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POPULATION GENETIC DIVERSITY AND STRUCTURE IN *Ziziphora tenuior* L.: IDENTIFICATION OF POTENTIAL GENE POOLS

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Ziziphora tenuior L. is a medicinal plant species of the genus *Ziziphora* (Labiatae) that grows in different areas of Iran. In order to study the population genetic structure in *Ziziphora tenuior*,we collected 107 plant specimens from 20 geographical populations that are located in 17 provinces. ISSR molecular markers were used for genetic diversity analysis. The populations studied revealed intra- and inter-population genetic variability. AMOVA test showed significant genetic difference among the studied populations. STRUCTURE plot identified two main gene pools for *Ziziphora tenuior* in Iran. These populations showed isolation by distance and restrict gene flow occurred among them.

Keywords: Genetic diversity, gene flow, isolation by distance, Ziziphora *tenuior*.

INTRODUCTION

The genus *Ziziphora* L. (family Labiatae) contains four species (*Z. clinopodioides* Lam., *Z. captitata* L., *Z. persica* Bunge. and *Z. tenuior* L.) that are widespread all over Iran (VERDIAN-RIVI, 2008). *Z. tenuior*L. (Kakuti in Farsi) is distributed in a different parts of Iran. It has an attractive odor and the local communities use it to make tea. *Z. tenuior* is a common teapot herb and used for treatment of fever, dysentery, coughing, diarrhea, painful menstruation, bladder stone, abortifacient and stomach tonic (NAGHIBI *et al.* 2005; AZADMEHR *et al.*,2014).

The *Ziziphora* species are the source of essential oils, flavonoids, caffeoyl derivatives, fatty acids and sterols. Many literature surveys indicated that the oils of *Ziziphora* species are rich

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in pulegone (PIRBALOUTI *et al.*, 2013). It has been reported that essential oil content differs in different populations of *Z. tenuior* (PIRBALOUTI *et al.*, 2013). Molecular studies in the genus *Ziziphora* is confined to RAPD (Random amplified polymorphic) analysis of few populations only (AL-RAWASHDEH, 2011).

Due to chemical and medicinal importance of *Z. tenuior* and extensive use of this valuable plant species by locals, it is of immediate importance to have proper plan for conservation of this species in the country. Therefore, we studied population structure, genetic variability and morphological diversity of 20 geographical populations in *Z. tenuior* for the first time.

We used ISSR (Inter simple sequence repeats) molecular markers, as these markers are stable, highly reproducible, easy to work with and are known to be useful in genetic diversity analysis and population structure studies (SHEIDAI *et al.*, 2012; 2013; 2014).

MATERIALS AND METHODS

Hundred and seven accessions of *Z. tenuior* species were collected from 20 geographical populations in Iran (Table 1). The voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU) (Table 1, Fig. 1).

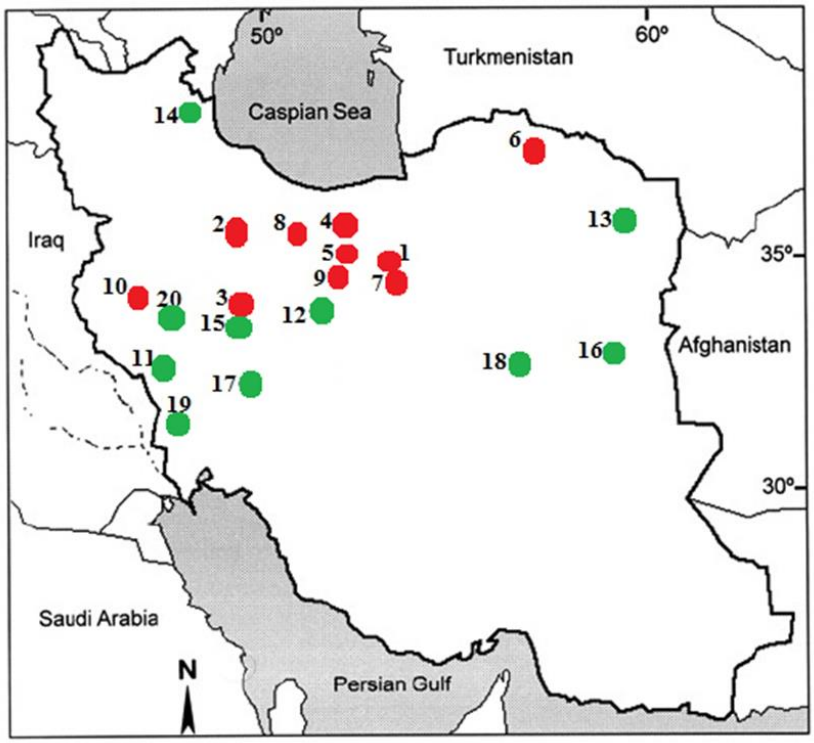

Fig. 1. Distribution map of the studied *Z. tenuior* populations. (Red-colored and green-colored circles are related with two different gene pools identified in this study).

Table 1. Populations of Z. tenuior, their locality and voucher number.

Pop No.	Province	Locality	Voucher No.	Altitude	Longitude	Latitude
1	Semnan	Shahrud. Beginning of the cloud forest	HSBU-2014400	1870	364257.4	550319.6
2	Qazvin	Qazvin. Takestan. Village Bashgole	HSBU-2014401	1900	360044.4	493240.5
3	Markazi	Arak. Between Mamoniyeh and Vidar. Beginning of the VArdeh road	HSBU-2014402	1505	351544.9	501823.4
4	Mazandaran	Mazandaran. Haraz road. 4 km to Rineh	HSBU-2014403	2072	355246.7	525933.1
5	Tehran	Firouzkouh. Homand. Cold water	HSBU-2014404	2200	353580.5	52107.8
6	North Khorasan	North Khorasan . 45 km to Bojnurd	HSBU-2014405	1348	372308	580428.4
7	Semnan	Semnan. Deh Sufian village mountains	HSBU-2014406	2550	354907.4	52339
8	Alborz	40 km to Karaj-Chalus. Shahrestanak. Sheleng village	HSBU-2014407	2150	355954.6	511874.2
9	Tehran	Tehran. Sorkhe Hesar National Park	HSBU-2014408	1380	354128.6	513403.3
10	Kurdistan	Kurdistan. Sanandaj	AUH512	1557	351952	465828.9
11	Kermanshah	Kermanshah. Harsin	AUH511	1568	341613.4	473623.8
12	Qom	Qom. Salafchegan	HSBU-2014409	1452	343605.8	502532.6
13	Razavi Khorasan	Razavi Khorasan. Mashhad. Khalaj road. Chin Kalagh mountains	T1328	1196	361114.3	593329.8
14	Ardabil	Ardabil	T1341	1346	381533.4	481720
15	Markazi	Arak. Mahallat to Khomein	HSBU-2014410	1733	325203.7	502507
16	South Khorasan	South Khorasan. Birjand. Bahlgerd	T26342	1876	324613.1	592242
17	Esfahan	Esfahan. Sofeh mountain	T1321	2007	323427	513847.5
18	Between South Khorasan and Yazd	Between South Khorasan and Yazd. Tabas mountains	T1338	834	315913	564449
19	Ilam	Ilam	T1586	2026	331922.4	464653.5
20	Hamedan	Hamedan. Tuyserkan mountain. Saryab range	T1303	1840	343253	482649

Morphological studies

Morphological characters studied are presented in Table 2.

DNA extraction and ISSR assay

Fresh leaves were collected randomly from plant specimens and dried in silica gel powder. Genomic DNA was extracted using CTAB activated charcoal protocol (SHEIDAI *et al*., 2013).The quality of extracted DNA was examined by running on 0.8% agarose gel.

Ten ISSR primers; $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9T$, UBC807, UBC811, $(GA)_9A$ and $(GT)_7CA$ custom synthesized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM $MgCl_2$ 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications, reactions were performed in Techne thermocycler (Germany) with the

following program: 5 min initial denaturation step at 94 °C, 30 s at 94 °C; 1 min at 57 °C and 1min at 72 °C. The reaction was completed with 7 min extension step at 72 °C.

The amplification products were visualized by running on 2% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Table 2. Morphological characters studied

	Morphological characters
1	Stem color
2	Flower cycle
3	Trichome stem density
4	Plant height(cm)
5	Basal leaf length(mm)
6	Basal leaf length(mm)
7	Basal leaf length/Basal leaf length
8	Basal petiole length(mm)
9	Lower leaf length of stem(mm)
10	Lower leaf width of stem(mm)
11	Lower leaf length of stem/Lower leaf width of stem
12	Floral leaf length of stem(mm)
13	Floral leaf width of stem(mm)
14	Floral leaf length of stem/Floral leaf width of stem
15	interval between the base of main inflorescence to root(cm)
16	inflorescence length(cm)
17	Pedicle length(mm)
18	Calyx length(mm)
19	Calyx width(mm)
20	Calyx length/Calyx width
21	Calyx teeth length(mm)
22	Corolla length(mm)
23	Corolla tube length(mm)
24	Petal length(mm)
25	Corolla tube length/Petal length
26	Stamen length(mm)
27	Staminode length(mm)
28	Stamen length/Staminode length
29	Style length(mm)
30	Seed length(mm)
31	Seed width(mm)
32	Seed length/Seed width

Data analyses

ANOVA (Analysis of variance) and CVA (Canonical variance analysis) were used to reveal significant difference for morphological characters among the studied populations.

Principal coordinate analysis (PCoA) was performed to group the plants specimens based on morphological characters and principal components analysis (PCA biplot) was used to identify the most variable morphological characters among the studied species (PODANI, 2000). Morphological data were standardized (mean = 0, variance = 1) for these analyses (PODANI, 2000).

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). Genetic diversity parameters were determined in each population. These parameters were Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (WEISING, 2005; FREELAND *et al.,* 2011). Nei's genetic distance was determined among the studied populations and used for clustering (FREELAND *et al.*, 2011; WEISING, 2005).

For grouping of the plant specimens, Neighbor Joining (NJ) clustering method and NeighborNet method of networking were performed after 100 times bootstrapping (FREELAND *et al.*, 2011; HUSON and BRYANT, 2006).

The Mantel test was performed to check correlation between geographical distance and the genetic distance of the studied species (PODANI, 2000). PAST ver. 2.17 (HAMER *et al.,* 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) programs were used for these analyses. Significant genetic difference among the studied populations and provinces were determined by: 1- AMOVA (Analysis of molecular variance) test (with 1000 permutations) by using GenAlex 6.4 (PEAKALL and SMOUSE, 2006), and 2- Nei's Gst analysis of GenoDive ver.2 (2013) (MEIRMANS and VAN TIENDEREN, 2004). Furthermore, populations' genetic differentiation was studied by G'st_est = standardized measure of genetic differentiation (HEDRICK, 2005), and D_est = Jost measure of differentiation (JOST, 2008).

In order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program, Hickory (ver. 1.0) (HOLSINGER *et al.,* 2003), was used to estimate parameters related to genetic structure (theta B value). Three runs were conducted with default sampling parameters (burn-in = 50,000, sample=250,000, thin = 50) to ensure consistency of results (TERO *et al*., 2003).

The genetic structure of geographical populations was studied by two methods. First we carried out Bayesian based model STRUCTURE analysis (PRITCHARD *et al.,* 2000). Data were scored as dominant markers (FALUSHET *et al*., 2007) and Evanno test was performed on STRUCTURE result to find proper number of *K* by using delta *K* value (EVANNO *et al.,* 2005).

Secondly, we performed K-Means clustering as done in GenoDive ver. 2. (2013). Here, the optimal clustering is the one with the smallest amount of variation within clusters. This is calculated by using the within-clusters sum of squares. The minimization of the within-groups sum of squares that is used in K-Means clustering is, in the context of a hierarchical AMOVA, equivalent to minimizing the among-populations-within-groups sum of squares, SSDAP/WG (MEIRMANS, 2012). We used two summary statistics to present K-Means clustering, 1- pseudo-F (CALINSKI and HARABASZ, 1974), and 2- Bayesian Information Criterion (BIC) (SCHWARZ, 1978). The clustering with the highest value for pseudo-F is regarded to provide the best fit, while clustering with the lowest value for BIC is regarded to provide the best fit (MEIRMANS, 2012).

Recently FRICHOT *et al*. (2013) introduced the statistical model called "latent factor mixed models (LFMM)" that tests correlations between environmental and genetic variation, while estimating the effects of hidden factors that represent background residual levels of population

structure. We used this method to check if ISSR markers used here, show correlation with environmental features of the studied species. The analysis was done by LFMM program Version: 1.2 (2013).

RESULTS AND DISCUSSION

Genetic diversity

We obtained reproducible bands from all ISSR primers. These bands were scored to form a data matrix for further analysis. Dentrented Correspondence Analysis (DCA) plot revealed (not shown) scattered distribution of the studied ISSR loci.It indicated that these loci are not linked and are suitable for population genetic structure analysis.

Genetic diversity parameters of the studied populations are presented in Table 3.The highest level of genetic polymorphism (46.88%), occurred in population No. 9, while the lowest level (10.94%) occurred in population No. 2. Population No. 9 also had the highest values for effective number of alleles (Ne = 1.284) and Shannon information index (I = 0.246).

Table 3. Genetic diversity parameters in the studied Z. tenuiorpopulations

Pop	N	Na	Ne	I	He	UHe	%P	Fst
Pop1	6	0.453	1.069	0.065	0.042	0.046	14.06%	0.03
Pop2	6	0.406	1.045	0.044	0.028	0.03	10.94%	0.19
Pop3	6	0.563	1.124	0.11	0.073	0.079	21.88%	0.33
Pop4	6	0.703	1.147	0.136	0.089	0.097	28.13%	0.43
Pop5	6	0.703	1.12	0.12	0.076	0.083	26.56%	0.79
Pop6	6	0.672	1.14	0.131	0.086	0.093	26.56%	0.58
Pop7	6	0.672	1.139	0.129	0.084	0.091	26.56%	0.06
Pop8	6	0.844	1.198	0.179	0.118	0.129	34.38%	0.66
Pop9	6	1.016	1.284	0.246	0.164	0.179	46.88%	0.70
Pop10	5	0.891	1.205	0.185	0.121	0.135	37.50%	0.58
Pop11	6	0.938	1.267	0.218	0.149	0.162	39.06%	0.75
Pop12	6	0.922	1.16	0.151	0.097	0.106	32.81%	0.49
Pop13	6	0.766	1.193	0.16	0.109	0.119	28.13%	0.55
Pop14	6	0.922	1.202	0.193	0.125	0.136	40.63%	0.01
Pop15	6	0.828	1.166	0.164	0.105	0.114	35.94%	0.04
Pop16	5	0.563	1.123	0.111	0.073	0.081	21.88%	0.64
Pop17	4	0.656	1.159	0.149	0.097	0.111	29.69%	0.72
Pop18	4	0.625	1.195	0.167	0.113	0.129	29.69%	0.49
Pop19	2	0.453	1.144	0.123	0.084	0.112	20.31%	0.64
Pop20	3	0.516	1.141	0.128	0.085	0.102	23.44%	0.50

Abbreviations:
N = Number of individuals, Na = No. of alleles, Ne = No. effective alleles, He = Gene diversity index, UHe = Unbiased gene diversity, %P = Percentage of polymorphism.
Population codes are according to Table 1.

Population genetic structure

AMOVA test revealed significant molecular difference (P = 0.01) among the studied populations. It also revealed that 59% of total genetic variability occurred among the studied populations while, 41% occurred within populations. Furthermore, pair-wise AMOVA test revealed that most of the paired samples comparison differed significantly from each other (P = 0.01).Similarly, significant values were obtained for the Hickory test (Theta B value = 0.40), Gst (0.562, P = 0.001), Hedrick, standardised fixation index (G'st = 0.657, P = 0.001) and Jost' differentiation index (D-est = 0.216, P = 0.001). These results revealed that the studied populations are genetically differentiated.

Nei' genetic identity and genetic distance were determined among the studied populations. The highest degree of genetic similarity (0.952) occurred between population 16 and population 17, followed by population 7 and population 8 (0. 0.947). The lowest degree of genetic similarity occurred between population1 and 12(0.632).

NJ tree and NeighborNet network of ISSR data produced similar results. Therefore, only NeighborNet network is presented and discussed here (Fig. 2). In general, the plant specimens of each population were placed together and formed a separate cluster. This was particularly true for populations 3-6. However, some of the populations showed mixed grouping with plants of the other populations due to within population genetic variability. This case was observed in populations 7-10, 16 and 17.

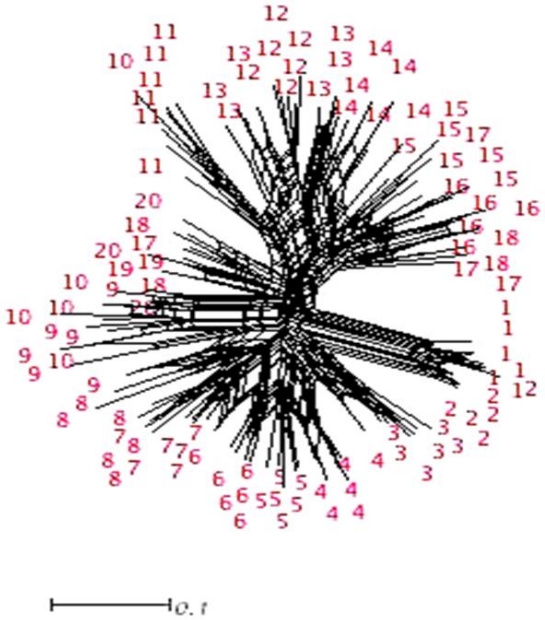

Fig. 2. NeighborNet network of ISSR data in *Ziziphora tenuior* populations. (Population codes are according to Table 1.).

The Mantel test produced significant correlation (r = 0.41, P < 0.01) between geographical distance and genetic distance of the studied populations that is called isolation by distance (IBD). Therefore, gene flow occurred between neighboring populations only.

Bayesian-based STRUCTURE analysis performed revealed genetic difference of the studied populations (Fig. 3). The genetic affinity of the populations in agreement with NJ tree and NeighborNet diagram presented before.

Evanno test produced k = 2 and STRUCTIRE plot based on k = 2 separated the studied populations in two main genetic groups. Populations 1-10 comprised the first group, while populations 11-20 formed the second group. Some degree of genetic admixture or ancestral gene flow was observed among these populations.

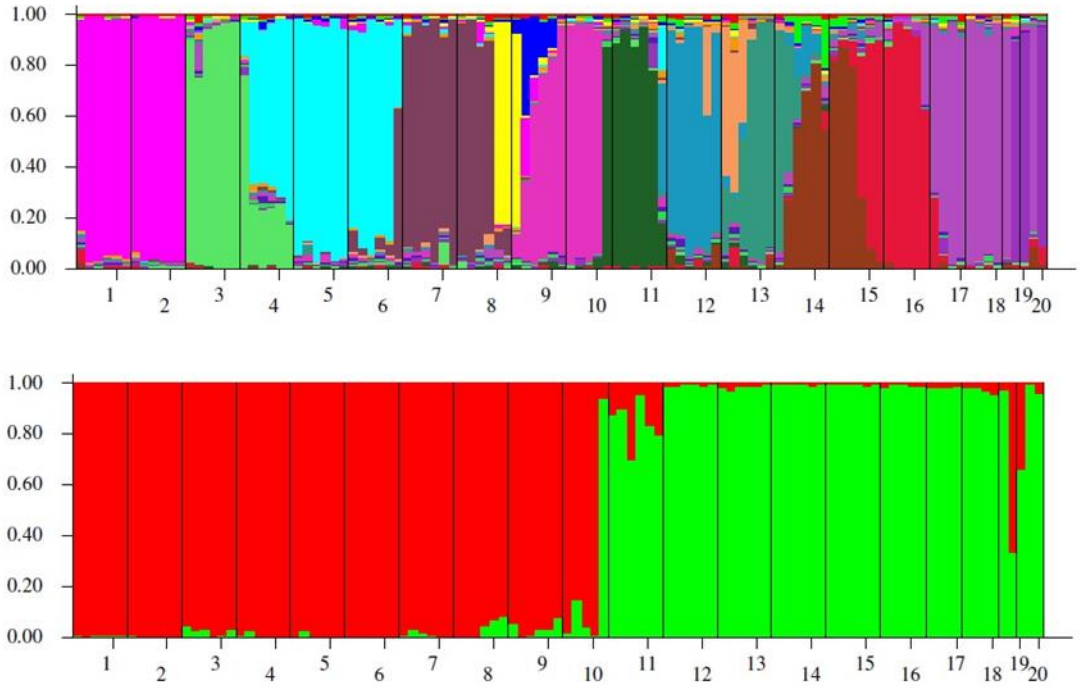

Fig. 3. STRUCTURE plot of *Ziziphora tenuior* populations based on k = 20 (top) and k = 2 (bellow).

The STRUCTURE result revealed that *Ziziphora tenuior* contains two main gene pools in the country. The first gene pool (indicated in red circle in Fig. 1) is comprised of populations that are mainly located in the western and center part of Iran along with a population in North-East of the country. The second gene pool (indicated in green circle in Fig.1) showed a higher concentration in western part and Eastern regions of the country.

K-Means clustering produced the optimum number of genetic groups K = 18 according to Calinski & Harabasz' pseudo-F and Bayesian Information Criterion. This is in agreement with AMOVA result that revealed significant molecular difference among the studied populations.

Pearson coefficient of correlation determined between gene diversity and geographical features (altitude, longitude and altitude) did not produce significant result. However, almost significant reduction in gene diversity and altitude was obtained ($r = -0.41$, $P = 0.07$).

Similar analysis performed separately for the two gene pools also did not produce significant correlation between gene diversity with geographical parameters. No correlation was found also between Fst value of the studied populations and geographical features.

Gene flow

The estimated gene flow, Nm ($Nm= 0.5(12 Gst)/Gst$), was 0.2747. The results of this analysis revealed that the genetic differentiation among populations throughout the entire distribution area is significant and that gene flow is restricted. This is in agreement with STRUCTURE analysis result.

STRUCTURE plot also revealed some amount of genetic admixture/ ancestral shared alleles between the studied populations. Moreover, population assignment test (based on maximum likelihood) that was performed on all 107 studied individuals revealed that allow degree of gene flow occurred between populations 1 and 2, populations 7 and 8, between 9 and 10, between 13, 14 and 15, and also between 17-20 (Table 4).

LFMM analysis revealed that ISSR loci 4-10, 20, 21, 26-28, 31, 40, 53, 55 and 62 were significantly correlated with the environmental factors studied ($\log10(p\text{-value}) >1.30$, $P <0.05$). Therefore, these loci are potentially adaptive for the studied populations. ISSR loci 31, 37, 55 and 62 had Nm value >1, and were involved in gene flow.

Morphometry

The ANOVA test (analysis of variance test) showed significant difference ($p < 0.05$) for quantitative morphological characters among the studied populations. Moreover, CVA plot of both quantitative and qualitative morphological characters separated the studied species in two distinct groups (Fig. 4).

Mantel test revealed significant correlation between morphological distance and geographical distance of the studied populations ($r = 0.4$, $P <0.01$). The grouping obtained for genetic data is very much similar to grouping obtained by CVA plot of morphological data (Fig. 4).

In CVA plot of morphological data, populations 1-6 were placed close to each other in one side of the plot, while populations 10-20 were placed close to each other, that is in agreement with ISSR grouping presented before. This result revealed that morphological divergence of the studied populations.

Habitat fragmentation generally is expected to reduce genetic diversity and to increase inter-population genetic divergence by restricting gene flow among fragmented populations.It is also expected to increase inbreeding andrandom genetic drift within populations (HOU and LOU, 2011). However, there are cases in which fragmentation did not result in reduced genetic diversity (PROBER *et al.*, 1990), due to various reasons like population size, gene flow and the time scale of fragmentation (CHEN, 2000).

Table 4. Population assignment test for studied populations. (Individuals that showed gene flow are only presented here).

Individual	Current	Inferred	Lik_max
1	Pop001	Pop002	-22.493
7	Pop002	Pop001	-12.484
41	Pop007	Pop008	-26.55
42	Pop007	Pop008	-9.754
43	Pop008	Pop007	-11.805
54	Pop009	Pop010	-21.164
56	Pop010	Pop009	-23.131
59	Pop010	Pop011	-29.983
78	Pop014	Pop013	-11.828
83	Pop014	Pop015	-38.611
84	Pop015	Pop014	-22.229
89	Pop015	Pop016	-23.583
90	Pop016	Pop015	-19.512
95	Pop017	Pop016	-28.318
96	Pop017	Pop014	-39.718
97	Pop017	Pop016	-41.83
98	Pop017	Pop015	-48.969
99	Pop018	Pop015	-50.579
100	Pop018	Pop015	-39.299
101	Pop018	Pop014	-48.002
102	Pop018	Pop015	-48.276
103	Pop019	Pop015	-40.462
105	Pop019	Pop009	-49.151
104	Pop020	Pop009	-44.551
106	Pop020	Pop015	-29.182
107	Pop020	Pop015	-38.853

The analysis of ISSR molecular markers indicated that *Ziziphora tenuior* has maintained a high overall genetic diversity (Ht =0.27) compared to that of other plants (HOU and LOU, 2011). Therefore, in spite of population fragmentationa high degree of genetic variability is present in this species.

AMOVA test also revealed that 59% of total genetic variability occurred among the studied populations while, 41% occurred within these populations.Sexual reproduction and open pollination in *Ziziphora tenuior* as well as restricted gene flow (ancestral or ongoing) might be the reasons for high within population genetic variability observed.

Genetic diversity is of fundamental importance in the continuity of a species as it provides the necessary adaptation to the prevailing biotic and abiotic environmental conditions, and enables change in the genetic composition to cope with changes in the environment (ÇALISKAN, 2012; SHEIDAI *et al*., 2013; 2014).

Mantel test revealed a pattern of isolation-by distance across the distribution range of *Ziziphora tenuior*. This pattern suggested that the dispersal of this species might beconstrained by

distance such that gene flow is most likely to occur between neighboring populations. As a result, more closely situated populations tend to be more genetically similar to one another (SLATKIN, 1993; HUTCHINSON and TEMPLETON, 1999; MEDRANO and HERRERA, 2008). This was particularly true for the two gene pools identifiedin *Ziziphora tenuior* populations in Iran, as these populations were grouped together in NJ tree and network diagram.

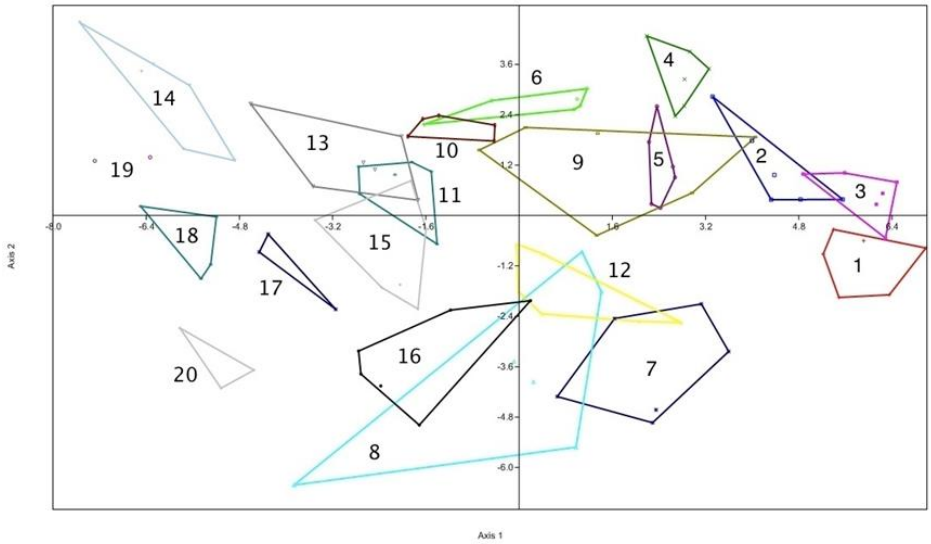

Fig. 4. CVA plot of morphological data in the studied populations.

Non-significant Pearson coefficient obtained between gene diversity and geographical features (altitude, longitude and latitude) within each gene pool suggest that although an isolation-by-distance pattern may be detected across the whole range of *Ziziphora tenuior*, the gene flow and the relationship between geographical and genetic distances have different patterns at different spatial scales. This is known to occur in some other plant species too (MEDRANO and HERRERA, 2008; HOU and LOU, 2011).

Significant correlation of some of the ISSR loci with environmental features as revealed by LFMM test, indicate that some of these populations have acquired adaptive alleles during divergence. These adaptive alleles along with the maintained genetic variability serve to adapt these plants to their present environment.

In conclusion, it seems that *Ziziphora tenuior* has two main gene pools in Iran. These populations are genetically and morphologically diverged from each other and may be in the way of speciation process. However, they have some degree of gene flow at least among neighboring populations that still preserve genetic continuity of the species.

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GENETIČKI DIVERZITET I STRUKTURA POPULACIJA *Ziziphora tenuior* L.: IDENTIFIKACIJA POTENCIJALNIH REZERVI GENA

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Izvod

Ziziphora tenuior L.je medicinska biiljka roda *Ziziphora* (Labiatae) koja raste u veoma različitim regionima Irana. Sakupljeno je 107 vrsta *Ziziphora tenuior* iz 20 različitih geografskih populacija lociranih u 17 provincija u cilju ispitivanja genetičke strukture. Korišćeni su ISSR molekularni markeri u ispitivanjima genetičkog diverziteta. Populaciona ispitivanja su pokazala intra - i inter populacionu varijabilnost. AMOVA test je pokazao značajne genetičke razlike ispitivanih populacija. STRUCTURE plot je identifikovao dva glavna rezervoara gena *Ziziphora tenuior* u Iranu. Populacije su prostorno izolovane i nije došlo do protoka gena među njima. ***Ključne reči*** : Genetički diverzitet, protok gena, prostorna izolacija, Ziziphora *tenuior*.

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