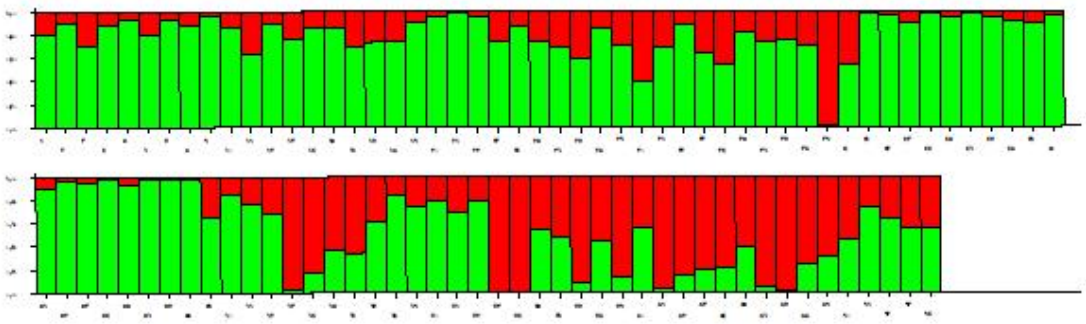

Fig. 5. Estimated genetic structure of 16 populations of *E. major* obtained using Bayesian model. Bar plots showing the arrangement of individuals based on its most probable. The individuals grouped into genetic clusters.

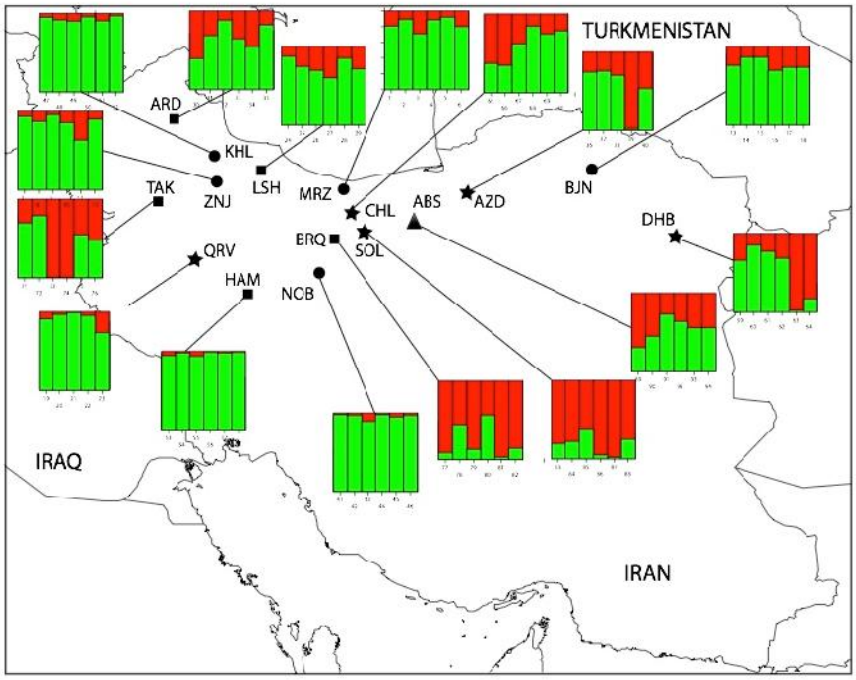

Fig. 6. The map indicating the relationship between geographical location and genetic structure of 16 natural populations of *Ephedra major* collected from west, northwest, central Alborz and north east of Iran. Pop.: population, MRZ: Marzanabad, ZNJ: Zanjan, BJN: Bojnord, QRV: Qorveh, LSH: Lowshan, ARD: Ardebil, AZD: Azadshahr, NOB: Nowbaran, KHL: Khalkhal, HMD: Hamedan, DHB: Dehbar, CHL: Chalous road, TKB: Takab, BRQ: Baraghan, SOL: Soleghan, ABS: Absard.

To develop proper and effective management strategies for preserving the natural population of plant species, having a comprehensive understanding genetic relationship among populations of the species is critical which later can be combined with the knowledge on reproductive biology (Ghafoor *et al*. 2007, Neel 2008, Silva *et al*. 2011). The level of genetic diversity is determinative in the stability of the population besides helping to gain a profound grasp of the evolutionary history of a species (Booy *et al*. 2000, Latzel *et al*. 2013). Further, populations that possess a considerable level of genetic diversity or those with differentiation can be utilized to conserve and manage plant populations by reviving genetic diversity (Hamrick & Godt 1996, Prieto *et al*. 2015). Preservation of depleting genetic resources is urgently required to ascertain the long term demands and to ensure a sustainable supply of raw materials for drug and pharmaceutical industries. Taking the importance of such understandings into account, in this investigation, we utilized molecular markers, ISSR to assess the genetic variability and population structure in 16 natural populations of *E. major* collected from western, northwestern, central Alborz and northeast of Iran covering highly heterogeneous environments. The generated molecular data indicated a significant level of diversity at the population level where the degree of genetic diversity varied significantly among populations possibly owing to the location of collected specimens from populations since a number of environmental and anthropological factors (e.g., excessive animal grazing and cutting shrubs) may endanger the natural generation process. Additionally, stems of *E. major* are valuable sources of medicinal constitutes (e.g. ephedrine), thus the risk of overexploitation of its populations by local inhabitants is notably high, as it has occurred in *E. gerardiana* L. (Pant *et al*. 2006, Bhatti & Vashishtha 2008). In a study on the genetic diversity of endangered *E. gerardiana* employing ISSR, Meena *et al*. (2016) unmasked a significant level of polymorphism among populations (P = 62.43%, H = 0.22, I = 0.33).

Analogously, genetic diversity in *Ephedra foliata* observed to be high (P = 49.44%, H = 0.18, I = 0.26) (Meena *et al*. 2016). In this study, the genetic diversity parameters (P = 67.96%, H = 0.35, I = 0.23), except for I, were higher than previous reports on *Ephedra* species. In consistence with our results, high genetic diversity using ISSR has been reported in many other gymnosperms (Liu *et al*. 2013, Wang *et al*. 2016, Chen *et al*. 2017, Semerdjieva *et al*. 2020) analyzed using ISSR markers.

Having such a high level of genetic diversity may have conferred significant environmental adaptability to *E. major* (Zhu *et al*. 2013) that can be located in heterogeneous geography. Gymnosperms mainly reported having high degrees of genetic diversity with a low level of population differentiation (Hamrick *et al*. 1992). Nonetheless, *Gnetum parvifolium* (Warb.) C.Y. Cheng ex Chun, a close relative of *Ephedra*, exhibited significant of genetic diversity and moderate intra-population genetic differentiation (Huang *et al*. 2010), which is predictable outcomes for perennial woody, relatively high longevity, outcrossing, and wind-pollinated species (Hamrick *et al*. 1992, Yang *et al*. 2019). In addition to environmental factors, characteristics of mating system, growth habit, dispersal strategies of pollen and seed can indeed influence genetic diversity in a species (Sun 1996, Ohsawa *et al*. 2008). The high level of genetic diversity in some populations of this study, Dehbar and Bojnord may reflect their small size and the fact that the decrement of population size has recently taken place. On the other hand, it has been suggested that an intact large population may have a higher level of genetic diversity as it was observed in populations of *E. foliata* (Meena *et al*. 2016). Gymnosperms are majorly relying on wind for pollination, whereas some are insect-pollinated, and *Ephedra* (e.g. *E. major*) uses both wind and insect for pollination (Rydin & Bolinder 2015, Semerdjieva *et al*. 2020).

In other species of *Ephedra*; *E. foliata*, an unbalanced ratio between male over female plants has been reported therefore when chances for the appropriate seed setting is low, efficient seed production could be challenging (Singh 2004). Comprehensive field and genetic investigations to generate reliable information on the mating system of *E. major* are needed, since, for example, embryo abortion and therefore empty seed formation in conifer species are common owing to inbreeding depression (Williams & Savolainen 1996, Bower *et al*. 2007).

In the present study, within-population genetic diversity was significantly higher than among population genetic diversity which indicates that, genetic diversity mainly is preserved within populations and it's the major source of variation. High similarity among populations than within individuals can reflect that individuals formed the population had a high degree of inter-population gene flow (Huang *et al*. 2014, Zhang *et al*. 2015, Zhao *et al*. 2017). High intra-population genetic diversity in *E. major* could be caused by its dependence on wind and insects for pollination, out-crossing, and pollen exchange among various populations. Generally, in on vegetative propagation (Posselt 2010, Shi *et al*. 2008). A higher percentage of intra-population genetic diversity in this study also can suggest that, plants from different had little, if none, gene exchange, while plants in one area had high gene exchange causing high intra-population genetic diversity against low inter-population genetic diversity. Using the clustering method, UPGMA, revealed the existence of relatively obvious geographical pattern among natural populations where individuals mainly placed in population and populations with close geographical distance clustered together. This pattern can mirror the critical role of environmental variables in shaping the genetic diversity of populations. Additionally, several other factors such as geographical limitation, location-specific environmental properties, habitat fragmentation, and erosion, can be responsible for this genetic differentiation (Tomimatsu & Ohara 2003, Oostermeijer & De Knegt 2004). While Bayesian clustering indicated a high admix population, however, to some extent, populations based on the geographical origin clustered together (Dhir & Singhvi 2012). Gene

flow is a chief determinate in genetic diversity among and within populations that can be affected by geographical limitations, the information on gene flow in *E. major* populations can greatly contribute to improving our understanding of *E. major* species in Iran.

The high genetic diversity and relatively high genetic differentiation in natural populations of *E. major* all indicate the high adaptability of this species to a wide spectrum of climatic ranges. Additionally, the clustering of populations similar to their geological origin is another evidence for the dominant effect of climatic variables on controlling the distribution of *E. major* populations. Significant intra-population genetic diversity also reflects the influence of high out-crossing nature and sexual propagation of this species. The revealed admixed population structure call for more research on gene flow among these populations of *E. major*. This study seems to be the first attempt on using molecular markers, ISSR analyses genetic diversity, and population structure. To further unmask the underlying relationships among populations of *E. major* in Iran, utilizing more ISSR and increasing the number of the sampled population are highly recommended.

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