

## POPULATION GENETIC STUDY IN *Epilobium minutiflorum* (Onagraceae) IN IRAN

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The genus *Epilobium* has about 200 species in which taxonomic subdivisions are difficult but possible. Species *E. minutiflorum* due to its fluffy white inflorescence and small miniature flowers and beaked seeds, it is one of the most prominent species of the genus *Epilobium* in the region. We have no data on the population genetic structure of this species in the Iran. Therefore a population genetic and morphological investigation was performed through light on genetic and morphological variability in this taxa. We used SCoT molecular markers for population genetic investigation. Genetic diversity analyses revealed a moderate genetic variability between *E. minutiflorum* populations, while PCoA showed some degree of genetic admixture among populations. AMOVA produced significant genetic difference among populations. Morphometric analysis showed that high degree of overlap among the studied populations. However, the results showed that SCoT marker has a good discrimination power and can differentiate the studied populations. This marker can be used to evaluate genetic diversity and identify genotypes of *E. minutiflorum* populations.

*Key words:* *Epilobium*, Iran, morphological, SCoT, UPGMA

### INTRODUCTION

The genus *Epilobium* L. (Family Onagraceae) is the largest genus of Onagraceae, comprising approximately 170 species of mainly temperate herbs (RAVEN, 1976; 1988; HOCH *et al.*, 1992). The genus is significant for its morphological, ecological and biological diversity. Therefore, they have been placed in 8 sections (BAUM *et al.*, 1994). The sections are highly distinctive showing variation in vegetative and floral morphology, anatomy, palynology,

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cytology, phytochemistry, biogeography, ecology, and breeding systems (reviewed in RAVEN, 1976; 1988). Taxonomically morphological characters of the genus are inflorescence leaf, calyx, capsule, and the flower (WALTER *et al.*, 2007).

Morphological variations observed may be related to phenotype plasticity (LEWIS *et al.*, 1962; FITTER, 1980; KITCHENER *et al.*, 1998).

Molecular data may provide useful and additional support for species delimitation where morphological characters are cryptic or overlapping among the species complexes and in where speciation is recent (BROADHURST *et al.*, 2004; MILLAR *et al.*, 2011).

In order to assist indicate of genetic diversity molecular markers used should be able to illustrate the level of variation between taxa and therefore complement morphological assessment of species and populations boundaries. Such a combined approach should enable more reliable taxonomic judgments (BYRNE, 2003).

Different molecular markers have been used to study species delimitation and population structure in plant species (SHEIDAI *et al.*, 2012; 2013; 2014; MINAEIFAR *et al.*, 2015).

SCoT molecular markers are known to be capable of illustrating genetic variability among populations, varieties, subspecies and species well enough (SABOORI *et al.*, 2019; TABASI *et al.*, 2020; LAAME-JUIBARY *et al.*, 2021).

*E. minutiflorum* is one of the species of *Epilobium* that distribution starts from the center of Turkey and extends in Asia to the east of the Himalayas and has the highest distribution in Iran and Afghanistan. It is also distributed in Iran in the north, northwest, west, center and northeast. It has a great variety in vegetative form in the form of simple non-branched stems and small size with many and large branches. Also the shape and size of the leaves, stem color and density of hairs are variable, so that in some cases it has close similarities with the species *E. tetragonum* L., *E. palustre* L., *E. confusum* L. and in some areas with *E. palustre* collected from a habitat becomes. There is no report on the biosystematics of populations of *E. minutiflorum* in Iran, therefore the present study is morphological and molecular analysis of 58 individuals from 12 populations *E. minutiflorum* considers Iran for the first time and tries to show the degree of genetic diversity between populations and the relationship between genetic distance, morphological characters and their geographical distance in this species. In this paper we present a molecular phylogenetic study of *E. minutiflorum* populations based on SCoT markers and then utilize the results to elucidate genetic diversity in this the specie.

## MATERIAL AND METHODS

### *Plant materials*

For the present study, 58 plant accessions were collected from 12 geographical populations. These populations were located in eight provinces of Tehran, Kerman, Arak, Azerbaijan, Golestan, Albourz, Markazi, Chaharmahal and Bakhtiari of Iran. Details of localities are provided in ( Table 1). The voucher specimens are deposited in herbarium of Shahid Beheshti University (HSBU).

### *Morphological study*

Morphological characters studied were: plant height, length of stem leaf, width of stem leaf, Length /width ratio of stem, length of corolla, length of calyx, width of calyx, Length/width

ratio of calyx, Length / width ratio of petal, length of stigma, length of anther, length of capsule, width of capsule, length of seed, length of stamen, length of style, Length /width ratio of capsule, Length / Length ratio of stamen, length /width ratio of inflorescence leaf and Length /width ratio of inflorescence.

Unweighted paired group with arithmetic average (UPGMA) and Principal component analyses (PCA) for grouping of the populations based on morphological characters by using were performed PAST ver. 2.17 (HAMMER *et al.*, 2012) respectively.

*Table 1. The studied populations, their localities and voucher numbers.*

Pop.	Province	Locality	Altitude (m)	Latitude	Longitude	Voucher no.
1	Tehran	Suleqan	1655	51°26'44"	35°81'99"	87
2	Kerman	Seh Konj	2330	57°43'08"	29°99'77"	88
3	Tehran	Jajrood	1463	51°69'79"	35°75'07"	89
4	Arak	Senedjan	1858	49°61'84"	34°04'14"	90
5	Azerbaijan	Miyaneh	1369	48°15'89"	36°91'46"	91
6	Azerbaijan	Quri Gol	1914	46°69'36"	37°92'09"	92
7	Arak	Maadan	1894	49°67'93"	34°05'75"	93
8	Tehran	Darakeh	1801	51°38'22"	35°81'53"	94
9	Golestan	Azad shahr	246	55°17'91"	37°07'15"	95
10	Albourz	Karaj	1634	51°08'82"	35°94'69"	96
11	Markazi	Tafresh	1670	49°95'46"	34°74'38"	97
	Chaharmahal va					
12	Bakhtiari	Farsan	2073	50°55'41"	32°27'49"	98

#### *Molecular study*

Fresh leaves were randomly collected from plant specimens and dried in silica gel powder. The genomic DNA was extracted using CTAB-activated charcoal protocol (KRIZMAN *et al.*, 2006). The extraction procedure was based on activated charcoal and polyvinylpyrrolidone (PVP) for binding of polyphenolics during extraction and under mild extraction and precipitation conditions. This promoted high-molecular-weight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 0.8% agarose gel.

PCR reactions were performed in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; 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).

#### *SCoT assay*

For this study, two SCoT primers, SCoT-2(5'-ACCATGGCTACCACCGGC-3') and SCoT-41(5'-CAATGGCTACCACTGACA-3') were used. Amplification reactions were performed in a Techne thermocycler (Germany) with the following program: 4 min for initial denaturation step at 95°C, 1 min at 94°C, 30 second at 52°C, and 1 min at 72°C. The reaction was completed by a final extension step of 5 min at 72°C. The amplification products were examined using 2% agarose gel electrophoresis and fluorescence staining on KBC power load (Kowsar

Biotech Company, Iran). The fragments size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

#### Data analyses

A total of 20 bands were generated from two SCoT primers. SCoT bands obtained from two primers were treated as binary characters and coded accordingly (presence = 1, absence = 0). Genetic diversity parameters like: The percentage of allelic polymorphism, allele diversity, Nei' gene diversity (He) and Shannon's information index (I) (WEISING, 2005) were determined. We used GenAlex 6.4 for these analyses (PEAKALL *et al.*, 2006).

Genetic differentiation of the studied populations was studied by AMOVA with 1000 permutations as performed in GenAlex 6.4 (PEAKALL *et al.*, 2006).

Different ordination and clustering methods were applied on standardized data like Neighbor Joining (NJ) and Ward clustering as well as Principal coordinate analysis (PCoA), multi-dimensional scaling (MDS) and UPGMA were used for population grouping, after 1000 times bootstrapping/ and or permutations (PODANI, 2000; FREELAND *et al.*, 2011). Data analyses were performed by using PAST ver. 2.17 (HAMMER *et al.*, 2012) respectively.

## RESULTS

#### Morphometry

Different clustering methods (PCA and UPGMA dendrograms) based on 39 morphological characters in identical populations produced similar results. Therefore, only UPGMA dendrogram is presented (Fig 1). The populations were located in two main clusters. Individuals from two populations 3 and 5 were formed the first group at the left part of the UPGMA dendrogram. The Individuals of all populations are located (1- 12) in the second group at the right part of the UPGMA dendrogram, Therefore, the studied *Epilobium minutiflorum* populations were not delimited based on morphological characters.

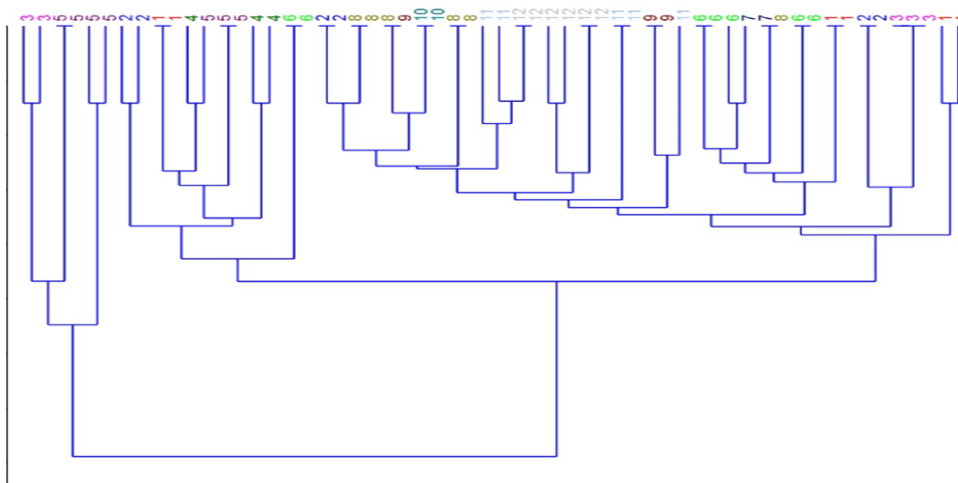


Fig. 1. UPGMA dendrogram of *Epilobium minutiflorum* populations based on morphological data.

Total number of bands, common and private bands in 12 studied populations is presented in Table 2. Despite the low number private bands in these populations, G<sub>st</sub> and other differentiation statistics determined for 20 loci obtained by SCoT analysis (Table 2) revealed the most of them have very good discrimination power and can differentiate the studied populations. The occurrence of private bands indicates the presence of specific sequences in local populations.

Table 2. The SCoT bands pattern in *Epilobium minutiflorum* populations studied.

Population	1	2	3	4	5	6	7	8	9	10	11	12
No. Bands	13	5	16	10	7	6	5	8	5	6	4	4
No. Bands Freq. $\geq$ 5%	13	5	16	10	7	6	5	8	5	6	4	4
No. Private Bands	0	1	2	0	0	0	0	0	0	0	0	0
No. LComm Bands ( $\leq$ 25%)	6	0	7	4	1	1	0	2	0	0	0	0
No. LComm Bands ( $\leq$ 50%)	9	3	11	6	4	4	0	3	0	1	0	0

AMOVA indicated significant genetic difference among *Epilobium minutiflorum* populations (Phi<sub>pt</sub> = 0.935, P = 0.001).

AMOVA produced significant genetic difference based on SCoT data among the most studied populations. It showed that the percentages of molecular variance among populations is 93% and within populations is 7%. Genetic differentiation parameters estimate, also supported AMOVA and produced significant difference among the most populations studied.

Nei, Genetic distance obtained for the studied populations based on SCoT data revealed that distance between populations varied from 0.000 to 1.579.

We presented only the loci with at least 0.77 G<sub>st</sub> value/ or above 0.14 Nm (Table 3) indicating their migration and shared value. The mean G<sub>st</sub> value 0.95 for all SCoT loci, indicates that these molecular markers have a good discriminating power and can be used in *Epilobium minutiflorum* genetic diversity.

The studied *Epilobium minutiflorum* populations almost showed a high degree of genetic similarity (Mean = 0.01) (Table 3). The highest degree of genetic distance (1.576) occurred between populations 3 and 11, 3 and 12 while the lowest degree (0.000) between populations 5 and 6, 11 and 12 (Table 4).

Genetic diversity parameters determined in 12 studied populations of *Epilobium minutiflorum* are presented in (Table 5). The highest value for the gene diversity (H<sub>e</sub> = 0.062), polymorphism percentage (P% = 15.00) in population 2 (Kerman population). The lowest value for the same parameters (0.000, 0.000%, respectively) occurred in the populations 6, 7, 8, 9, 10, 11, 12 (Azerbaijan, Arak, Tehran, Golestan, Albourz, Markazi, Chaharmahal and Bakhtiari populations, respectively).

Table 3. Discriminating power of SCoT markers in *Epilobium minutiflorum* populations studied

Locus	Sample Size	Ht	Hs	Gst	Nm*
Locus1	60	0.4406	0.0890	0.7981	0.1265
Locus2	60	0.3982	0.0345	0.9133	0.0475
Locus3	60	0.2778	0.0000	1.0000	0.0000
Locus4	60	0.4461	0.1007	0.7743	0.1458
Locus5	60	0.3750	0.0000	1.0000	0.0000
Locus6	60	0.4972	0.0654	0.8684	0.0757
Locus7	60	0.3750	0.0000	1.0000	0.0000
Locus8	60	0.0000	0.0000	****	****
Locus9	60	0.2778	0.0000	1.0000	0.0000
Locus10	60	0.4861	0.0000	1.0000	0.0000
Locus11	60	0.3750	0.0000	1.0000	0.0000
Locus12	60	0.1528	0.0000	1.0000	0.0000
Locus13	60	0.2778	0.0000	1.0000	0.0000
Locus14	60	0.1528	0.0000	1.0000	0.0000
Locus15	60	0.3750	0.0000	1.0000	0.0000
Locus16	60	0.4995	0.0250	0.9500	0.0263
Locus17	60	0.1528	0.0000	1.0000	0.0000
Locus18	60	0.3750	0.0000	1.0000	0.0000
Locus19	60	0.4444	0.0000	1.0000	0.0000
Locus20	60	0.3750	0.0000	1.0000	0.0000
Mean	60	0.3377	0.0157	0.9534	0.0244

Ht total heterozygosity, Hs subpopulation heterozygosity, Gst analog of Fst, Nm number of migrants \*Not determined.

Table 4. Nei genetic distance versus genetic identity of *Epilobium minutiflorum* populations. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Pop ID	1	2	3	4	5	6	7	8	9	10	11	12
1	****	0.470	0.723	0.566	0.313	0.315	0.513	0.703	0.550	0.601	0.499	0.499
2	0.755	****	0.329	0.581	0.770	0.771	0.781	0.576	0.731	0.679	0.783	0.783
3	0.324	1.111	****	0.567	0.308	0.310	0.304	0.414	0.259	0.310	0.207	0.207
4	0.569	0.544	0.568	****	0.647	0.647	0.662	0.463	0.615	0.564	0.564	0.564
5	1.163	0.262	1.176	0.436	****	1.000	0.703	0.506	0.656	0.606	0.706	0.706
6	1.157	0.261	1.170	0.436	0.000	****	0.697	0.500	0.650	0.600	0.700	0.700
7	0.667	0.248	1.192	0.413	0.352	0.361	****	0.817	0.968	0.917	0.917	0.917
8	0.353	0.552	0.882	0.769	0.682	0.693	0.203	****	0.850	0.900	0.800	0.800
9	0.598	0.313	1.352	0.487	0.421	0.431	0.033	0.163	****	0.950	0.950	0.950
10	0.509	0.386	1.170	0.572	0.501	0.511	0.086	0.105	0.051	****	0.900	0.900
11	0.695	0.245	1.576	0.572	0.348	0.357	0.086	0.223	0.051	0.105	****	1.000
12	0.695	0.245	1.576	0.572	0.348	0.357	0.086	0.223	0.051	0.105	0.000	****

MDS analysis, supported PCoA ordination and five main genetic groups have been achieved (Fig 2). In The left group, some samples of populations partially overlaps with other samples and these group are not well separated. The other populations in other groups are well separated.

The grouping of the populations by NJ tree and Ward clustering produced similar results. Therefore, the Ward dendrogram is only presented here (Fig 3). Almost all plants of each population were grouped together in a distinct cluster. This result is in agreement with AMOVA result and revealed the populations, genetic divergence in *Epilobium minutiflorum*. Moreover, it showed the use of SCoT molecular markers in population genetic studies of this species. Two

major clusters were formed. The populations 1-3-4 were placed in the first major cluster and showed are well separated. The populations 11 and 12 showed higher degree of genetic affinity and were placed close to each other in the second major cluster.

Table. 5. Genetic diversity parameters in the studied populations ( $N$ = number of samples,  $N_a$ = number different alleles,  $N_e$  = number of effective alleles,  $I$ = Shannon ' s information index,  $H_e$  gene diversity,  $UHe$  = unbiased gene diversity,  $P\%$ = percentage of polymorphism).

Pop		N	$N_a$	$N_e$	I	$H_e$	uHe	%P
Pop1	Mean	6.000	0.800	1.040	0.053	0.031	0.034	15.00%
Pop2	Mean	6.000	0.400	1.106	0.091	0.062	0.068	15.00%
Pop3	Mean	5.000	0.950	1.109	0.086	0.059	0.065	15.00%
Pop4	Mean	3.000	0.550	1.021	0.024	0.015	0.018	5.00%
Pop5	Mean	7.000	0.400	1.008	0.013	0.007	0.007	5.00%
Pop6	Mean	7.000	0.300	1.000	0.000	0.000	0.000	0.00%
Pop7	Mean	3.000	0.300	1.021	0.024	0.015	0.018	5.00%
Pop8	Mean	6.000	0.400	1.000	0.000	0.000	0.000	0.00%
Pop9	Mean	3.000	0.250	1.000	0.000	0.000	0.000	0.00%
Pop10	Mean	3.000	0.300	1.000	0.000	0.000	0.000	0.00%
Pop11	Mean	5.000	0.200	1.000	0.000	0.000	0.000	0.00%
Pop12	Mean	6.000	0.200	1.000	0.000	0.000	0.000	0.00%

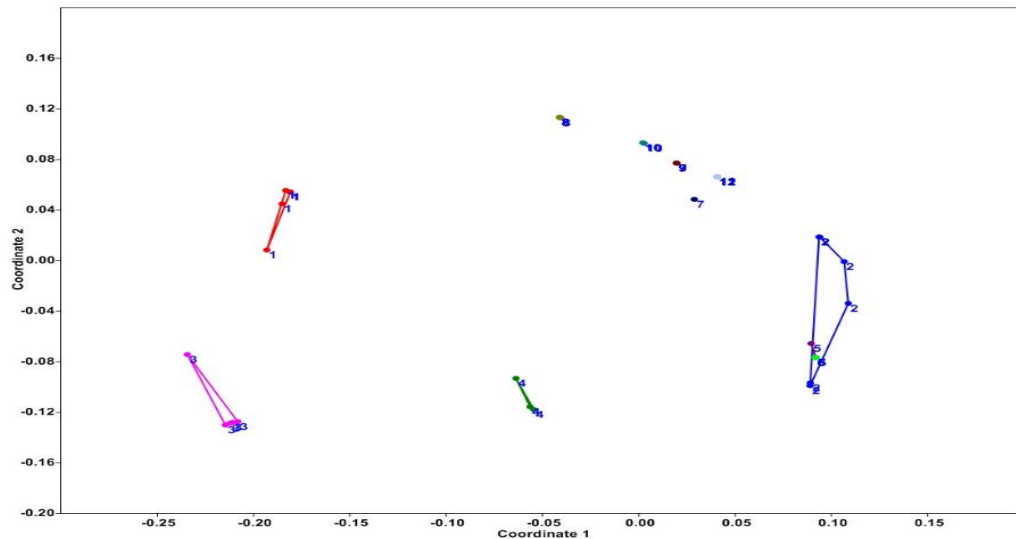


Fig. 2. MDS plot of the studied *Epilobium minutiflorum* populations (The number of samples is included) based on SCoT data, showing genetic relatedness of the populations. Populations 1–12 are according to Table 1.

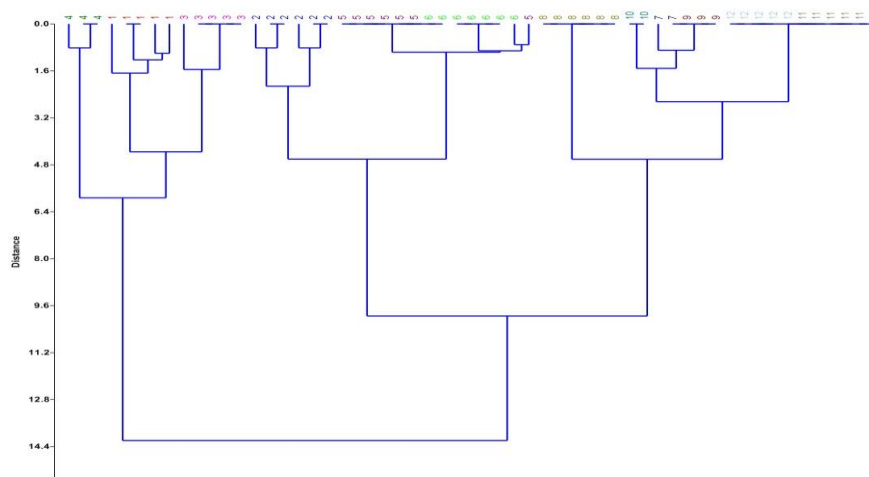


Fig. 3. Ward tree of SCoT data of the selected populations of *E. minutiflorum*. Populations 1–12 are according to Table 1.

UPGMA clustering of SCoT data (Fig 4) separated *Epilobium minutiflorum* populations. This is in agreement with others SCoT data results. It also showed close affinity between the populations 11 and 12 that is in accordance with Ward result too.

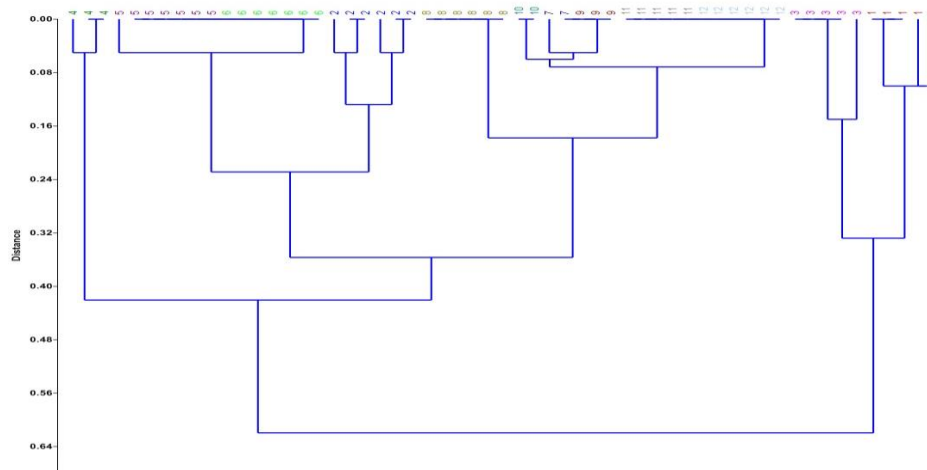


Fig. 4. UPGMA clustering of *Epilobium minutiflorum* populations. (Populations 1-12 are according to Table 1).



PCoA grouping of the *Epilobium minutiflorum* populations based on SCoT data (Fig 5), placed the studied specimens in 5 major groups. Individuals from seven populations 5-7, 9-10, 2 and 12 were intermixed and formed the first major group at the left part of the PCoA plot. The individuals of populations 1, 2, 4, and 8 are separately included in other parts of the PCoA plot. These results indicate that a limited degree of genetic admixture has occurred in some of the studied populations.

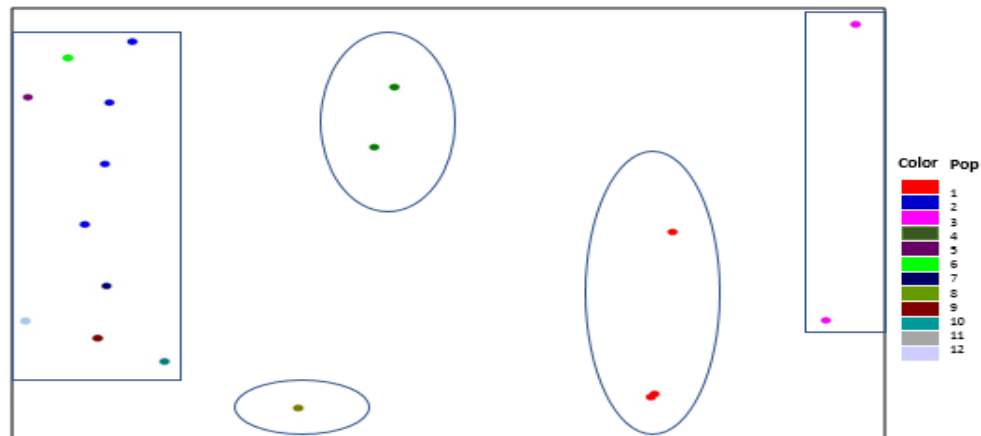


Fig. 5. PCoA plot of the studied populations based on SCoT data. Populations 1–12 are according to Table 1.

#### DISCUSSION

The sect. *Epilobium* included about 150 species, and about 90% of the genus species (RAVEN, 1976). This section has been delimited by combination of cytological ( $n = 18$ ), and morphological features like leaves opposite below, flowers actinomorphic, floral tube present, petals deeply notched, pollen shed in tetrads, etc. However it is known that these characters are not unique to this section only and may occur in the other sections of the genus *Epilobium* too (BAUM *et al.*, 1994). Therefore, it is concluded that the sect. *Epilobium* is not monophyletic (BAUM *et al.*, 1994).

*Epilobium minutiflorum* is one of the sect. *Epilobium* that recognize by fluffy white inflorescence and small miniature flowers and beaked seeds.

The present study revealed morphological variability of the studied *Epilobium minutiflorum* populations and showed some degree of morphological overlap among populations. Therefore morphometry could not differentiate and did not separate the populations. Population genetic study produces important information on the genetic structure of plants, the stratification versus gene flow among the species populations, genetic divergence of the populations, etc. (SHEIDAI *et al.*, 2014). However, assessing the discriminating power of SCoT markers in *Epilobium minutiflorum* populations studied revealed that these populations are highly differentiated ( $G_{st} = 0.95$ ). Gene flow ( $N_m$ ) among the studied populations was 0.02 that suggesting restricted gene flow among populations ( $N_m \ll 1$ ).

The SCoT marker technique is also efficient for genetic characterization even at the varietal level of a populations. For example, LAAME-JUIBARY *et al.* (2021) used 23 cultivars of sweet oranges available in Iran using SCoT marker. Similarly, TABASI *et al.* (2020) distinguished 20 populations of Persian walnut (*Juglans regia L.*) in Iran using SCoT markers. SABOORI *et al.* (2019) used SCoT molecular markers determined genetic fingerprinting of date palm (*Phoenix dactylifera L.*) cultivars.

Genetic study revealed that there are different genetic groups in the studied populations. Morphological study of the selected studied population showed that we have affinity between populations, Therefore, we suggest using other characters to differentiate populations of this species.

As a general result obtained of our study, SCoT marker is a good marker to evaluate genetic diversity, identify genotypes and can be used for populations delimitation and existence genetic diversity of *Epilobium minutiflorum*. However, the further molecular studies is necessary in confirm it.

### CONCLUSIONS

The present study showed the efficiency of SCoT molecular markers in evaluating the genetic diversity of *E. minutiflorum*. These markers can be used for genetic analysis of *Epilobium* species others. SCoT molecular markers have a good discrimination power and can differentiate the populations.

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## GENETIČKO PROUČAVANJE *Epilobium minutiflorum* (Onagraceae) U IRANU

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### Izvod

Rod *Epilobium* ima oko 200 vrsta kod kojih su taksonomske podele teške, ali moguće. Vrsta *E. minutiflorum* zbog svoje paperjaste bele cvasti i malih minijaturnih cvetova i kljunastog semena, jedna je od najistaknutijih vrsta roda *Epilobium* u regionu. Nemamo podataka o populacijskoj genetičkoj strukturi ove vrste u Iranu. Stoga je populaciono genetičko i morfološko ispitivanje sprovedeno kroz genetičke i morfološke varijabilnosti u ovim taksonima. Koristili smo SCoT molekularne markere. Analize genetičke raznovrsnosti otkrile su umerenu genetsku varijabilnost između populacija *E. minutiflorum*, dok je PCoA pokazao određeni stepen genetskih primesa kod populacija. AMOVA je proizvela značajnu genetsku razliku među populacijama. Morfometrijska analiza je pokazala visok stepen preklapanja među proučavanim populacijama. Međutim, rezultati su pokazali da SCoT marker ima dobru moć diskriminacije i može razlikovati proučavane populacije. Ovaj marker se može koristiti za procenu genetičke raznovrsnosti i identifikaciju genotipova populacija *E. minutiflorum*.

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