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Population genetic structure and diversity of *Teucrium polium* in Iran using ISSR markers

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Abstract

Teucrium polium is a Mediterranean native subshrub with large distribution range and high medicinal values which overexploitation of its population has become a serious danger and conservation attempts is needed. Having the genetic information of its population can help to clarify the complicated phylogenetic relationships of species within the sections, genetic conservation and establishing breeding programs to develop cultivars and ultimately preventing declining the populations of *T. polium*. The genetic diversity parameters and populations structures of 16 populations of *T. polium* at the local scale in the Alborz mountain range of Iran were assessed using inter-simple sequence repeats (ISSR) primers. The averages of polymorphism (P%), Nei's genetic diversity (*H*), and Shannon's Information Index (*I*) were 33.24%, 0.118 and 0.179, respectively. The population of Asara to Gach-Sar presented the highest P%; 43.28%, *H*; 0.163 and *I*; 0.243. AMOVA analysis indicated that, a large portion of genetic variation as within population (77%), and a relatively high genetic differentiation (*Gst*: 0.311) and gene flow (*Nm*: 1.107) among populations were observed. UPGMA tree and PCoA plot of ISSR data divided the populations into three genetic groups to a significant extent based on the geographical origins. Similarly, the results showed that, STRUCTURE analysis grouped the populations into three clusters with significant geographical affinity. *Teucrium polium* exhibited a strong structure and genetic differentiation with low to moderate genetic diversity.

Keywords: Genetic differentiation, genetic diversity, *Lamiaceae*, outcrossing STRUCTURE analysis

ROSTANIHA

ساختار ژنتیکی جمعیتی و تنوع *Teucrium polium* در ایران با استفاده از نشانگرهای ISSR*

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خلاصه

Teucrium polium L. متعلق به تیره نعناییان (*Lamiaceae*)، درختچه‌ای کوچک، بومی مدیترانه، با دامنه گسترش وسیع و ارزش دارویی بالاست که برداشت بی‌رویه آن از جمعیت‌های طبیعی تبدیل به تهدید جدی گردیده و نیازمند اقدامات محافظتی می‌باشد. داشتن اطلاعات ژنتیکی جمعیت‌های آن می‌تواند برای روشن شدن روابط پیچیده فیلوژنتیک گونه‌ها درون بخش‌ها، حفاظت از منابع ژنتیکی و ایجاد برنامه اصلاحی جهت توسعه ارقام و نهایتا جلوگیری از کاهش جمعیت‌های *T. polium* استفاده گردد. پارامترهای تنوع ژنتیکی و ساختار جمعیتی ۱۶ جمعیت *T. polium* در سطح محلی در رشته کوه البرز ایران با استفاده از نشانگر مولکولی ISSR مورد بررسی قرار گرفت. میانگین‌های درصد چندریختی (P%)، ضریب تنوع نی (*H*) و ضریب شاخص شانون (*I*) به ترتیب ۳۳/۲۴، ۰/۱۱۸ و ۰/۱۷۹ بود. جمعیت آسارا به گچسر بالاترین مقادیر درصد چندریختی، ضریب تنوع نی و ضریب شاخص شانون به ترتیب ۴۳/۲۸، ۰/۱۶۳ و ۰/۲۴۳ را نشان داد. تجزیه و تحلیل واریانس مولکولی (AMOVA) سطح بالایی از تنوع ژنتیکی درون جمعیت‌ها (۷۷٪) را نشان داد و تمایز ژنتیکی نسبتا بالا (*Gst*: ۰/۳۱۱) و جریان ژنی (*Nm*: ۱/۱۰۷) در بین جمعیت‌ها مشاهده شد. نتایج تحقیق حاضر همچنین نشان داد که تجزیه و تحلیل درخت UPGMA و نقشه PCoA از داده‌های ISSR جمعیت‌های عمدتا مرتبط با منشا جغرافیایی را می‌توان به سه گروه ژنتیکی تقسیم کرد. ساختار جمعیتی و تمایز ژنتیکی قوی با سطح تنوع ژنتیکی متوسط به پایین در *T. polium* مشاهده گردید.

واژه‌های کلیدی: آنالیز ساختار، دگرگشنی، تمایز ژنتیکی، تنوع ژنتیکی، نعناییان

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Introduction

The genus *Teucrium* L. (*Lamiaceae*; subfam. *Ajugoideae*) has 240–300 species worldwide grouped into nine sections, half of them belonging to *T.* sect. *Polium* (Mill). Schreb. (Tutin *et al.* 1972, Navarro & El Oualidi 2000, Harley *et al.* 2004, Govaerts & Faden 2016). The basic sectional arrangement of the genus is mostly relies on morphological traits such as the calyx and inflorescence types with varying characteristics (Abdollahi *et al.* 2003, Salmaki 2017). Jamzad (2012) reported 12 *Teucrium* species from Iran, five of them from the section *Polium. Teucrium polium* L. is the most important and taxonomically complicated species of the *T.* sect. *Polium* subsect. *Polium.* It is a Mediterranean native sub-shrub outbreeding species with wide-distribution range in steppe, arid, and semi-desert areas of Iran (Boulila *et al.* 2010, Eshratifar *et al.* 2011), has leaves with crenate margin and short petioles, and white to pinkish white flowers entirely covered by trichomes (Navarro & El Oualidi 2000, El Oualidi *et al.* 2002). *Teucrium polium* is markedly diverse, both morphologically and ploidally. Having an outcrossing mating system has facilitated the generation of a high rate of hybridization and polyploidy in *T. polium*, which has significantly contributed to the ambiguity of sectional and interspecies relationships at the phylogenetic level (Puech 1990, El Oualidi *et al.* 2002, Soltis *et al.* 2016).

Teucrium polium has traditionally been utilized as an important medicinal herb for its diuretic, tonic, antipyretic, antispasmodic, antifungal, antirheumatic, and antioxidant, carminative and antibacterial activities. It has been reported to be an effective remedy for intestinal and gastrointestinal issues and lacerations, in addition to its influence on regulation insulin in diabetic patients (Bahramikia & Yazdanparast 2012, Djabou *et al.* 2012, Bukhari *et al.* 2015). In traditional Iranian medicine, using *T. polium* as tea has been advised to treat several disorders including stomach pain, digestion issues, flu and insulin-dependent type II diabetes. Therefore, during the last decades a large body of scientific reports have indicated and confirmed the presence of above mentioned medicinal values (Rizk *et al.* 1986, Iriadam *et al.* 2006, Bahramikia *et al.* 2009, Stankovic *et al.* 2012, Hassan 2017). Due to its wide application in Iran, particularly in the rural communities, preclinical studies to evaluate its possible negative effects in high dosage have indicated that, care should be taken in application quantity (Rafieian-Kopaei *et al.* 2014).

The species is declared as "Least Concern" regarding the IUCN (2001) criteria, which means it has thriving populations with a wide spectrum of distributional range. But, being an important source for traditional medicine can jeopardized *T. polium* populations (Bahramikia *et al.* 2009). Since there is no commercial cultivation for most of the medicinal herbs, natural populations are the sole source to fulfill the increasing demand by herbal practitioners and native people; hence many of the natural population of valuable medicinal plants are endangered in the recent decades owing over the collection as well as habitat destruction by the expansion of cities. Additionally, overgrazing of live stocks is another important source of threat (Sheibani *et al.* 2018, Bakhshipour *et al.* 2019, Mafakheri & Kordrostami 2020). Thus, *T. polium* is not an exception and extensive use of its natural populations in the short-run causes decline in population size and eventually might become extinct. Thus preliminary studies are required to provide insight information on population status and level of genetic diversity to take necessary conservation measures.

Given the broad distribution of *T. polium* species over a variety of environments may be responsible for broad phenotypic/genotypic tolerances or to have evolved local and differential genotypic accommodations to each habitat variant (Mayer *et al.* 1994, Boyd *et al.* 2009). Therefore, morphological characteristics are potent tools to improve the resolution at the section level to generate reliable information on natural plant populations. DNA-based molecular markers have proven to be a potent tool that can serve scholars to understand the backbone and ongoing conditions of populations. Several molecular markers are available to depend on the theme of the study;

ROSTANIHA

for instance, inter simple sequence repeats (ISSRs) does not require prior information on the species of interest besides being significantly cost-efficient and easy to use with high polymorphisms, repeatability, and abundance throughout the genome (Godwin *et al.* 1997, Wang 2002, Souframanien *et al.* 2004, Vijayan 2005, Jugran *et al.* 2013). ISSRs have been applied to assess various aspects of genetic diversity and population structure in wild population either at the section level to delaminate species or study variation within and among the population, innumerably, for instance, ISSR in a combination of morphological data was used to evaluate genetic diversity of declining populations of *Humulus lupulus* L. (Mafakheri *et al.* 2020), genetic diversity in populations *Stylosanthes scabra* Vogel. (Costa *et al.* 2018), *Mallotus oblongifolius* Miq. (Yan, W. *et al.* 2019), Coffee germplasm (Yan, L. *et al.* 2019), *Rhododendron triflorum* Hook. f. (Xu *et al.* 2017), and *Chamomilla recutita* L. (OkoĚ *et al*. 2013), just to name a few. Application of PCR-based markers on *T. polium* except for Norouzi Ghare Tapeh *et al.* (2018) who employed ISSR to investigate genetic variation and structure of eight population, the other studies on genetic diversity in *T. polium* populations in Tunisia (Boulila *et al.* 2010), Iran (Pesaraklu *et al.* 2013), and Jordan (Al-Rawashdeh 2015) involved random amplified polymorphic DNA (RAPD). Additionally, there is one report on using chloroplast and nuclear internal transcribed spacer (ITS) regions to assess genetic variation among natural populations of two subspecies of *T. polium* (Djabou *et al.* 2012).

Given the importance of *T. polium* from a medicinal perspective and usefulness of genetic diversity on its populations for not only conservation purposes but also for establish and develop breeding programs as well as help in clarifying the systematic position of section *polium*, here we aimed to conduct a comprehensive study on genetic diversity and population structure of 16 populations of *T. polium* in north west, north and north east of Iran using ISSR markers. Also, despite of the previous studies on *T. polium* populations in Iran, there is the absence of comprehensive study to relatively high number of populations which we tried to fulfil in this investigation by collecting high number of populations.

Materials and Methods

- Plant materials and DNA extraction

After an extensive survey through the east, west, and central Alborz regions, a total of 16 populations of *Teucrium polium* were located during June 2017 (Table 1). A fixed number of six individuals were taken from each population, and overall 96 individuals were collected. Leave samples from populations were silica gel-dried for molecular study. Voucher specimens of each population, are kept in the Herbarium of Islamic Azad University, Tehran, Iran (IAUH) (Table 1).

ROSTANIHA

Table 1. Sampling locality, voucher number along with related data of the studied *Teucrium polium* populations in Iran

Locality	Longitude	Latitude	Altitude (m)	Collector	Voucher No.
Khorassan (N) prov.: Ashkhaneh, 22 km from Torghabeh to Ashkhaneh	56° 0.31′	37° 20.35′	978	Mohajer Tabrizi	IAUH00 0015169
Khorassan (RZ) prov.: Ghochan, 46 km from Ghochan to Dargaz	58° 33.38′	37° 25.9′	1850	Mohajer Tabrizi	IAUH00 0015170
Khorassan (RZ) prov.: Mashhad, Dehgheybi	59 ° 29.55′	36° 5.5′	1570	Mohajer Tabrizi	IAUH00 0015171
Semnan prov.: Shahrood, Mojen	54° 39.56′	36° 27.36′	2113	Mohajer Tabrizi	IAUH00 0015172
Golestan prov.: Gorgan, Tuskastan forest,Saraliabad	54° 43.2′	36° 47.30′	758	Mohajer Tabrizi	IAUH00 0015173
Mazandaran prov.: Kiasar, Koard Mir	53° 43.36′	36° 14.38′	1705	Mohajer Tabrizi	IAUH00 0015174

Table 1 (contd)

Alborz prov.: Karaj, 12 km from Asara to Gach-Sar	51˚ 18.6′	36˚ 1.16′	2057	Mohajer Tabrizi	IAUH00 0015175
Mazandaran prov.: Kelardasht, Rudbarak	51˚ 7.51′	36˚ 28.32′	1633	Mohajer Tabrizi	IAUH00 0015176
Mazandaran prov.: Nur, Galand Rud	51˚ 54.34′	36˚ 26.32′	663	Mohajer Tabrizi	IAUH00 0015177
Mazandaran prov.: Nowshahr, Chenarbon	51˚ 40.52′	36˚ 23.24′	1616	Mohajer Tabrizi	IAUH00 0015178
Tehran prov.: Gilavand, 10 km from Rudehen to Gilavand	51˚ 59.40′	35˚ 41.51′	2018	Mohajer Tabrizi	IAUH00 0015179
Tehran prov.: Firozkuh, Gadok	52˚ 55.37′	35˚ 49.56′	2190	Mohajer Tabrizi	IAUH00 0015180
Qazvin prov.: Abyek, Aghchari	50˚ 35.6′	36˚ 7.1′	1805	Mohajer Tabrizi	IAUH00 0015181
Zanjan prov.: Abbar, 9 km from Badamestan to Abhar	48˚ 51.35′	36˚ 46.38′	2045	Mohajer Tabrizi	IAUH00 0015182
Ardabil prov.: Khalkhal, 12 km from Khalkhal to Asalem	48˚ 36.3′	37˚ 35.12′	2042	Mohajer Tabrizi	IAUH00 0015183
Ardabil prov.: Ardabil, 28 km from Ardabil to Germi	48˚ 13.25′	38˚ 29.42′	1577	Mohajer Tabrizi	IAUH00 0015184

- DNA extraction and ISSR-PCR

Genomic material of silica gel-dried leaves taken from populations was extracted using mini plants kits (Zofagen, Germany) (Doyle 1987). To examine the extracted DNA for quality, spectrophotometer, and for quantity, 1% agarose gel electrophoresis was utilized. Using eight ISSR primers, initial testing was carried out which all of them viz. $(CAA)_5$, $(AGA\ GAG)_2AGAGT$, $(ACA\ CAC)_2ACACT$, $(CAC\ ACA)_2GC$, $(GACA)_4$, $(AGA\ GAG)_2AGAGT$, $(ACA\ CAC)_2ACACYT$ and $(CAC\ .ACA)_2CACARG$ (Biolegio, Netherland) (Abd El-Hady *et al.* 2010, Agarwal *et al.* 2015) were provided consistent binding. Polymerase chain reaction (PCR) was carried out in a final volume of 13 µl per reaction composed of 6.5 µl master mix, 4.75 µl double distilled water, 0.75 µl extracted DNA, 0.5 µl of each primer, and 0.5 µl DMSO. PCR reaction was performed by a LabCycler Basic thermocycler (Sensoquest, Göttingen, Germany) at 5 min of initial denaturation at 94 ˚C, afterward, 35 cycles of 40 s at 94 ˚C, 1 min annealing with different temperatures for primers (37.8 ˚C, 48.1 ˚C, 47 ˚C, and 42.1 ˚C), and final expansion 1 min at 72 ˚C. The reaction was completed with the final extension step: 7 min at 72 ˚C. By using 1% Agarose gel, the success of PCR reaction was confirmed. A mixture of fluorescent dyes (Fam, NED, PET, and VIC) was made and used for labeling products to make the identification possible on ABI 3730 capillary system with the internal size standard of GeneScan ROX 500 (applied genetic).

- Data analysis

The outcome data of ISSR markers collected and aligned with the aim of GeneMarker Ver. 1.95 (GeneMarker, SoftGenetics, State College, Pennsylvania). Only reproducible and completely clear bands were chosen and manually scored by binary coding symbols; present (1) or absent (0). Then, to ensure the presence of peaks, each of the specimens was tested using > 200 signal intensity. Also, in each sample, 3 replicate were considered to prove the fidelity of peaks. The genetic diversity indices including the percentage of polymorphic loci (P%), number of different allels (*Na*), number of effective alleles (*Ne*), number of private bands (*Np*), Nei's gene diversity (*H*), Shannon information index (*I*), total gene diversity (*Ht*), gene diversity within populations (*Hs*), and Nei's genetic identity (Nei's *I*) obtained by employing GenAlEx 6.5 (Peakall & Smouse 2006) and coefficient of genetic differentiation (*Gst* = (*Ht-Hs*)/Ht), gene flow (*Nm*) and were acquired utilizing POPGENE

ROSTANIHA

1.32 software (Kimura & Crow 1964, Nei 1973, Nei 1978, Loveless *et al.* 1984, Sherwin *et al.* 2006).

Using GenAlEx 6.5 (Peakall & Smouse 2006), the test for hierarchical analysis of molecular variance (AMOVA) assessing the interpopulation and intrapopulation distribution of genetic variation. The same program was used to perform the principal coordinate analysis (PCoA). Further analysis was carried out to study the genetic relationship between populations, UPGMA phylogenetic tree built based on Nei's genetic distance to determine population genetic structure using PAST Ver. 4.03 software (Hammer *et al.* 2001). The genetic structure of populations and individuals was evaluated based on Bayesian analysis model as implemented in STRUCTURE Ver. 2.3. (Pritchard *et al.* 2000) used to identify the proper number of population genetic clusters (k), and individuals from each of the assumed populations to each of the genetic clusters. Ten independent replicates ran for k 1–8. A burn-in period of 25,000 initiated per ran and a Markov Chain Monte Carlo (MCMC) replication number set to 50,000. Evanno method was used to determine the most likely number of 'k' based on DK (Evanno *et al.* 2005).

Results

- DNA marker polymorphism

The average number of bands (*NB*) per population was 49.06, which maximum *NB* observed in the population of Mojen (65), while both populations of Germi and Gadok showed the minimum (34). The Aghchari population exhibited the highest number of private bands (*Np*: 6) where the mean Np of the populations was 2.06, and several populations presented the absence of *Np* (Saraliabad, Kord Mir, and Gadok).

- Genetic diversity and differentiation

Genetic diversity statistics calculated based on information acquired from 95 individuals of 16 populations of *T. polium* were relatively varied (Table 2). The percentage of polymorphic loci (P%) ranged from 19.40% (Dehgheybi) to 43.28% (Gach-Sar) with an average of 33.40% at the population level. The average values of the observed number of different alleles (*Na*) and the effective number of alleles (*Ne*) among the population were 0.724 and 1.195, respectively. The Gach-Sar populations observed to have the maximum values of Na and Ne (0.881 and 1.28); however, the population of Aghchari indicated the same *Ne*.

A relatively static pattern among the population regarding genetic diversity parameters did appear, since the Gach-Sar population with superior values for P%, *Na* and *Ne* had the highest values of Shannon's information index (*I*: 0.243) and Nei's genetic diversity (*H*:0.163), whereas the averages of the two parameters at the population level were 0.179 and 0.118. The Population of Dehgheybi represented the lowest values for *I* and *H* (0.107 and 0.071); the same applies for Unbiased Nei's gene diversity (*uHe*) in this population (0.86). The Gach-Sar population, with 0.196 indicated the highest value of *uHe* among populations (Table 2).

ROSTANIHA

Table 2. Genetic diversity parameters of 16 studied populations of *Teucrium polium* using ISSR markers

Population	NB	Np	P%	Na	Ne	I	H	uHe
Pop 1	51	2	31.34	0.694	1.185	0.169	0.112	0.135
Pop 2	55	4	30.60	0.716	1.167	0.159	0.104	0.125
Pop 3	35	1	19.40	0.455	1.121	0.107	0.071	0.086
Pop 4	65	2	43.10	0.94	1.255	0.24	0.158	0.189
Pop 5	59	0	41.04	0.851	1.239	0.219	0.145	0.174
Pop 6	46	0	32.09	0.664	1.174	0.167	0.109	0.131
Pop 7	60	3	43.28	0.881	1.28	0.243	0.163	0.196
Pop 8	57	1	42.54	0.851	1.244	0.226	0.149	0.179
Pop 9	48	3	32.84	0.687	1.219	0.187	0.127	0.152
Pop 10	40	3	28.36	0.582	1.172	0.154	0.103	0.123
Pop 11	44	1	32.84	0.657	1.207	0.183	0.123	0.147
Pop 12	34	0	22.39	0.478	1.126	0.118	0.078	0.094
Pop 13	61	6	42.54	0.881	1.219	0.216	0.14	0.168
Pop 14	57	4	41.04	0.836	1.227	0.215	0.141	0.169

Table 2 (contd)

Pop 15	39	2	27.61	0.567	1.159	0.146	0.097	0.116
Pop 16	34	1	20.90	0.463	1.129	0.116	0.078	0.097
Mean	49.06	2.06	33.24	0.700	1.195	0.179	0.118	0.142

Number of bands (*NB*), number of private bands (*Np*), percentage of polymorphic loci (P%), number of different allels (*Na*), number of effective alleles (*Ne*), Nei's gene diversity (*H*), Shannon information index (*I*), Unbiased Nei's gene diversity (*uHe*). Pop.: Population code; Pop 1, Ashkhaneh; Pop 2, Ghochan; Pop 3, Dehgheybi; Pop 4, Mojen; Pop 5, Tuskestan forests to Saraliabad; Pop 6, Kord Mir; Pop 7, Asara to Gach-Sar; Pop 8, Rudbarak; Pop 9, Galand Rud; Pop 10, Chenarbon; Pop 11, Gilavand; Pop 12, Gadok; Pop 13, Aghchari; Pop 14, Abhar; Pop 15, Khalkhal-Asalem; Pop 16, Ardebil to Germi.

The results of further analysis on genetic indices at the species level are given in table 3. Averaging total gene diversity (*Ht*) for *T. polium* populations was 0.132. The value for intrapopulation gene diversity (*Hs*) was 0.091, which is more than 50% of the *Ht* (Table 4). The parameters of the coefficient of genetic differentiation (*Gst*) and gene flow (*Nm*) with 0.311 and 1.107, respectively, were exhibited higher than average values since *Gst* between 0.05–0.15 is defined as moderate and *Gst* values above 0.30 as high (Boulila *et al.* 2010). Thus *Gst* reflected the presence of an extreme genetic differentiation among the population. The average gene flow >1 reflects the high gene flow (Xu *et al.* 2017). Similar to Hs, AMOVA analysis (Fig. 1) indicated the major distribution of total genetic diversity in within-population (77%) while the share of among the population (15%) and among regions (8%) was notably lower. Unlike Gst, AMOVA results revealed the a weak genetic differentiation among populations (P, 0.001, PhiPT = 0.229). To assess the level of genetic similarity among populations, Nei's genetic identity (Nei's *I*) for each pair of populations was calculated (Table 4). This value varies between minimum of 0.0 (no genetic similarity) to a maximum 1.0 (complete genetic similarity) (Nei 1973). The Nei's *I* parameter among the population was significantly high with min. 0.853 (Germi/Ghochan) and max. 0.977 (Gadok/Kord Mir), indicating that populations are highly genetically similar.

Table 3. Genetic diversity and differentiation parameters at the population level for 16 populations of *Teucrium polium*

Ht	Hs	Gst	Nm
0.132	0.091	0.311	1.107

Total gene diversity (*Ht*), gene diversity within populations (*Hs*), coefficient of genetic differentiation (*Gst* = (*Ht*-*Hs*)/*Ht*), gene flow (*Nm*).

Table 4. Average values of Nei's genetic identities (Nei's *I*) between pairs of 16 populations of *Teucrium polium*. The means obtained from all the pairwise comparisons for a particular population

Pop	ASH	GHO	DGH	MJN	TSK	KRD	ASA	RUB	GLR	CHB	GIV	GDK	AGH	ABR	KHL	ARD
Pop 1																
Pop 2	0.939															
Pop 3	0.958	0.939														
Pop 4	0.934	0.882	0.900													
Pop 5	0.954	0.901	0.925	0.933												
Pop 6	0.926	0.877	0.905	0.958	0.942											
Pop 7	0.925	0.891	0.903	0.920	0.912	0.913										
Pop 8	0.930	0.886	0.922	0.938	0.941	0.963	0.920									
Pop 9	0.919	0.886	0.923	0.917	0.942	0.932	0.907	0.957								
Pop 10	0.914	0.871	0.905	0.919	0.925	0.954	0.922	0.969	0.957							
Pop 11	0.910	0.863	0.910	0.933	0.929	0.946	0.920	0.960	0.936	0.943						
Pop 12	0.933	0.880	0.909	0.951	0.949	0.977	0.915	0.975	0.943	0.950	0.965					
Pop 13	0.924	0.863	0.911	0.915	0.932	0.929	0.942	0.938	0.922	0.940	0.949	0.942				
Pop 14	0.932	0.889	0.931	0.937	0.947	0.955	0.934	0.967	0.955	0.956	0.969	0.969	0.955			
Pop 15	0.917	0.873	0.926	0.912	0.934	0.939	0.928	0.958	0.943	0.962	0.955	0.944	0.955	0.966		
Pop 16	0.905	0.853	0.906	0.902	0.921	0.929	0.906	0.954	0.936	0.969	0.934	0.935	0.934	0.949	0.971	

Pop.: Population code; Pop 1, ASH: Ashkhaneh; Pop 2, GHO: Ghochan; Pop 3, DGH: Dehgheybi; Pop 4, MJN: Mojen; Pop 5, TSK: Tuskestan forests to Saraliabad; Pop 6,KRD: Kord Mir; Pop 7,ASA: Asara to Gach-Sar; Pop 8, RUB: Rudbarak; Pop 9, GLR: Galand Rud; Pop 10, CHB: Chenarbon; Pop 11, GIV: Gilavand; Pop 12, GDK: Gadok; Pop 13, AGH: Aghchari; Pop 14, ABR: Abhar; Pop 15, KHL: Khalkhal-Asalem; Pop 16, ARD: Ardebil to Germi.

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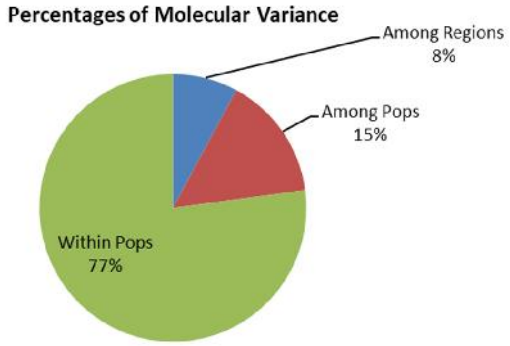

Fig. 1. AMOVA test showing the percentage of molecular diversity in *Teucrium polium.*

- Population genetic structure

The clustering analysis method, the unweighted paired-grouping method with arithmetic averages (UPGMA) used to assess the genetic relationship among populations and divided them into four clusters (Fig. 2). Cluster I, as the largest one, encompassed eight populations group according to their geographical origins, populations from Aghchari, Abhar and Gach-Sar in sub-group 1, Gilavand and Saraliabad populations in sub-group 2, and Kord Mir and Gadok populations in sub-group 3, and the Mojen population alone placed in the fourth sub-group. The cluster II, encompassed four populations of Khalkhal-Asalem, Germi, Chenarbon, and Rudbarak. Populations from the far northeast of central Alborz: Ashkhaneh, Ghochan, and Dehgheybi placed in cluster III. Ultimately, cluster IV, contained only one population, Galand Rud. Overall, the populations are mainly grouped based on geographical closeness. The genetic relationship of the populations also evaluated utilizing principal co-ordinate analysis (PcoA) which the generated plot (Fig. 3) was able to position in three relatively distinct groups (I: the central Alborz, II: the northwest, III: the northeast) similar to UPGMA close to their physical locations.

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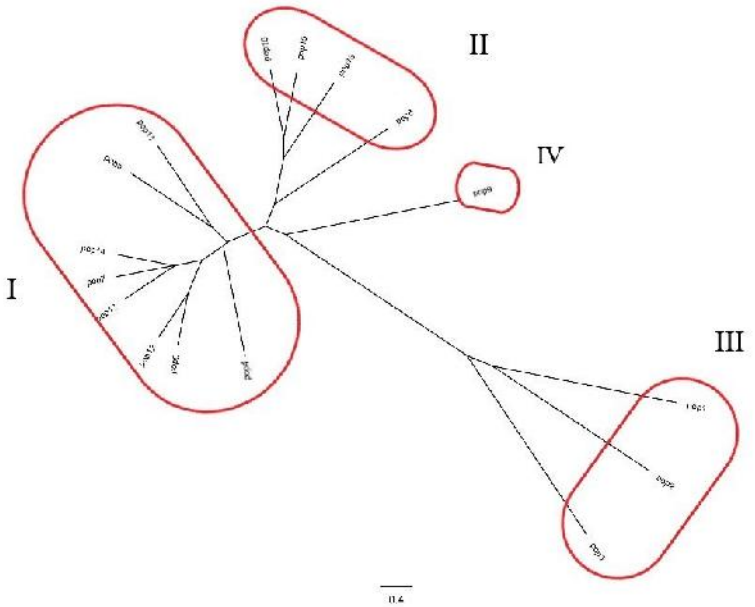

Fig. 2. UPGMA tree of 16 populations of *Teucrium polium* based on Nei's genetic distance. Pop.: population code; Pop. 1, Ashkhaneh; Pop. 2, Ghochan; Pop. 3, Dehgheybi; Pop. 4, Mojen; Pop. 5, Tuskestan forests to Saraliabad; Pop. 6, Kord Mir; Pop. 7, Asara to Gach-Sar; Pop. 8, Rudbarak; Pop. 9, Galand Rud; Pop. 10, Chenarbon; Pop. 11, Gilavand; Pop. 12, Gadok; Pop. 13, Aghchari; Pop. 14, Abhar; Pop. 15, Khalkhal-Asalem; Pop. 16, Ardebil to Germi.

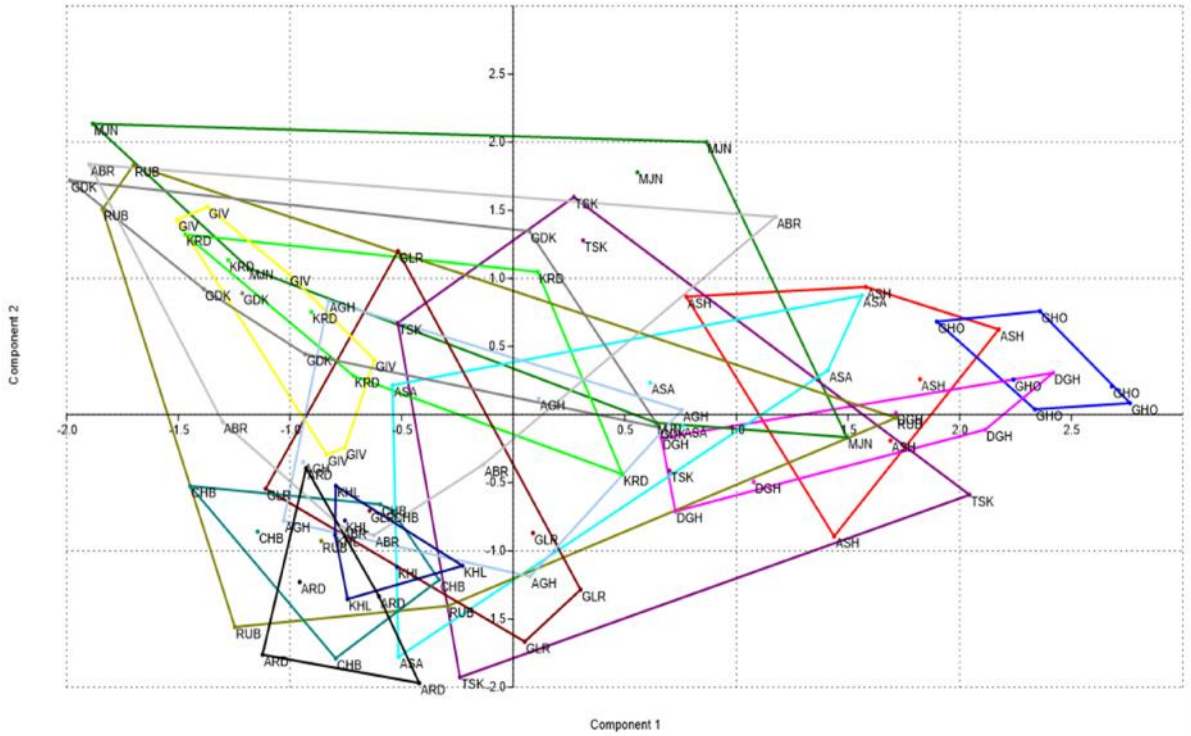

Fig. 3. Principal co-ordinate analysis of 16 *Teucrium polium* populations studied using ISSR. Pop.: population code; ASH, Ashkhaneh; GHO, Ghochan; DGH, Dehgheybi; MJN, Mojen; TSK, Tuskastan forest,Saraliabad; KRD, Kord Mir; ASA, Asara to Gach-Sar; RUB, Rudbarak; GLR, Galand Rud; CHB, Chenarbon; GIV, Gilavand; GDK, Gadok; AGH, Aghchari; ABR, Abhar; KHL, Khalkhal-Asalem; ARD, Ardabil to Germi.

To confirm the accuracy of the clustering pattern STRUCTURE analysis was performed. The optimal number of genetic groups of the populations found to be k=3, which was obtained using STRUCTURE analysis and the Evanno test to detect the actual number of genetic groups (Fig. 4). The generated plot indicated significant geographical affiliation among individuals, in which to a large extent, by increasing geographical distance of populations from central Alborz, the homogeneity of populations increased, for instance, populations of the far northeast populations (Ashkhaneh, Ghochan, and Dehgheybi) and populations from northwest and north (Rudbarak, Galand Rud, Asalem, Ardabil to Germi) observed to have the least shared alleles. Although populations of central Alborz and its eastside were notable genetically admixture populations of Kord Mir, Gilavand, and Gadok presented the dominance of the third genetic group. In consistent with UPGMA, the STRUCTURE was successful in dividing populations in to three groups close to their physical locations. The considerably genetically mixed populations of central Alborz can explain the high gene flow (1.107) exists between populations.

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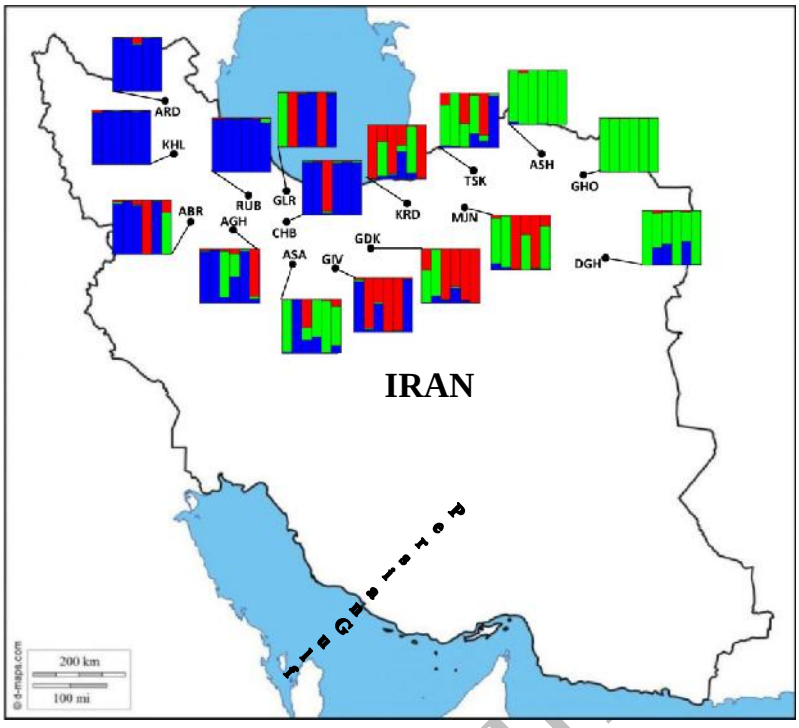

Fig. 4. STRUCTURE plot of *Teucrium polium* populations based on the genetic information generated by ISSR markers. Pop.: population code, ASH, Ashkhaneh; GHO, Ghochan; DGH, Dehgheybi; MJN, Mojen; TSK, Tuskastan forest,Saraliabad; KRD, Kord Mir; ASA, Asara to Gach-Sar; RUB, Rudbarak; GLR, Galand Rud; CHB, Chenarbon; GIV, Gilavand; GDK, Gadok; AGH, Aghchari; ABR, Abhar; KHL, Khalkhal-Asalem; ARD, Ardabil to Germi.

POSTAMI

Discussion

- Genetic variation

The DNA markers (ISSR) utilized in this study revealed a relatively high polymorphism (P%) and genetic diversity (*H*) among populations of *T. polium* (33.24% and 0.132, respectively), that were lower than the level previously reported by Boulila *et al.* (2010) in Tunisian *T. polium* populations (46.19%). Investigating the genetic diversity of *Teucrium* L. species in Libya using ISSR and RAPD markers Marzouk and El-Badan (2018) observed the highest P% (32%) in *T. polium* and *T. fruticans*. Sözen *et al.* (2017) reported higher H (0.263) in *T. leucophyllum*. A similar study on eight *T. polium* populations in Iran revealed a considerably higher total genetic diversity (*Ht*) 0.352 versus 0.132 in our study (Norouzi Ghare Tapeh *et al.* 2018). The outcomes of this research indicate a moderate degree of genetic diversity among populations of *T. polium* in Iran. It should be noted that none of the above described studies had equal or higher population numbers.

The strength of a given species in dealing with different intensity of environmental changes mainly tied to genetic diversity that determines its survival and evolutionary capabilities (Huenneke & plants 1991, Hughes *et al.* 2008). The genetic variation is the consequence of a long evolutionary process and mirrors the adaptability of a plant species. Quite a few factors can influence the level of genetic diversity, namely mating system, life-history traits (reproduction methods, lifespan, seed dispersal, and quantity of produced seeds and size), and gene flow ((Nybom *et al.* 2000, Guo *et al.* 2016). Perennial plant species comparatively due to having a longer lifespan and those with the outcrossing breeding system compared to self-pollinating or asexually propagating ones often maintain higher degree of genetic variation (Hamrick & Godt 1996, Barrière & Félix 2005, Mable & Adam 2007, Vandepitte *et al.* 2010). *T. polium* is not only outcrossing but also a perennial plant species; however, its important life history characteristics did not reflect its low-moderate genetic diversity among the population in Alborz Mountain as it was asserted from this

study. Given the fact that natural populations of this species is medicinally highly valuable with significant economic value and consumption, quantity is the major, if not only source; it can be speculated that over collection of natural populations is the possible responsible of weak genetic diversity. Extrinsic factors, in particular over the collection of plant species, have been repeatedly reported as the main cause of genetic diversity reduction in various edible or medicinal herbs (Moustafa *et al.* 2015, Blambert *et al.* 2016, Ramírez-Rodríguez & Amich 2017). Excessive collection of a specific species from its populations leads to a reduction in density and abundance that increases the physical space between individuals and, consequently, smaller population size that may lower the gene flow and genetic variation (Young *et al.* 1996, Aguilar *et al.* 2008). Nonetheless the negative effect of overharvesting on population is highly species-specific, and the type of plant material interest. In the case of *T. polium*, the collection approach can be significantly damaging, particularly if the clipping intensity 75% in each harvesting time (Ahmadi *et al.* 2016), since aerial parts are targeted, and harvest can take place several times. Thus, seed production is prevented or considerably low, and more importantly, consecutive clipping of sub-shrubs yearly eventually depletes the underground parts from stored assimilated compounds that can threaten the vital rates of the populations (Malinowski *et al.* 2003, Mondragón 2009).

The *T. polium* dependency on cross-pollination to a considerable extent can explain the high distribution of genetic variability within-population (AMOVA: 77%, *H's*: 0.091 >50% of *Ht*) in this study, which in agreement with the earlier investigations on *T. polium* (Boulila *et al.* 2010, Norouzi Ghare Tapeh *et al.* 2018) and *T. leucophyllum* (Sözen *et al.* 2017). Whereas asexually propagated, self-pollinated, and self-compatible plant species has the majority of the genetic diversity among populations (Culley & Wolfe 2001, Honnay & Jacquemyn 2007, Stöcklin *et al.* 2009).

- Genetic differentiation and structure

The cumulative impacts of genetic divergence, shifts, population segregation, gene flow, and breeding system through the course of evolution of a given species shape the genetic structure (Loveless *et al.* 1984, Schaal *et al.* 1998, Mafakheri *et al.* 2020). The level of gene differentiation (*Gst*) of the *T. polium* populations was 0.311, relatively higher than average Gst reported for perennial and cross-pollinated plant species (*Gst*: 0.19 and 0.22, respectively). Interestingly, a proportionately high gene flow (Nm: 1.107) was observed. The estimated *Gst* and *Nm* values are congruent with the previous report in cross-pollinating species (Loveless *et al.* 1984, Hu *et al.* 2010, Ghafouri *et al.* 2018, Li *et al.* 2018). High Gst often associated with self-pollinated species which has low gene flow since there's a reverse relationship between gene differentiation and gene flow (Govindaraju 1989). Additionally, a large portion of total genetic diversity is partitioned within populations indicating the possible influence of the breeding system. The *Gst* value indicating a significant genetic differentiation among populations, which is in contrast to the result supporting the existence of sufficient gene flow, as a value greater than >1 is a prerequisite to halt the genetic divergence among populations. The contrasting outcomes can be clarified with this interpretation that *T. polium* populations expose to overharvesting due to its high medicinal values (Bahramikia & Yazdanparast 2012). Hence, these circumstances generate the possibility of population size reduction and isolation that facilitate the gene differentiation; it seems the role of pollen exchange paly is more important considering the medium to low seed production and notably low germination rate of seeds (2.2%) (Shakeri-Almoshiri *et al.* 2009) and increased max. 32% after GA3 treatment (Kochaki & Azizi 2005). Moreover, outcrossing and insect-pollination in this species can assist the share of alleles between populations (Brunet & Sweet 2006) and maintain gene flow high, also human or animal-mediated seed dispersal may contribute in the transportation of seeds from one population to another (Gáspár *et al.* 2019). Alborz Mountains are covered with valleys and slops that can isolate populations

ROSTANIHA

and enhance the chance of gene differentiation. The cluster analysis, UPGMA, and Pcoa and STRUCTURE strongly support this explanation by grouping populations into three relatively distinct groups with high geographical affinity. In previous reports on genetic structure of *T. polium* Boulila *et al.* (2010) observed a relatively greater *Gst* (0.38) with a considerably low number of populations or Norouzi Ghare Tapeh *et al.* (2018) who reported a similar but lower *Gst* (0.235) and a potent genetic structure. In consistence with this study, populations of *Myrtus communis* L. with similar *Gst* (0.311) and *Nm* (1.11) presented strong genetic structure (Ghafouri *et al.* 2018), the same can be applied to Hu *et al.* (2010) and Koelling *et al.* (2011). Although the results of those studies are in agreement with ours, but considering the number of populations, the type of molecular marker and ecological dissimilarities compressions are not easily justifiable. The genetic differentiation of *T. polium* populations is supported by strong genetic structure and geographical affiliation with homogeneous populations in the far northeast and northwest and heterogeneity in central Alborz.

Conclusion

The outcomes of this study support the effectivity of ISSR utilization in revealing the essential parameters of genetic diversity and, more importantly, determining the genetic structure. This is the first extensive study respecting the number population on *T. polium* provides a notable understanding of the vulnerability and strength of populations regarding the degree of genetic variability in addition to demonstrating the partitioning of genetic diversity. The considerable gene differentiation and potent genetic structure were also exhibited. This information on *T. polium* can be mostly exploited to improve the systematic situation of this species. Also, given its medicinally and economically important the breeding projects can be established to develop cultivars. Most importantly, these results call for urgent attention to further studies on polyploidy levels among this species in Iran and investigating gene flow among populations.

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ROSTANIHA

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