ISSN 0095-4527, Cytology and Genetics, 2018, Vol. 52, No. 4, pp. 309–321. © Allerton Press, Inc., 2018.

Morphometric and ISSR-Analysis of Local Populations of *Geranium molle* L. from the Southern Coast of the Caspian Sea[1]

S. Esfandani-Bozchaloyi[a], *, M. Sheidai[a], M. Keshavarzi[b], and Z. Noormohammadi[c]

[a] *Department of Plant Sciences, Faculty Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran*

[b] *Department of Plant Sciences, Faculty of Biological Science, Alzahra University, Tehran, Iran*

[c] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

**e-mail: somayehesfand@yahoo.com*

Received March 2, 2017

Abstract—*Geranium molle* is known as Dovefoot *Geranium* or Awnless *Geranium*. Dovefoot *Geranium* is a low-growing herb with pink flowers and sharply toothed leaves. Dovefoot *Geranium* is native to Eurasia and has been introduced to many habitats of the world. This species is very similar to *G. robertianum* but its palmate-like leaves and bilobed petals show differences. This plant is considered to be anodyne, astringent and vulnerary. We have no information on its population genetic structure, genetic diversity, and morphological variability in Iran. Therefore, due to the importance of these plant species, we performed a combination of morphological and molecular data for this species. For this study, we used 132 randomly collected plants from 18 geographical populations in 4 provinces. Genetic diversity parameters were determined in these populations. STRUCTURE analysis and K-Means clustering identified 14 gene pools in the country and revealed isolation by distance among the studied populations. The Mantel test showed correlation between genetic and geographical distance. AMOVA revealed a significant genetic difference among populations and showed that 40% of total genetic variation was due to within-population diversity. The consensus tree of both molecular and morphological data identified divergent populations. These data may be used in future breeding and conservation of this important medicinal plant in the country.

Keywords: Geranium molle, populations, genetic polymorphism, ISSR-analysis, morphometric characteristics, gene flow

DOI: 10.3103/S0095452718040102

INTRODUCTION

Population genetics produce significance data on the levels of genetic variation, the partitioning of genetic variability intra and inter populations, inbreeding, gene flow, self-pollination versus outcrossing, effective population size and population bottleneck. Population genetics data can help us to choose effective management strategies for conservation of endangered and/or invasive species [1, 2]. The genus *Geranium* L. comprises more than 400 species in different temperate habitats of the world [3]. The generic delimitation and a complete description of the genus are provided by Aedo [3–5]. *Geranium* is classified in 3 subgenera and 18 sections [4–7]. The genus has 23–25 species in Iran. These species are grouped in two subgenera and 8 sections [8–10].

Diagnostic features in intrageneric level related to fruit discharge methods, mericarp margin and leaves shape. *Geranium molle* L. belongs to section *Batrachioidea* of the subgenus *Robertium* [7, 11, 12], which is distributed in Europe, northern Africa, and western Asia [4, 5]. It is mainly found in northern parts of Iran at the southern coast of Caspian Sea. This species is a common weed of gardens, roadsides, pastures, disturbed sites and waste areas. It shows a great invasion in natural habitats, although generally seen as a potential environmental weed. *G. molle* is very similar to *G. robertianum* L., both species are in the subgenus *Robertium* that is characterized by the "carpel-projection" type of fruit discharge. *G. molle* differs from *G. robertianum* in having palmate leaves, bilobed petals, and smaller flowers [13]. Dovefoot *Geranium* is an annual or perennial herb with a low rate of growth. It has multiple stems and basal leaves. Branched stems grow erect to 9–60 cm tall. The stems are reddish towards the base and densely covered with white hairs. The hairy leaf stalks are longer in basal leaves and shorter in the cauline leaves. The leaves are dull green with ciliate edges. Self-fertilized flowers occur from April to September. It can tolerate a wide range of soil types but requires dry to very dry and well drained soils [14, 15]. There are no edible uses for *G. molle*. But it is considered to be anodyne, astringent and vulnerary. It has minerals, vitamins, antioxidants and fibre.

[1] The article is published in the original.

Table 1. Location and herbarium accession numbers of the studied populations of *G. molle* collected in Iran and East Azerbaijan

Population	Locality	Longitude	Latitude	Altitude, m	Voucher no.
1	Mazandaran, Siah bisheh to Chalus	36°14′14″	51°18′07″	1807	HSBU 201648
2	Guilan, Bandar Anzali, Road sid	37°27′48″	49°33′20″	−23	HSBU 201649
3	Mazandaran, Amol	36°27′42″	52°21′00″	103	HSBU 201650
4	Mazandaran, Nur	36°34′24″	52°00′50″	−20	HSBU 201651
5	Mazandaran, Noshahr, Kheyrud kenar Forest	36°38′05″	51°29′05″	−16	HSBU 201652
6	Mazandaran, Babol	36°20′53″	52°30′20″	606	HSBU 201653
7	Guilan,Gole rodbar	37°09′55″	49°55′49″	15	HSBU 201654
8	Mazandaran, Noshahr,	36°35′04″	51°48′24″	−29	HSBU 201655
9	Mazandaran, Shirgah	36°14′47″	52°54′20″	466	HSBU 201656
10	Mazandaran, SangDeh Forest	36°01′11″	53°13′26″	1720	HSBU 201657
11	Guilan, Siahkal, Ezbaram	37°07′48″	49°54′04″	63	HSBU 201658
12	Guilan, Siahkal, Ezbaram	37°07′08″	49°54′11″	165	HSBU 201659
13	Mazandaran, Qaem shahr	36°28′17″	52°51′10″	51	HSBU 201660
14	Golestan, Ramian	37°0′8′23″	55°8′50.73″	1320	HSBU 201661
15	East Azerbaijan, kaleybar, cheshme ali akbar	38°22′08″	48°65′07″	1370	HSBU 201662
16	East Azerbaijan, kaleybar, Shojabad	38°52′393″	47°25′92″	1133	HSBU 201663
17	Mazandaran, Tonekabon, Shirod	37°08′23″	55°8′50.73″	−16	HSBU 201664
18	Mazandaran, Tuska Cheshmeh	36°38′1952″	53°48′56.9″	1427	HSBU 201665

Molecular markers are useful in identifying the maximally diverse parental genotypes through an evaluation of genetic diversity which is useful in cultivar identification and breeding. Among the various molecular markers, Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) are simple and quick techniques and have become popular as their application does not need any prior information about the target sequences in the genome, high-efficiency and sharp sensibility, and these techniques have now been widely used for line identification and genetic diversity. These markers have been used in DNA fingerprinting, conservation biology, identification and determination of species relationships in many plants [16, 17]. *G. molle* grows in different regions of Iran and comprises many local geographical populations. Therefore, the present study was performed for the first time in the country to investigate the genetic structure and diversity of these local populations. The results of present study may have positive effect on conservation strategy and future breeding of this medicinally important plant. We used ISSR molecular markers in the present study as these markers were shown to be informative for genetic diversity and population structure studies [18–20].

MATERIALS AND METHODS

Plant Materials

For morphometric studies we used 132 plant specimens (5–12 samples from each populations), and for ISSR analysis, we used 109.

Different references were used for the correct identification of species (*G. molle*), [4, 8, 10, 21, 22]. Details of sampling sites are mentioned (Table 1, Fig. 1). Voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU).

Morphological Studies

In total 80 morphological (42 qualitative, 38 quantitative) characters were studied. 5–12 plants from each populations were randomly studied for morphological analyses (Table 2).

Fig. 1. Map of distribution of populations *G. molle* in Iran and East Azerbaijan region.

DNA Extraction and ISSR Analyses

Fresh leaves were used randomly from 5–10 plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA [20]. The quality of extracted DNA was examined by running on 0.8% agarose gel. 10 ISSR primers; $(AGC)_5GT$, $(CA)_7GT$, $(AGC)_5GG$, UBC810, $(CA)_7AT$, $(GA)_9C$, UBC807, UBC811, $(GA)_9T$ and $(GT)_7CA$ commercialized by UBC (the University of British Columbia) were used. PCR reactions were carried in a 25 µl 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 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 52—57°C and 2 min at 72°C. The reaction was completed by final extension step of 7–10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

DATA ANALYSES

Morphological Studies

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa [23]. For grouping of the plant specimens, the UPGMA (Unweighted paired group using average) and Ward (Minimum spherical characters) as well as ordination methods of MDS (Multidimensional scaling) and PCoA (Principal coordinate analysis) were used [23]. ANOVA (Analysis of variance) were performed to show morphological difference among the populations while, PCA (Principal components analysis) biplot was used to identify the most variable morphological characters among the studied populations [23]. PAST version 2.17 [24] was used for multivariate statistical analyses of morphological data.

Table 2. Evaluated morphological characters for populations of *G. molle*

No	Characters	No	Characters
1	Plant height (mm)	20	Mericarp length (mm)
2	Length of stem leaves petiole (mm)	21	Mericarp width (mm)
3	Length of stem leaves (mm)	22	Mericarp length/ Mericarp width (mm)
4	Width of stem leaves (mm)	23	Seed length (mm)
5	Length of stem leaves/Width of stem leaves(mm)	24	Seed width (mm)
6	Width of stem leaves/Length of stem leaves (mm)	25	Seed length/ Seed width(mm)
7	Number of segment stem leaves (mm)	26	Stipules length (mm)
8	Length of basal leaves petiole (mm)	27	Stipules width (mm)
9	Length of basal leaves (mm)	28	Stipules length/ Stipules width (mm)
10	Width of basal leaves (mm)	29	Bract length (mm)
11	Length of basal leaves/Width of basal leaves (mm)	30	Bract width (mm)
12	Width of basal leaves/Length of basal leaves (mm)	31	Bract length / Bract width (mm)
13	Number of segment basal leaves	32	Pedicel length (mm)
14	Calyx length (mm)	33	Peduncle length (mm)
15	Calyx width (mm)	34	Rostrum length (mm)
16	Calyx length/Calyx width (mm)	35	Style length (mm)
17	Petal length (mm)	36	Stamen filament length (mm)
18	Petal width (mm)	37	Fruit length (mm)
19	Petal length/Petal width (mm)	38	Number of flowers per inflorescence
39	Type root	60	Bract shape
40	Vegetation-forms	61	Stipules shape
41	State of stem strength	62	Bract and Stipules hair density
42	State of stem branches	63	Bract and Stipules hair
43	Leave shape	64	Shape of segments cauline leaves
44	Phyllotaxy	65	Shape of calyx
45	Leaf tips	66	Calyx apex
46	Shape of segments basal leaves	67	Petal shape
47	Stamen filament color	68	State of petale ligule
48	Stigma hair	69	Shape of petal lobes
49	Mericarp shape	70	State of petale ligule hair
50	Mericarp surface	71	Stamen filament hair
51	Mericarp hair	72	Mericarp hair density
52	Mericarp Rostrum hair	73	Mericarp color
53	Sepale hair	74	Seed color
54	Sepale hair density	75	Seed shape
55	Peduncle and pedicel hair	76	Seed surface ornamentation
56	Anthers colour	77	Peduncle and pedicel hair density
57	Stem hair	78	Petioles hair
58	Stem hair density	79	Petioles hair density
59	Leaf hair	80	Leaf hair density

Molecular Analyses

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were determined [25, 26]. Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking [26, 27].

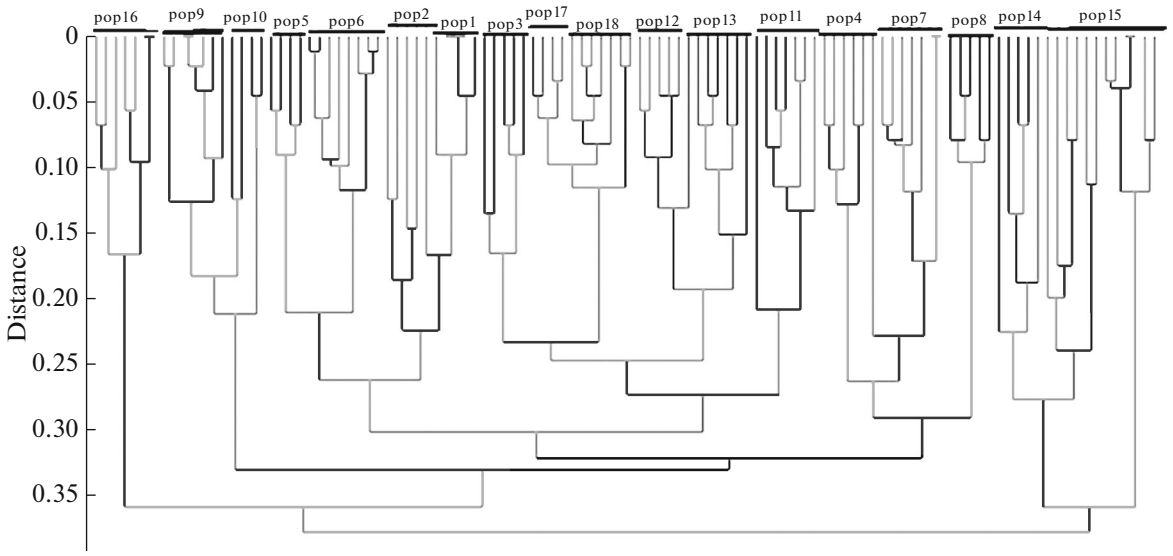

Fig. 2. UPGMA tree of morphological data in *Geranium molle* populations studied.

The Mantel test was performe as implemented in PAST ver. 2.17 [23, 24]. For this, Nei genetic distance was determined for ISSR data, while Geographic distance of PAST was determined for geographical data. It is calculated based on the sum of the paired differences among both longitude as well as latitude coordinates of the studied populations.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 [28] and Nei,s Gst analysis as implemented in GenoDive ver.2 (2013) [29] were used to show genetic difference of the populations. Moreover, populations, genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation [30] and D_est = Jost measure of differentiation [31].

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis [32] and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers [33]. The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value [34]. In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k [35].

Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: $Nm = 0.5(1 - Gst)/Gst$. This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

RESULTS

Morphometry

In present study 132 plant samples were collected from 18 geographical populations. ANOVA test revealed significant difference in quantitative morphological characters among the studied populations ($P < 0.05$). Clustering and PCoA plot of *G. molle* populations based on morphological characters produced similar results therefore only UPGMA is presented and discussed (Fig. 2). In general, two major clusters were formed in UPGMA tree 1–13, 16 populations showed morphological similarity and were placed in the first major cluster. The population no. 15 has subdivision on two subclusters, but one of subclusters showed higher degree of morphological similarity with population no. 14 and formed the second major cluster.

The result showed morphological difference/divergence among most of the studied populations. This morphological difference was due to quantitative characters only. For example, population no. 16 has the shortest stem and basal-leaf length (1 cm), the shortest basal-leaf width (1.5 cm), the narrowest petal and calyx length (3.5 mm), among the studied populations. Similarly, population no. 3 had the longest stem-leaf length (2 cm), the broadest basal-leaf width (4 cm) and the longest petal length (6.3 mm), besides character as (petiole length of Basal leaves), separate population no. 7 form the other, while pedicel length separate population no. 6 from the other populations.

Populations' Genetic Diversity

Genetic diversity parameters determined in 18 geographical populations of *G. molle* are presented in Table 3. The highest value of percentage polymor-

Table 3. Genetic diversity parameters in the studied populations of *G. molle* (N = number of samples, Na = Number of different alleles, Ne = number of effective alleles, I = Shannon's information index, He = genetic diversity, UHe = unbiased gene diversity, P (%) = percentage of polymorphism, populations)

Pop	N	Na	Ne	I	He	UHe	%P
pop1	5	0.427	1.037	0.043	0.026	0.029	10.11%
pop2	5	0.831	1.185	0.172	0.113	0.125	34.83%
pop3	5	0.596	1.167	0.144	0.097	0.108	25.84%
pop4	6	0.685	1.144	0.134	0.087	0.095	26.97%
pop5	4	0.629	1.128	0.118	0.078	0.089	22.47%
pop6	8	0.506	1.133	0.112	0.076	0.081	19.10%
pop7	7	0.708	1.165	0.143	0.095	0.103	28.09%
pop8	5	0.629	1.087	0.085	0.054	0.060	17.98%
pop9	7	0.629	1.097	0.095	0.060	0.065	21.35%
pop10	4	0.764	1.189	0.169	0.113	0.129	30.34%
pop11	7	0.787	1.161	0.151	0.098	0.105	32.58%
pop12	6	0.921	1.239	0.209	0.139	0.151	41.57%
pop13	6	0.663	1.113	0.114	0.072	0.078	25.84%
pop14	5	0.888	1.180	0.174	0.112	0.124	37.08%
pop15	11	1.315	1.312	0.289	0.190	0.199	58.43%
pop16	7	0.753	1.171	0.148	0.100	0.107	26.97%
pop17	5	0.472	1.061	0.060	0.039	0.043	12.36%
pop18	6	0.517	1.063	0.069	0.043	0.047	15.73%

phism (58.43%) was observed in East Azerbaijan kaleybar (population no. 15) which shows high value for genetic diversity (0.19). and Shanon information index (0.28). Population Mazandaran, Siah bisheh to Chalus (no. 1) has the lowest value for percentage of polymorphism (10.11%) and the lowest value for Shanon, information index (0.043), and He (0.026).

Population Genetic Differentiation

AMOVA (PhiPT = 0.59, P = 0.010) revealed significant difference among the studied populations. It also revealed that, 40% of total genetic variability was due to within population diversity and 60% was due to among population genetic differentiation (Table 4, Fig. 3). Pairwise AMOVA produced significant difference among the studied populations. Moreover, we got high values for Hedrick standardized fixation index after 999 permutation (G'st = 0.373, P = 0.001) and Jost, differentiation index (D-est = 0.435, P = 0.001). These results indicate that the geographical populations of *G. molle* are genetically differentiated from each other.

Table 4. AMOVA test of the studied populations *G. molle*

Source	df	SS	MS	Est. Var.	%
Among Pops	17	990.045	58.238	8.683	60%
Within Pops	91	534.671	5.876	5.876	40%
Total	108	1524.716		14.559	100%

Non-metric MDS plots of ISSR data (Fig. 4) showed both intra- and inter-population genetic diversity. The plots showed higher within population genetic diversity in the population no. 15 (East Azerbaijan kaleybar), supporting genetic diversity parameters obtained (Table 3). Genetic divergence and separation of populations 1–3, 5, 11, 8 as well as 15 and 16 from the other populations is evident in MDS plot of ISSR data after 900 permutations (Fig. 4). The other populations as population nos. 12 and 13, show some genetic similarity and overlapping as well as populations nos. 17 and 18, the overlapping occurs to a lesser extent for populations nos. 9 and 10, populations nos. 6 and 14, pop. nos. 4 and 7.

Mantel test after 5000 permutations produced significant correlation between genetic distance and geographical distance in these populations ($r = 0.41$, $P = 0.0002$). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *G. molle*.

Population's Grouping Based on Genetic Data

The pairwise comparisons of "Nei genetic identity" among the studied populations *G. molle* (Table 5)

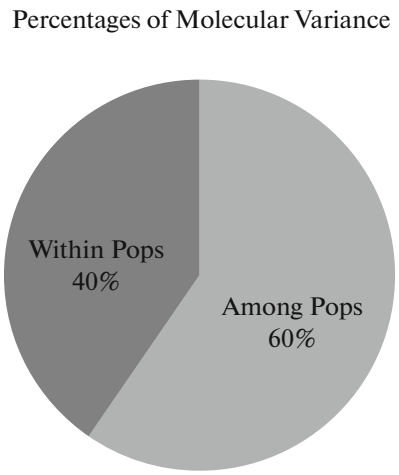

Fig. 3. AMOVA test of *G. molle* populations based on ISSR data.

have shown a higher a genetic similarity (0.95) between populations Mazandaran, Tonekabon, Shirod (pop. no. 17) and Mazandaran, Tuska Cheshmeh (pop. no. 18), while the lowest genetic similarity value (0.62) occurs between East Azerbaijan kaleybar (pop. no. 16) and Mazandaran, Noshahr populations (pop. no. 8).

Populations Genetic Similarity

Grouping of the studied populations by NJ tree is presented in Fig. 5. In general three major clusters were formed. Populations of Golestan, Ramian and East Azerbaijan kaleybar populations (pop. nos. 14–16) formed the first major cluster and showed higher degree of genetic similarity, supporting our morphological result presented before (Fig. 2). The second major cluster contained two sub-clusters, plants of populations nos. 9 and 10 comprised the first sub-cluster both from Mazandaran, second sub-cluster was formed by populations nos. 1, 2 and 5, 6. The other populations comprised the third major cluster and were distributed into two subclusters. Populations nos. 7, 8 and 4 which comprised the first sub-cluster, while populations nos. 11–13 and 17, 18, 3 comprised the second sub-cluster.

Among-population differentiation in phenotypic traits and allelic variation is expected to occur as a consequence of isolation, drift, founder effects and local selection [36].

We have almost complete separation of the studied population in the network, supporting AMOVA result. The populations nos. 1–6 and 11, 16 are distinct and stand separate from the other populations with great distance. The populations nos. 7 and 8, as well as populations 12, 13 and 17, 18 show closer genetic similarity and are placed close to each other. Also, the other populations as population nos. 9, 10 and 14, 15 show some genetic similarity (Fig. 6).

Populations Genetic Structure

The Evanno test produced delta $k = 14$ as the best number of genetic groups. A STRUCTURE plot based on $k = 14$ in presented in Fig. 7. The STRUCTURE plot has revealed the allele combination difference among the studied populations and the occurrence of genetic admixture among them.

This genetic grouping is in agreement with Neighbor Net diagram (Fig. 6), presented before. Therefore, 14 gene pools are identified for *Geranium molle* in the country. Although the studied populations contained some specific alleles for example populations nos. 1–6 and 11, 16 (differently colored segments in Fig. 7), they shared some similar alleles too. For example, it

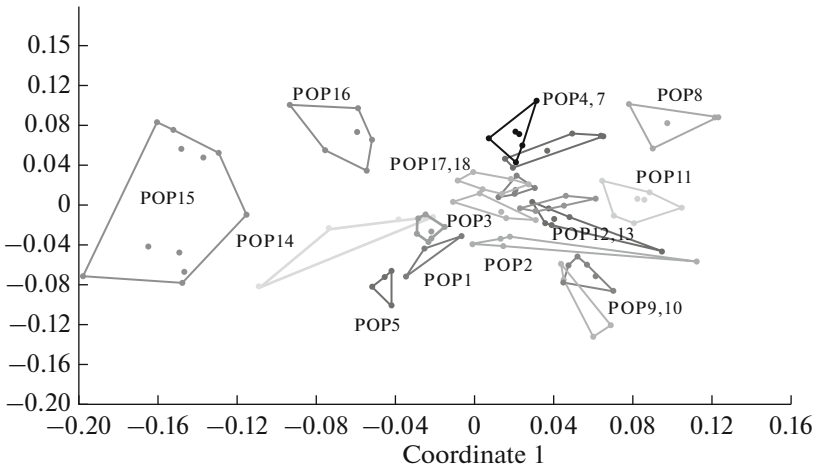

Fig. 4. MDS plot of *G. molle* populations based on ISSR data. Note: Population numbers are according to Table 1.

Table 5. Pairwise Population Matrix of Nei Genetic Identity among the studied populations *G. molle*

pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	pop9	pop10	pop11	pop12	pop13	pop14	pop15	pop16	pop17	pop18	
1.000																		pop1
0.874	1.000																	pop2
0.802	0.848	1.000																pop3
0.751	0.779	0.839	1.000															pop4
0.730	0.800	0.819	0.782	1.000														pop5
0.852	0.853	0.844	0.814	0.750	1.000													pop6
0.712	0.831	0.798	0.828	0.762	0.794	1.000												pop7
0.624	0.758	0.748	0.714	0.691	0.681	0.828	1.000											pop8
0.727	0.810	0.804	0.743	0.765	0.742	0.745	0.699	1.000										pop9
0.712	0.803	0.831	0.747	0.769	0.819	0.742	0.709	0.890	1.000									pop10
0.743	0.794	0.841	0.817	0.822	0.768	0.773	0.735	0.796	0.774	1.000								pop11
0.786	0.871	0.854	0.774	0.815	0.800	0.806	0.738	0.799	0.801	0.863	1.000							pop12
0.792	0.819	0.858	0.794	0.802	0.786	0.798	0.721	0.781	0.772	0.858	0.916	1.000						pop13
0.780	0.855	0.862	0.800	0.774	0.862	0.797	0.662	0.745	0.778	0.758	0.851	0.817	1.000					pop14
0.701	0.771	0.789	0.708	0.730	0.777	0.765	0.666	0.728	0.737	0.747	0.796	0.799	0.853	1.000				pop15
0.729	0.750	0.764	0.733	0.731	0.765	0.756	0.621	0.745	0.739	0.740	0.730	0.748	0.743	0.740	1.000			pop16
0.787	0.833	0.844	0.814	0.796	0.805	0.764	0.712	0.731	0.739	0.821	0.841	0.809	0.796	0.682	0.760	1.000		pop17
0.759	0.808	0.850	0.780	0.787	0.814	0.794	0.723	0.760	0.772	0.816	0.845	0.832	0.801	0.719	0.814	0.946	1.000	pop18

showed genetic similarity between populations nos. 7 and 8 (similarly colored), as well as 17, 18 and 12, 13. The plants of population no. 9 had some similar alleles for population no. 10: population no. 14 had some similar alleles for population 15. Moreover, mean Nm value of 0.23 was obtained for the studied populations that showed low value of gene flow, supporting STRUCTURE plot result.

However, reticulogram obtained based on the least square method (Fig. 8), revealed some amount of shared alleles among populations nos. 16 and 17, 18 and between nos. 9, 10 and 11 also between no. 15 and 7, 8 and between nos. 1 and 5, 6. This result is in agreement with grouping we obtained with Neighbor-Net, as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are in agreement in showing high degree of genetic stratification within *G. molle* populations.

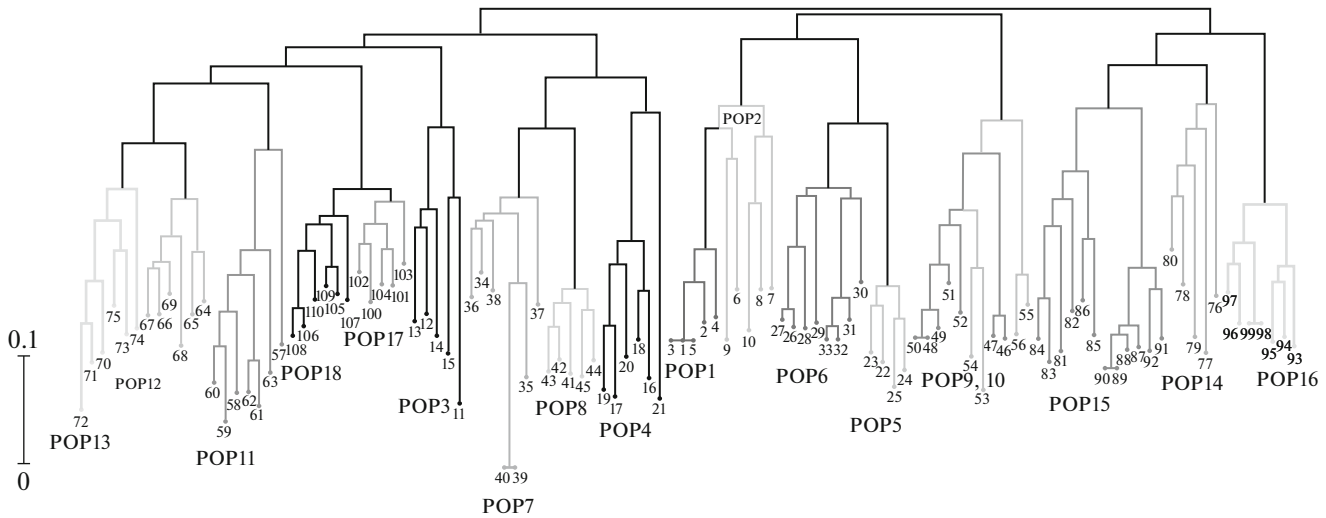

Fig. 5. NJ tree of ISSR data in *G. molle* populations studied.

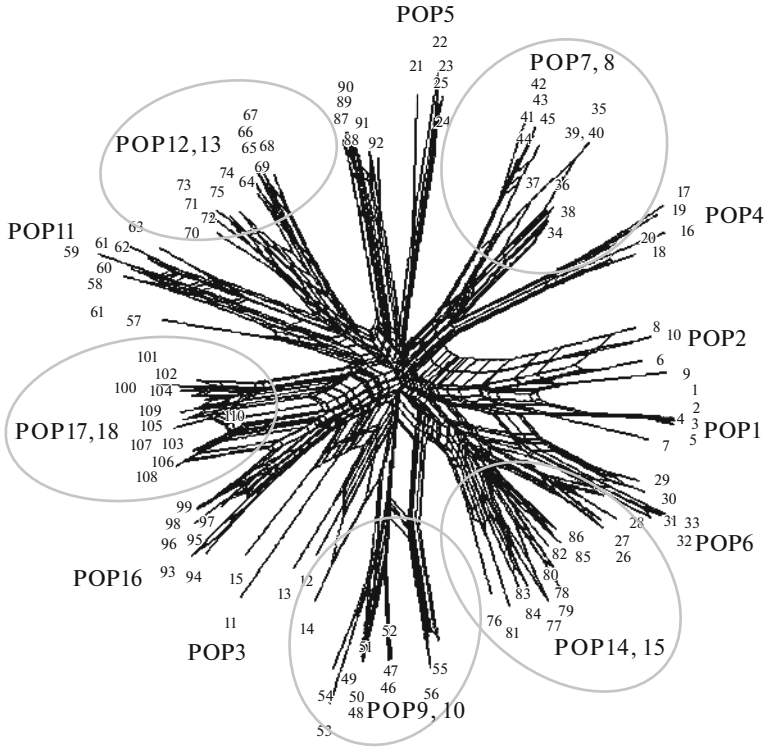

Fig. 6. Neighbor Net diagram of ISSR data.

A consensus tree was obtained for both ISSR and morphological trees (Fig. 9), to reveal the populations that are diverged based on both morphological and molecular features.

DISCUSSION

The distribution of plant species is determined by various factors, such as physiological limitations, ecological niche, and geological history [38]. Plant populations in the center of their distribution generally sustain stable conditions in a desirable environment for the species, while location at the margin of their distribution are exposed to less suitable location for establishment and to greater competition with other species than at their distribution center [39, 40].

Population genetic study provides valuable information about genetic structure of plants, the stratification versus gene flow among the species populations, genetic divergence of the populations, etc. [20]. These information have different applications, and from pure understanding of biology of the species to conservation of endangered species, choosing of proper parents for hybridization and breeding and phylogeography and mechanism of invasion [26].

Geranium molle mainly found in northern parts of Iran at the southern coast of Caspian Sea. The present study revealed interesting data about its genetic variability, genetic stratification and morphological divergence in north part of Iran.

The studied populations have a low level of genetic diversity (He = 0.026–0.190). The Genetic diversity is of fundamental importance in the continuity of a species as it is used to bring about the necessary adaptation to the cope with changes in the environment [19, 20, 41]. Degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/cross breeding brings about higher level of genetic variability in the studied taxon [26]. *G. molle* is mainly self-pollinating species [37], therefore, low level of genetic variability within populations in this species might be related to its selfing nature. The mean Nm = 0.23 was obtained for investigated ISSR loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by Neighbor-Net diagram and STRUCTURE analyses.

Similarly, the lower level of genetic variability occurred in *Geranium* species with limited geographical distribution and probably more selfing capabilities. *G. stepporum* Davis and *G. tuberosum* L. had the lowest level of genetic polymorphism (2.15%). Each of these species have a confined geographical distribution in the country and occur only in one province and low genetic variability may also occur due to small size of the populations 10<, in *G. mascatense* had the lowest

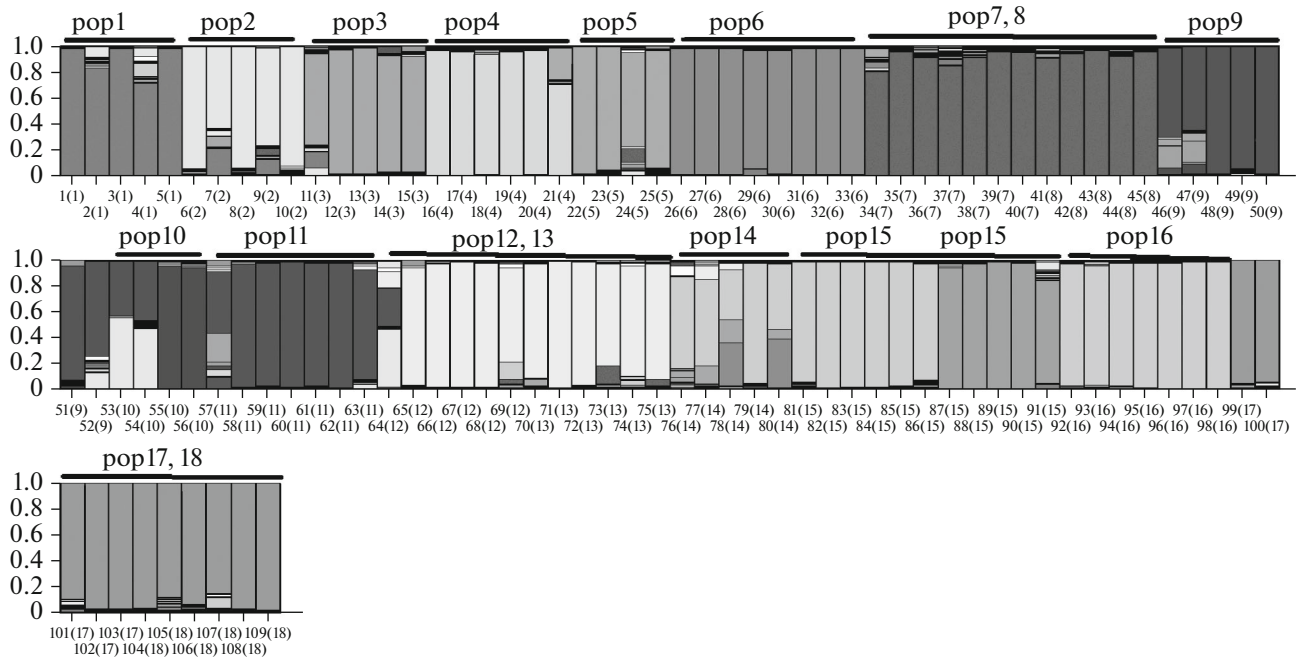

Fig. 7. STRUCTURE plot of *G. molle* populations based on k = 16 of ISSR data.

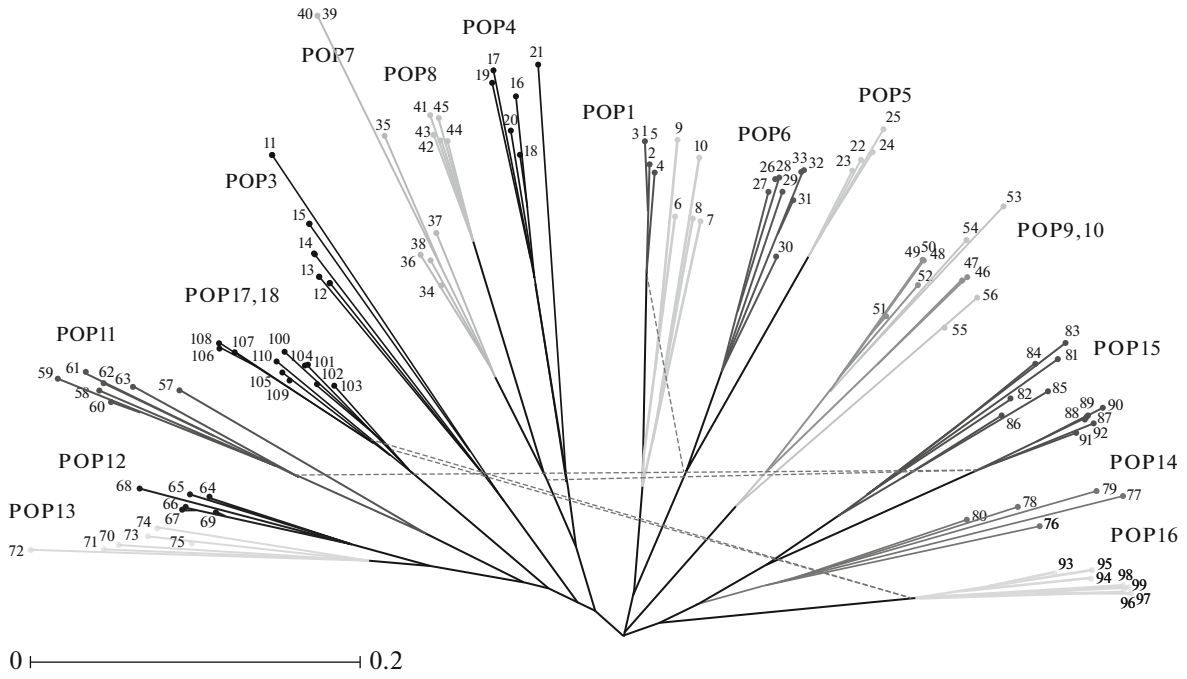

Fig. 8. Reticulogram of *G. molle* populations based on least square method analysis of ISSR data. (Numbers below branches are population number according to Table 1).

level of genetic polymorphism (2.15%) not only have small size of the populations 10<, but also is inbreeding due to annual with small flowers [42].

Low genetic variability may also occur due to small size of the populations and genetic drift [37]. These species tend to perform inbreeding as also evidenced

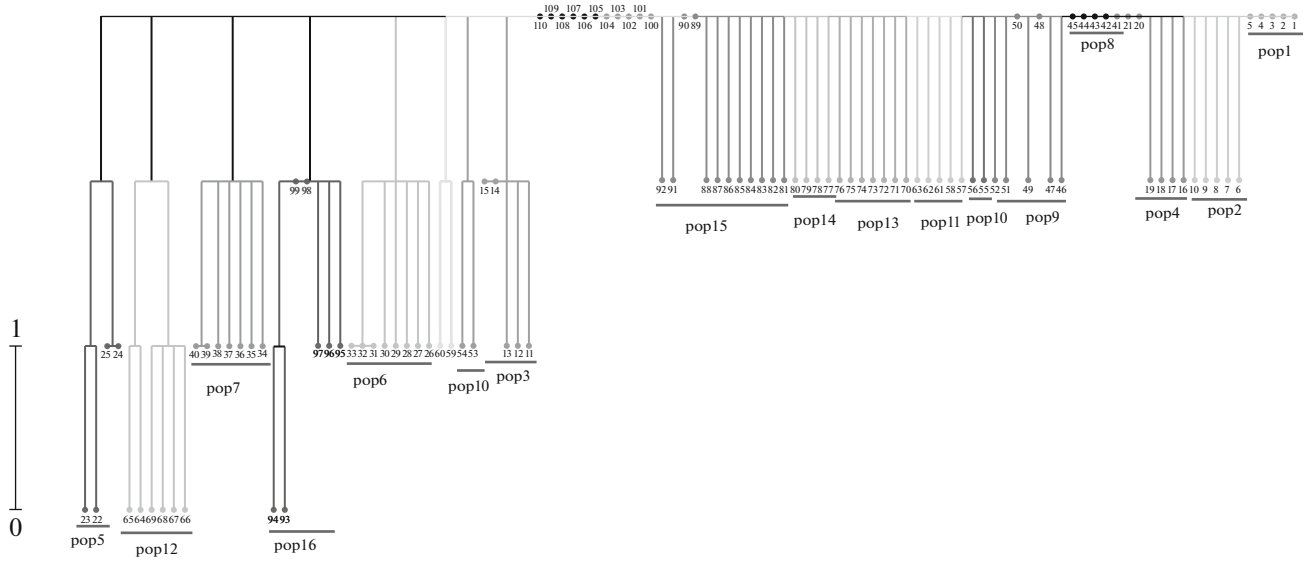

Fig. 9. Consensus tree of morphological and molecular data in *G. molle* populations.

by very low Nm value and IBD obtained for the studied species. However, limited gene flow was not solely due to geographical distance among the species, but some of the species, which grew in adjacent areas with overlapping zones, did not form any hybrids or intermediate forms as evidenced by morphological and ISSR clusters obtained [42].

Another well-known feature of self-pollinating species is high among-population genetic and morphological divergence. This happens due to limited amount of gene flow or its complete absence among geographical population in a single species [26]. The present study also revealed significant morphological and genetic difference among *G. molle* populations, quite in agreement with the mentioned assumption. This is particularly supported by STRUCTURE plot that identified 14 separate genetic groups within this populations and by consensus tree of both morphological and genetic data. Different mechanisms like isolation, drift, founder effects and local selection may act to bring about among population differentiation and therefore, populations differ in phenotypic traits and allelic composition [36].

The present population divergence may be under influence of isolation-by distance across the distribution range of the studied *G. molle* populations. The dispersal of these populations might be constrained by distance and 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 [43–45]. The populations, divergence may be accompanied by local adaptation. When we use multilocus molecular markers (such as SSR, AFLP, RAPD, ISSR, etc.) for population genetic studies we understand that these are neutral molecular markers (they are not directly acting as adaptive genes), but they may be linked to a gene or a genetic region with adaptive value [26]. Therefore, combination of genetic divergence, limited gene flow and local adaptation have played role in diversification of *G. molle* population in the country. An ecotype is a distinct set of genotypes (or populations) within a species, resulting from adaptation to local environmental conditions, but which are capable of inter breeding with other ecotypes of the same species [46]. Ecotypes are populations adapted to local conditions, at a variety of spatial and temporal scales. Creation of a ecotype is very important because this type of variation has adaptive value for each species and increases biodiversity in the ecosystems. Matching ecotypes to local conditions increases restoration success. A practical value of recognizing ecotypic variation in grasses is in identifying the most suitable ecotypes for conservation, restoration, renovation, landscaping, and bioremediation [47–51].

Based on the above definition, some ecotypes were distinguished within the studied populations. For example some of these populations had distinct morphological traits and also genetic content, such as populations nos. 3, 5–7 and 12, 16 were genetically differentiated (Fig. 9). A consensus tree was obtained for both ISSR and morphological trees divergence of almost the most populations at molecular level as well as morphological characteristics (Fig. 9). Detailed comparison of the characteristics in these populations revealed noticeable morphometric differences described above for populations nos. 16, 3, 7, 5. But we should state that, the studied populations differed in quantitative morphological characters and we do not know how much of the morphological difference

among the studied populations is genetically controlled; they may be under influence of environmental conditions. Therefore we do not attempt to suggest new taxonomic forms bellow the species level for this taxon and consider them as different ecotypes only.

ACKNOWLEDGMENTS

I would like to thank Mokhtar Hasanzadeh for his assistance in collection of plant from the wild.

REFERENCES

1. Chen, X.Y., Effects of fragmentation on genetic structure of plant populations and implications for the biodiversity conservation, *Acta. Ecol. Sin*., 2000, vol. 20, pp. 884–892.
2. Ellis, J.R. and Burke, J.M., EST-SSRs as a resource for population genetic analyses, *Heredity*, 2007, vol. 99, no. 2, pp. 125–132.
3. Aedo, C., Revision of *Geranium* subgenus *Erodioidea* (Geraniaceae), *Syst. Bot. Monogr.*, 1996, vol. 49, pp. 1–104.
4. Aedo, C., Garmendia, F.M., and Pando, F., World checklist of *Geranium* L. (Geraniaceae), *Anales. Jard. Bot. Madrid*, 1998, vol. 56, pp. 211–252.
5. Aedo, C., Aldasoro, J.J., and Navarro, C., Taxonomic revision of *Geranium* L., sections Divaricata Rouy and Batrachioidea W.D.J. Koch (Geraniaceae), *Ann. Miss. Bot. Gard.*, 1998, vol. 85, pp. 594–630.
6. Mabberley, D.J., *Mabberley's Plant Book: A Portable Dictionary of Plants, Their Classification and Uses*, Cambridge: Cambridge Univ. Press, 2009.
7. Yeo, P.F., Fruit-discharge-type in *Geranium* (Geraniaceae): its use in classification and its evolutionary implications, *Bot. J. Linn. Soc.*, 1984, vol. 89, no. 1, pp. 1–36. org/ doi 10.1111/j.1095-8339.1984.tb00998.x
8. Janighorban, M., *Flora of Iran, Geraniaceae*, The Research Institute of Forests and Rangelands, 2009, vol. 62.
9. Onsori, S., Salimpour, F., and Mazooji, A., The new record of *Geranium linearilobium* Dc. based on anatomy and micromorphological study of pollen and seed, in Iran, *J. Plant Environ. Physiol.*, 2010, vol. 5, no. 3, pp. 21–30.
10. Schonbeck-Temesy, E., Geraniaceae, in *Flora Iranica*, Rechinger, K.H., Ed., Graz, Austria: Akademische Druck, 1970, vol. 69, pp. 30–58.
11. Yeo, P.F., The classification of Geraniaceae, in *Proceeding of the International Geraniaceae Symposium, Univ. of Stellenbosch, RSA*, Vorster, P., Ed., 1990, pp. 1–22.
12. Price, R.A. and Palmer, J.D., Phylogenetic relationships of the Geraniaceae and Geraniales from rbcL sequence comparisons, *Ann. Missouri Bot. Gard.*, 1993, vol. 80, no. 3, pp. 661–671.
13. Chen, C. and Wang, C., *Geranium molle* L. (Geraniaceae), a newly naturalized plant in Taiwan, *Coll. Res.*, 2005, vol. 18, pp. 11–14.
14. Pilorge, E. and Mircovich, C., Weed control strategies using GMO herbicide tolerant oilseed rape, in *Proc. 10th Int. Rapes. Congress, Canberra, Australia*, 1999.
15. Roberts, H.A. and Boddrell, J.E., Seed survival and seasonal emergence in some species of *Geranium, Ranunculus* and *Rumex* (abstract), *Ann. Appl. Biol.*, 1985, vol. 107, no. 2, pp. 231–238. org/ doi 10.1111/j.1744-7348.1985.tb01566.x
16. Martin, J.P. and Sanchez-Yelamo, M.D., Genetic relationships among species of the genus *Diplotaxis* (Brassicaceae) using inter-simple sequence repeat markers, *Theor. Appl. Genet.*, 2000, vol. 101, no. 8, pp. 1234–1241. doi 10.1007/s001220051602
17. Manica-Cattani, M.F., Zacaria, J., Pauletti, G., Atti-Serafini, L., and Echeverrigaray, S., Genetic variation among South Brazilian accessions of *Lippia alba* Mill. (Verbenaceae) detected by ISSR and RAPD markers, *Braz. J. Biol.*, 2009, vol. 69, no. 2, pp. 375–80.
18. Azizi, N., Sheidai, M., Mozafarian, V., and Noormohammadi, Z., Genetic, cytogenetic and morphological diversity in *Helicrysum leucocephalum* (Asteraceae) populations, *Biologia*, 2014, vol. 69, no. 5, pp. 566–573.
19. Sheidai, M., Seif, E., Nouroozi, M., and Noormohammadi, Z., Cytogenetic and molecular diversity of *Cirsium arvense* (Asteraceae) populations in Iran, *J. Jpn. Bot.*, 2012, vol. 87, pp. 193–205.
20. Sheidai, M., Ziaee, S., Farahani, F., Talebi, S.M., Noormohammadi, Z., and Hasheminejad-Ahangarani Farahani, Y., Intra-specific genetic and morphological diversity in *Linum album* (Linaceae), *Biologia*, 2014, vol. 69, no. 1, pp. 32–39. org/ doi 10.2478/s11756-013-0281-4
21. Zohary, M., *Flora Palaestina. Platanaceae to Umbelliferae*, Jerusalem, Israel: The Israel Academy of Sciences and Humanities, 1972.
22. Davis, P.H., Geraniacea, in *Flora of Turkey and East Aegean Islands*, 1965, vol. 2, pp. 450–488.
23. Podani, J., *Introduction to the Exploration of Multivariate Data*, Leiden: Backhuyes Publ., 2000.
24. Hammer, Q., Harper, D.A.T., and Ryan, P.D., PAST: Paleontological Statistics software package for education and data analysis, *Palaeontol. Electr.*, 2001, vol. 4, no. 1, pp. 1–9.
25. Weising, K., Nybom, H., Wolff, K., and Kahl, G., *DNA Fingerprinting in Plants. Principles, Methods, and Applications*, 2nd ed., Boca Raton: CRC Press, 2005.
26. Freeland, J.R., Kirk, H., and Peterson, S.D., *Molecular Ecology*, 2nd ed., UK: Wiley-Blackwell, 2011.
27. Huson, D.H. and Bryant, D., Application of phylogenetic networks in evolutionary studies, *Mol. Biol. Evol.*, 2006, vol. 23, no. 2, pp. 254–267. doi 10.1093/molbev/msj03
28. Peakall, R. and Smouse, P.E., GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research, *Mol. Ecol. Notes*, 2006, vol. 6, pp. 288–295.
29. Meirmans, P.G. and Van Tienderen, P.H., GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms, *Mol. Ecol. Notes*, 2004, vol. 4, pp. 792–794.

30. Hedrick, P.W., A standardized genetic differentiation measure, *Evolution*, 2005, vol. 59, no. 8, pp. 1633–1638.

31. Jost, L., GST and its relatives do not measure differentiation, *Mol. Ecol.*, 2008, vol. 17, pp. 4015–4026.

32. Pritchard, J.K., Stephens, M., and Donnelly, P., Inference of population structure using multilocus genotype data, *Genetics*, 2000, vol. 155, no. 2, pp. 945–959.

33. Falush, D., Stephens, M., and Pritchard, J.K., Inference of population structure using multilocus genotype data: dominant markers and null alleles, *Mol. Ecol. Notes*, 2007, vol. 7, no. 4, pp. 574–578.

34. Evanno, G., Regnaut, S., and Goudet, J., Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study, *Mol. Ecol.*, 2005, vol. 14, no. 8, pp. 2611–2620. doi 10.1111/j.1365-294X.2005.02553.x

35. Meirmans, P.G., AMOVA-based clustering of population genetic data, *J. Heredity*, 2012, vol. 103, no. 5, pp. 744–750. doi 10.1093/jhered/ess047

36. Jolivet, C. and Bernasconi, G., Molecular and quantitative genetic differentiation in European populations of *Silene latifolia* (Caryophyllaceae), *Ann. Bot.*, 2007, vol. 100, no. 1, pp. 119–127. doi 10.1093/aob/mcm088

37. Baker, H.G., *Geranium purpureum* Vill. and *G. robertianum* L. in the British flora. II: *Geranium purpureum, Watsonia*, 1955, vol. 3, pp. 160–167.

38. Heuertz, M., Hausman, J.F., Hardy, O.J., Vendramin, G.G., Franscaria-Lacoste, N., and Vekemans, X., Nuclear microsatellites reveal contrasting patterns of genetic structure between western and southeastern European populations of the common ash (*Fraxinus excelsior* L.), *Evolution*, 2004, vol. 58, pp. 976–988.

39. Lawton, J.H., Range, population abundance and conservation, *Trends Ecol. Evol.*, 1993, vol. 8, no. 11, pp. 409–413.

40. Hampe, A. and Petit, R.J., Conserving biodiversity under climate change: the rear edge matters, *Ecol. Lett.*, 2005, vol. 8, no. 5, pp. 461–467.

41. Caliskan, M., *Genetic Diversity in Plants*, InTech, Chapters published under CC BY 3.0 license, 2012. doi 10.5772/2640

42. Esfandani, S., Sheidai, M., and Noormohammadi, Z., Species delimitation and population structure analysis in *Geranium* subg. *Geranium* (Geraniaceae) with the use of ISSR molecular markers, *Biologia*, 2017, vol. 72, no. 7.

43. Hutchison, D.W. and Templeton, A.R., Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability, *Evolution*, 1999, vol. 53, no. 6, pp. 1898–1914. doi 10.1111/j.1558-5646.1999.tb04571.x

44. Medrano, M. and Herrera, C.M., Geographical structuring of genetic diversity across the whole distribution range of *Narcissus longispathus*, a habitat-specialist, Mediterranean narrow endemic, *Annal. Bot.*, 2008, vol. 102, no. 2, pp. 183–194. doi 10.1093/aob/mcn086

45. Slatkin, M., Isolation by distance in equilibrium and non-equilibrium populations. *Evolution*, 1993, vol. 47, no. 1, pp. 264–279.

46. Hufford, K.M. and Mazer, S.J., Plant ecotypes: genetic differentiation in an age of ecological restoration, *Trends Ecol. Evol.*, 2003, vol. 18, no. 3, pp. 147–55. doi 10.1016/S0169-5347(03)00002-8

47. Gibson, D.J., *Grasses and Grassland Ecology*, Oxford, UK: Oxford Univ. Press, 2009.

48. Esfandani-Bozchaloyi, S., Sheidai, M., Keshavarzi, M., and Noormohammadi, Z., Genetic diversity and morphological variability in *Geranium purpureum* Vill. (Geraniaceae) of Iran, *Genetika*, 2017, vol. 49, no. 2, pp. 543–557. doi 10.2298/GENSR1702543B

49. Esfandani-Bozchaloyi, S., Sheidai, M., Keshavarzi, M., and Noormohammadi, Z., Species delimitation in *Geranium* sect. Batrachioidea: morphological and molecular, *Act. Bot. Hung.*, 2017, vol. 59, nos. 3–4, pp. 319–334. org/ doi 10.1556/034.59.2017.3-4.3

50. Esfandani-Bozchaloyi, S., Sheidai, M., Keshavarzi, M., and Noormohammadi, Z., Genetic and morphological diversity in *Geranium dissectum* (Sec. Dissecta, Geraniaceae) populations, *Biologia*, 2017, vol. 72, no. 10, pp. 1–10. doi 10.1515/biolog-2017-0124

51. Esfandani-Bozchaloyi, S., Sheidai, M., Keshavarzi, M., and Noormohammadi, Z., Analysis of genetic diversity in *Geranium robertianum* by ISSR markers, *Phytol. Balcanica*, 2017, vol. 23, no. 2, pp. 157–166.