Analysis of genetic diversity in *Geranium robertianum* by ISSR markers

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Abstract. *Geranium robertianum* is highly adaptable and shows various morphological differences, including the degree of hairiness, growth habit (erect or prostrate) and differences in flowering parts. It has a long history of medicinal use, including relief from toothache and nosebleeds. We have no information on its population genetic structure, genetic diversity and morphological variability in Iran. Therefore, owing to the importance of this plant species, we have studied genetic variability and population structure in seven geographical populations of *G. robertianum* (*Geraniaceae*). The results indicated that the geographical populations of *G. robertianum* are well differentiated both in genetic content and in morphological characteristics. A consensus tree based on the morphological and genetic data has separated some of the populations from the others, suggesting existence of ecotypes within this species.

Key words: Gene flow, genetic admixture, *Geranium robertianum,* network

Introduction

Population genetic analyses produce important data on the levels of genetic variation, partitioning of genetic variability within/between populations, gene flow, inbreeding, self-pollination versus outcrossing, effective population size, and population bottleneck. The obtained information can help in developing effective management strategies for endangered and/or invasive species (Chen 2000; Ellis 2007). *Geranium* L. (*Geraniaceae*) comprises 423 species in the world, which are classified into three subgenera and 18 sections (Aedo & al. 1998; Mabberley 2008). The genus is represented in Iran by 23–25 species. These species are grouped into two subgenera and eight sections (Schönbeck-Temesy 1970; Janighorban 2009; Onsori & al. 2010). Diagnostic features in infrageneric classification are related to fruit discharge methods, mericarp margin and leaves shape. *Geranium robertianum* L. is both a winter and a spring annual. The leaves are deeply dissected and light-green in color. In the late autumn the foliage turns red. The stems fork, and are brittle at the joints, they are pubescent and under high light conditions are red and up to 70 cm long. The pink flowers are perfect with five petals 8–15 mm long. The receptacle is elongated into a structure called "torus". The fruit is a capsule. Seeds are brown and about 2 mm in length. *G. robertianum* is a common and a more variable species in the British flora than G. *purpureum* (Baker 1955). Experiments described elsewhere have demonstrated that some of this

variability results directly from the influence of environmental factors, while the remaining part, some of it taxonomically important, is genetically determined. Although the populations of this species are often relatively uniform, there may be striking differences between them. Part of the intra-population uniformity can be related to the prevailing self-pollination in this species (as in G. *purpureum*) (Baker 1953). *G. robertianum* is predominantly a woodland plant, but may be found in shaded hedge banks, grasslands and more open vegetation, including in cracks in the rocks, and also as an epiphyte. *G. robertianum* has been introduced into many other parts of the world. It is recorded from the mountains in Japan and from Southwest China (Ohwi 1965; Yeo 1985) and Iran (Parsa 1951).

Several trends in the pollination mechanism can be observed in *Geranium,* with gradual transition between them. According to Philipp (1985), most perennial species of *Geranium* produce large and protandrous flowers, while a slight or null protandry is accompanied by increased selfing and reduction in flower size. Selfing here is related to annual or colonizer strategies, which occur in many other taxa (Baker 1955, 1967; Stebbins 1957, 1970; Ambruster 1993). Annual or biennial species with small flowers, such as *G. lucidum, G. pusillum*, *G. molle, G. dissectum*, *G. rotundifolium,* and *G. robertianum,* are expected to be automatically self-pollinated. This has been proved for *G. molle* and *G. dissectum*. Usually, large-flowered perennial species rely on insects for pollination. The flowers of *G. pratense* are pollinated by bees, honeybees and bumblebees. Some species of the genus *Geranium* (Cranesbill) are utilized as an antidiabetic, hemostatic, antihemorrhoidal, and antidiarrheal cure, and as a remedy for tonsillitis, cough, whooping cough, urticaria, dysentery, pain, fevers, and gastrointestinal ailments in some folk medicines (Baytop 1995; Calzada & al. 2005). The aerial parts of *G. purpureum* are consumed as food in Turkey (Kızılarslan 2008). *G. robertianum* is reported as containing no alkaloids (Raffauf 1996). The foliage contains high concentrations of calcium, sodium and iron (Grime & al. 1988) and has been reported to be rich in vitamin C. Allen (1989) and Hegnauer (1966) reported the polyphenolics quercetin, kaempferol, ellagic acid, caffeic acid, and ferulic acid, plus suspected gallic acid for *G. robertianum*.

ISSR combines the advantages of RAPD and SSR markers at the same level. Thus, it can show more polymorphism than RAPD and the reaction system is more sensitive, more stable, and has good repeatability (Wolfe & Liston 1998). This molecular marker has been widely used in studies on germplasm resource identification, phylogeny of species, plant taxonomy, evolution, and genetic diversity. It has also a number of limitations when compared with codominant markers. Its only negative point is its dominance in nature, which cannot identify heterozygotes in population.

Knowledge of genetic diversity for medicinal and economically important plants is essential for conservation and breeding purposes. If genetic diversity is very low in a taxon, this may exercise negative selective pressure on its population size and its eventual extinction. In such a case, genetic diversity should be increased by different means like hybridization, induced mutation, etc. (Sheidai & al. 2012, 2013). Therefore, the present study was performed for the first time in the country to investigate the genetic structure and diversity of these local populations. Its results may have positive effect on the conservation strategy and future breeding of this medicinally important plant. We have used ISSR molecular markers in the present study as these markers are known as informative in genetic diversity and population structure studies (e.g. Sheidai & al. 2012, 2013, Azizi & al. 2014, Esfandani Bozchaloyi & al. 2017a,b).

Material and methods

Plant material

In the present study, 69 plant samples were collected from seven geographical populations. Different references were used for the correct identification of *G. robertianum* species (Davis 1967; Schonbeck-Temesy 1970; Zohary 1972; Aedo & al., 1998; Janighorban 2009). Details of the sampling sites are given (Table 1, Fig. 1). Voucher specimens are deposited in the Herbarium of the Shahid Beheshti University (HSBU).

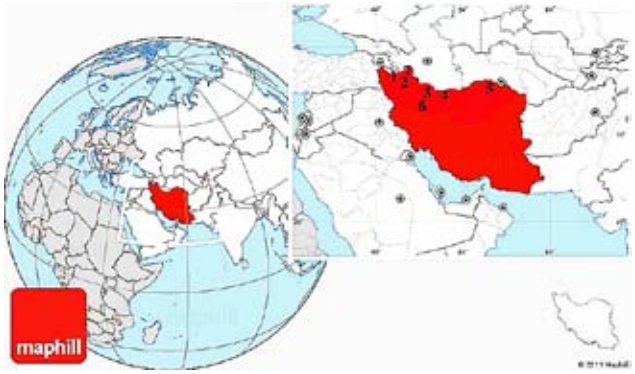

Fig. 1. Distribution map of *G. robertianum.*

Table 1. Populations studied, their locality and ecological features.

Sp.	Population	Locality	Longitude	Latitude	Altitude (m)	Voucher No.
G. robertianum	1	East Azerbaijan Kaleybar, roadside	38° 52'293"	47° 25' 92"	1133	HSBU 201619
G. robertianum	2	East Azerbaijan Kaleybar to Makidy valley	38° 52'393"	47° 24 92"	1127	HSBU 201626
G. robertianum	3	Guilan,Gole Rodbar	37° 09'5507"	49° 55'4977"	20	HSBU 201620
G. robertianum	4	Mazandaran, Tuska Cheshmeh	36° 38'1952"	53° 48'56.9"	1427	HSBU 201621
G. robertianum	5	Mashhad-Torghabeh-Arghavan valley	36° 18'3234"	59° 22'2634"	1300	HSBU 201622
G. robertianum	6	Giulan, Baharestan jungle	37° 09'2175"	49° 52'2121"	45	HSBU 201623
G. robertianum	7	East Azerbaijan Kaleybar	38° 52'493"	47° 2392"	1155	HSBU 201624

Morphological studies

Eighty morphological (42 qualitative, 38 quantitative) characters were studied altogether. Five plant specimens were randomly selected for morphological analyses.

DNA extraction and ISSR assay

Fresh leaves were taken randomly from 5–10 plants in each of the studied populations. They were dried by silica gel powder. CTAB-activated charcoal protocol was used to extract genomic DNA (Sheidai & 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, UBC 810, (CA) 7AT, (GA) 9C, UBC 807, UBC 811, (GA) 9T, and (GT) 7CA – commercialized by UBC (the University of British Columbia) were used. PCR reactions were carried out in a 25 µl volume of 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 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 amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step at 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 a final extension step of 7–10 min at 72 °C. The amplification products were observed by running on 1 % agarose gel, followed by ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Table 2. Evaluated morphological characters.

No	Characters
1	Plant height (mm)
2	Length of stem leaves petiole (mm)
3	Length of stem leaves (mm)
4	Width of stem leaves (mm)
5	Length of stem leaves / Width of stem leaves(mm)
6	Width of stem leaves/ Length of stem leaves (mm)
7	Number of segment stem leaves (mm)
8	Length of basal leaves petiole (mm)
9	Length of basal leaves (mm)
10	Width of basal leaves (mm)
11	Length of basal leaves / Width of basal leaves (mm)
12	Width of basal leaves / Length of basal leaves (mm)
13	Number of segment basal leaves
14	Calyx length (mm)
15	Calyx width (mm)
16	Calyx length/ Calyx width (mm)
17	Petal length (mm)
18	Petal width (mm)
19	Petal length / Petal width (mm)
20	Mericarp length (mm)
21	Mericarp width (mm)
22	Mericarp length/ Mericarp width (mm)
23	Seed length (mm)
24	Seed width (mm)
25	Seed length/ Seed width(mm)
26	Stipules length (mm)
27	Stipules width (mm)
28	Stipules length/ Stipules width (mm)
29	Bract length (mm)
30	Bract width (mm)
31	Bract length / Bract width (mm)
32	Pedicel length (mm)
33	Peduncle length (mm)
34	Rostrum length (mm)
35	Style length (mm)
36	Stamen filament length (mm)
37	Fruit length (mm)
38	Number of flowers per inflorescence
39	Type root
40	Vegetation-forms
41	State of stem strength
42	State of stem branches

Table 2. Continuation.

No	Characters
43	Leave shape
44	Phyllotaxy
45	Leaf tips
46	Shape of segments basal leaves
47	Stamen filament color
48	Stigma hair
49	Mericarp shape
50	Mericarp surface
51	Mericarp hair
52	Mericarp rostrum hair
53	Sepale hair
54	Sepale hair density
55	Peduncle and pedicel hair
56	Anthers colour
57	Stem hair
58	Stem hair density
59	Leaf hair
60	Bract shape
61	Stipules shape
62	Bract and stipules hair density
63	Bract and stipules hair
64	Shape of segments cauline leaves
65	Shape of calyx
66	Calyx apex
67	Petal shape
68	State of petal ligule
69	Shape of petal lobes
70	State of petal ligule hair
71	Stamen filament hair
72	Mericarp hair density
73	Mericarp colour
74	Seed colour
75	Seed shape
76	Seed surface ornamentation
77	Peduncle and pedicel hair density
78	Petioles hair
79	Petioles hair density
80	Leaf hair density

Data analyses

Morphological studies

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish the Euclidean distance among the pairs of taxa (Podani 2000). For grouping of the plant specimens, UPGMA (Unweighted Paired Group Average) and Ward (Minimum Spherical clusters), as well as ordination methods of MDS (Multidimensional Scaling) and PCoA (Principal Coordinate Analysis) were used (Podani 2000). ANOVA (Analysis of Variance) was 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 (Podani 2000). PAST version 2.17 (Hammer & al. 2012) was applied for multivariate statistical analyses of the morphological data.

Molecular analysis

The obtained ISSR bands were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Such parameters were determined like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Weising & al 2005; Freeland & al. 2011). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland & al. 2011; Huson & Bryant 2006). Correlation between geographical and genetic distance of the studied populations (Podani 2000) was checked with a Mantel test. These analyses were done with PAST ver. 2.17 (Hammer & al. 2012), ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software. AMOVA (Analysis of Molecular Variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall & Smouse 2006) and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (Meirmans & Van Tienderen 2004) were applied to show genetic difference of the populations. Furthermore, the population genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008).

Genetic structure of the populations was studied by a Bayesian-based model STRUCTURE analysis (Pritchard & al. 2000), and maximum likelihood-based method of K-Means clustering with GenoDive ver. 2. (2013). For STRUCTURE analysis, data were scored as dominant markers (Falush & al. 2007). Evanno's test was performed on STRUCTURE results to determine

the proper number of *K* by using delta *K* value (Evanno & al. 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provided the best fit for k (Meirmans 2012).

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

Results

Morphometry

In the present study, 69 plant samples were collected from seven geographical populations. The ANOVA test revealed a significant difference in the quantitative morphological characters among the studied populations ($P < 0.05$). MDS and PCoA plot of *G. robertianum* populations based on morphological characters produced similar results. Therefore, only Clustering and PCA plot are presented and discussed (Figs. 2, 3). The results showed morphological difference/ divergence among most of the studied populations. In general, two major clusters were formed in the UPGMA tree (Fig. 2); populations 1, 2, 3, 4, 5, 6 showed morphological similarity and were placed in the first major cluster, while population 7 formed the second major cluster. PCA analysis revealed that the first three components accounted for about 68 % of total variation. It also revealed that character No. 8 (basal leaf of petiole length) separated the populations 1, 2, 4 from the others. Character No. 1 (plant height)

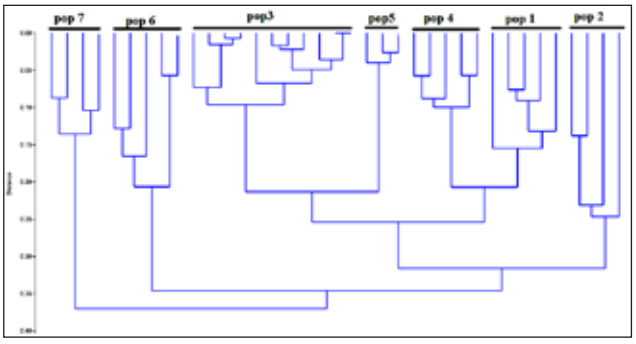

Fig. 2. UPGMA tree of morphological data in the studied *G. robertianum* populations.

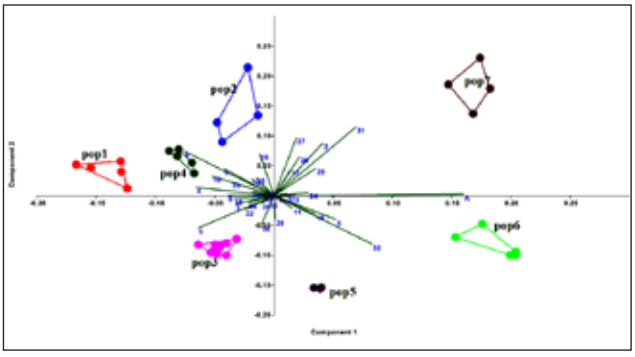

Fig. 3. PCA plot of the morphological characters in the studied *G. robertianum* populations. Different colors indicate the plant specimens (numbers 1–7) from each geographical population.

separated population 3 from others. Character No. 32 (pedicel length) separated population 6, and character No. 31 (ratio of bract length/ bract width) separated population 7 from others. The PCA plot supported the grouping made by the UPGMA tree (Fig. 3).

Population genetic diversity

Genetic diversity parameters determined in seven geographical populations of *G. robertianum* are presented in Table 3. The highest value of percentage polymorphism (47.14 %) was observed in Mazandaran, Tuska Cheshmeh (population 4), which shows high gene diversity (0.177) and Shannon's information index (0.262). Population 1 in East Azerbaijan, Kaleybar, roadside has the lowest value for percentage of polymorphism (14.29 %) and the lowest value of the Shannon's information index (0.074), and He (0.048).

Table 3. Genetic diversity parameters in the studied populations.

Pop No.	N	Na	Ne	I	He	UHe	P%
Pop1	5.000	0.500	1.079	0.074	0.048	0.054	14.29 %
Pop2	4.000	0.457	1.116	0.098	0.067	0.076	17.14 %
Pop3	11.000	0.957	1.214	0.202	0.130	0.136	44.29 %
Pop4	5.000	0.957	1.305	0.262	0.177	0.196	47.14 %
Pop5	3.000	0.843	1.258	0.211	0.145	0.174	35.71 %
Pop6	5.000	0.771	1.230	0.193	0.130	0.144	35.71 %
Pop7	4.000	0.671	1.099	0.094	0.061	0.070	18.57 %

(N = number of samples, Ne = number of effective alleles, I = Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P% = percentage of polymorphism).

Population genetic differentiation

AMOVA (PhiPT = 0.50, P = 0.010), and Gst analysis (0.599, p = 0.001) revealed a significant difference among the studied populations. It also revealed that 45 % of the total genetic variability was related to

within-population diversity and 55 % was related to among-populations genetic differentiation. Pairwise AMOVA showed a significant difference among the studied populations. Moreover, we got high values for Hedrick standardized fixation index after 999 permutations (G'st = 0.615, P = 0.001) and Jost differentiation index (D-est = 0.231, P = 0.001). These results indicate that the geographical populations of *G. robertianum* are genetically differentiated from each other.

Neighbor Joining (NJ) tree, PcoA and MDS plot produced similar results (Figs 4-6). PcoA and MDS plot revealed that the studied populations were placed in separate groups, which was in agreement with the AMOVA results and the close genetic affinity between populations 4 and 5. Grouping of the plant

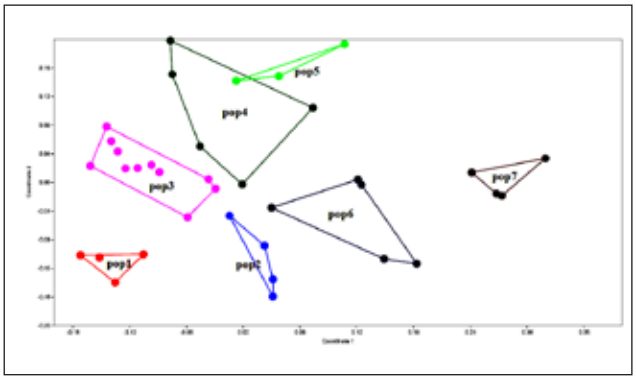

Fig. 4. MDS plot of *G. robertianum* populations based on ISSR data. Note: Population numbers are according to Table 1.

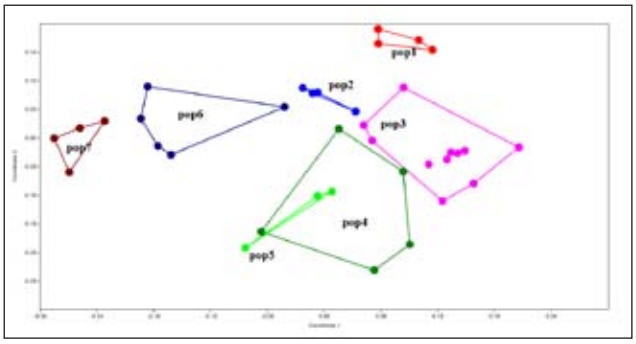

Fig. 5. PcoA plot of *G. robertianum* populations based on ISSR data. Note: Population numbers are according to Table 1.

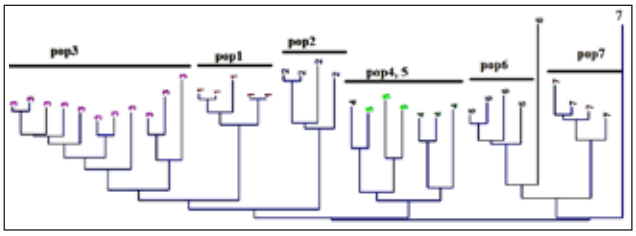

Fig. 6. NJ tree of *G. robertianum* populations based on ISSR data. Note: Population numbers are according to Table 1.

populations obtained by NJ tree produced similar results. In general, two major clusters were formed in the NJ tree (Fig. 6). Populations 1–5 formed the first major cluster and showed a higher degree of genetic affinity, while populations 6 and 7 formed the second major cluster.

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

A comparison between genetic identity and genetic distance in Table 4 showed genetic similarity (0.91) between the populations of Guilan, Gole Rodbar and Mazandaran, Tuska Cheshmeh (Nos 3, and 4), while the lowest genetic similarity value (0.61) occured between the East Azerbaijan Kaleybar, roadside and East Azerbaijan Makidy – Kaleybar populations (pop. 1 and 7).

Table 4. Nei's genetic identity (upper diagonal) and genetic distance (lower diagonal) among the studied populations.

Pop ID	1	2	3	4	5	6	7
1	****	0.7379	0.8194	0.7796	0.7000	0.7476	0.6183
2	0.3039	****	0.8384	0.8419	0.7822	0.8137	0.6662
3	0.1991	0.1763	****	0.9195	0.8150	0.8264	0.6619
4	0.2489	0.1720	0.0839	****	0.8997	0.8766	0.7148
5	0.3567	0.2456	0.2045	0.1057	****	0.7881	0.7082
6	0.2909	0.2061	0.1907	0.1317	0.2381	****	0.7581
7	0.4809	0.4062	0.4127	0.3357	0.3451	0.2770	****

Grouping of the studied populations by NJ tree is presented in Fig. 7. In general, two major clusters were formed. Populations 3 and 4 were placed close to each other and the length of the branch joining them indicates a significant genetic difference. Population 7 joined all other populations at a great distance, revealing its high degree of genetic difference and supporting our morphological and ISSR results presented above (Fig. 4).

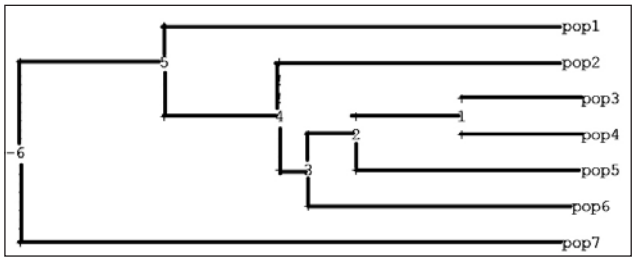

Fig. 7. NJ tree of populations based on genetic data.

Population genetic affinity

The NeighborNet diagram (Fig. 8) also revealed almost complete separation of the studied populations within the network, supporting the AMOVA results. Populations 1, 2 and 3 are distinct and stand separately from the other populations at a great distance. Populations 6 and 7 and populations 4 and 5 show a closer genetic affinity and are placed close to each other.

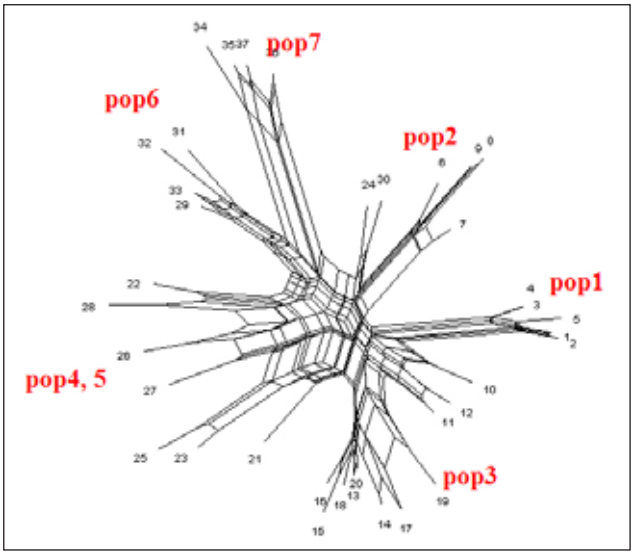

Fig.8. Neighbor-Net network of populations in *G. robertianum* based on ISSR data.

Population genetic structure

Evanno's test performed in STRUCTURE analysis produced k = 3 (Fig. 10). This genetic grouping is in agreement with the NeighborNet diagram results presented above. A STRUCTURE plot (Fig. 9) based on k = 3 revealed genetic difference in populations 1 and 2 (differently colored), as well as in populations 3 and 6, 7. However, it showed genetic affinity between populations 1 and 3 (similarly colored), as well as between populations 2 and 4, 5.

The mean Nm = 0.3 was obtained for all ISSR loci, which indicates a low gene flow among the populations and supports the genetic stratification as indicated by NeighborNet diagram and STRUCTURE

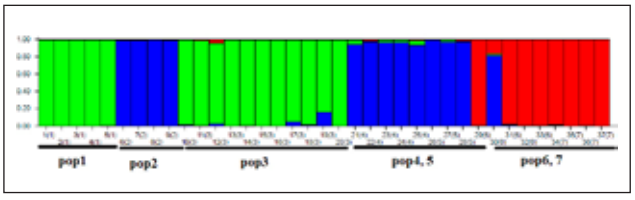

Fig. 9. STRUCTURE plot of *G. robertianum* populations based on k = 3 of ISSR data.

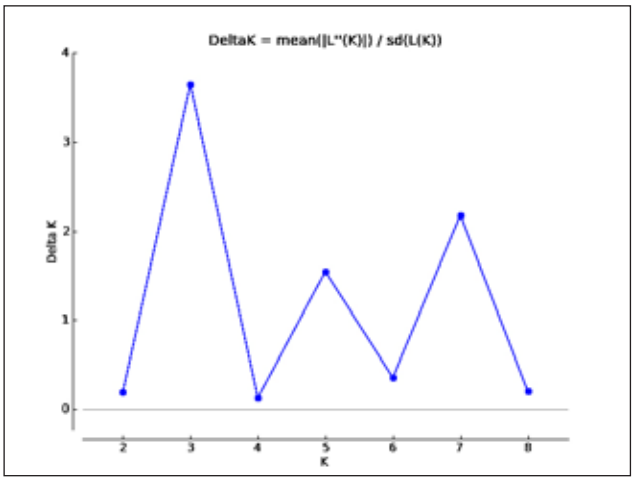

Fig. 10. Delta k plot of Evanno's test based on STRUCTURE analysis.

analyses. Population assignment test also agreed with Nm results and could not identify a significant gene flow among these populations. However, the reticulogram obtained on the basis of the least square method (Fig. 11) revealed some shared alleles among the populations 2 and 6, between 3 and 4, and also between 6 and 7. This result is in agreement with the grouping we obtained with the Neighbor-Net, as these populations were placed close to each other. As evidenced by the STRUCTURE plot based on admixture model, these shared alleles comprise a very limited part of the genomes in these populations and all these results are in agreement with the demonstrated high degree of genetic stratification within *G. robertianum* populations.

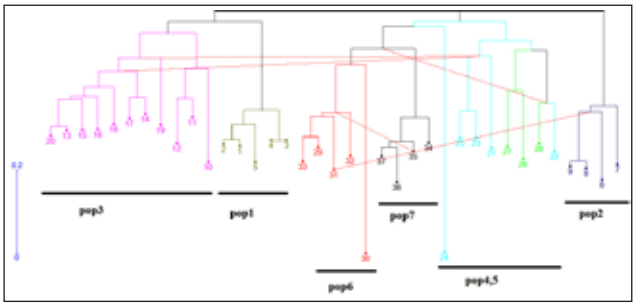

Fig. 11. Reticulogram of *G. robertianum* populations based on the least square method analysis of ISSR data.

(Numbers below the branches are population numbers according to Table 1).

A consensus tree was obtained for both ISSR and morphological trees (Fig. 12) to reveal that the populations 3, 6 and 7 diverged on the basis of morphological and molecular features. A detailed comparison of the characteristics of these populations has shown that,

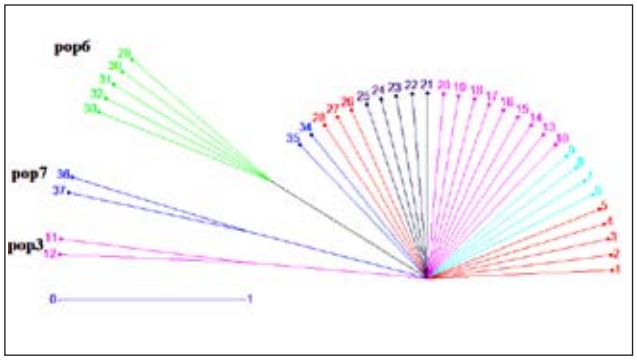

Fig. 12. Consensus tree of morphological and molecular data in *G. robertianum* populations.

Fig. 12. Consensus tree of morphological and molecular data in *G. robertianum* populations.

for example, population No. 6 had the greatest pedicle length (15 mm), the narrowest petal width (2 mm), the greatest stem-leaf length (2 cm), and the narrowest stem-leaf width (2 cm) among the studied populations. Population No. 7 had the greatest petal length (15 mm) and stems and petioles of deep dull red. The significant morphological differences among the geographical populations of a single species and separation of populations on the PCA or MDS plot seemed adequate reasons to consider these populations as separate ecotypes. According to Knaus (2008), "if we take the species to be the unit of distinction, the infra-taxa (the subspecies, the variety and the ecotype) are consequently non-distinct. The process in which a group of organisms diverge from being one cohesive group to becoming two or more distinct groups is the process of speciation". Stebbins (1993) also added the idea that "species are systems of populations, which resemble each other, yet contain genetically different ecotypes that could be arranged in a continuous series. These allopatric infraspecific categories are usually recognized as infra-taxa". Therefore, we consider specimens collected from Gilan, Gole Rudbar and Gilan, Baharestan, as an ecotype of *G. robertianum*. We will introduce these new taxa with their details in our next publication.

Discussion

Genetic study of the populations provides valuable information about the genetic structure of plants, stratification versus gene flow among the species populations, genetic divergence of the populations, etc. (Sheidai & al. 2014). This information has different applications: from pure understanding of biology of the species to conservation of endangered species, choosing the proper parents for hybridization and breeding, and phylogeography and mechanism of invasion (Freeland & al. 2011). *Geranium robertianum* is widespread in Iran and it has several medicinal applications (Proestos & al. 2006).

The studied populations had low to moderate level of genetic diversity. Genetic diversity is of fundamental importance for the continuity of a species as it brings about the necessary adaptation and ability to cope with changes in the environment (Sheidai & al. 2013, 2014). The degree of genetic variability within a species is highly correlated with its reproductive mode: the higher the degree of open pollination/ crossbreeding, the higher is the level of genetic variability in the studied taxon (Freeland & al. 2011). *Geranium robertianum* is mainly a self-pollinating species (Baker 1955), therefore, the low level of genetic variability within the populations in this species might be related to the closer breeding within this taxon.

In *Geranium,* several trends can be observed in the pollination mechanism, with gradual transition between them. According to Philipp (1985), most perennial species of *Geranium* produce large and protandrous flowers, while a slight or null protandry is accompanied by an increased selfing and a reduction in flower size. Here, selfing is related to annual or colonizer strategies, which occur in many other taxa (Baker 1955, 1967; Stebbins 1957, 1970; Ambruster 1993). Annual or biennial species with small flowers such as *G. lucidum, G. pusillum*, *G. molle, G. dissectum*, *G. rotundifolium,* and *G. robertianum* are expected to be automatically self-pollinated. This has been proved for *G. molle* and *G. dissectum*. Usually, large-flowered perennial species rely on insects for pollination. The flowers of *G. pratense* are pollinated by bees, honeybees and bumblebees. We used methods of indirect estimation of the gene flow and its occurrence among the populations may be due either to ancestral shared alleles, or to ongoing gene flow. The Nm value obtained on the basis of ISSR data revealed a very limited gene flow among the studied populations which was also supported by the STRUCTURE analysis, as *G. robertianum* has mostly a distinct genetic structure. Reticulation analysis also showed some gene flow for ISSR.

Low genetic variability may also occur due to the small size of the populations and genetic drift (Freeland et al. 2010). These species tend to inbreed as al-

so was evidenced by the very low Nm value and IBD obtained for the studied species. Therefore, the limited gene flow observed was not solely due to geographical distance, but might have been due to the strong tendency of inbreeding of the studied populations. The lower level of genetic variability in those populations came with limited geographical distribution and probably more selfing abilities.

The present population divergence may be influenced by the isolation-by-distance across the distribution range of the studied *G. robertianum* populations. Dispersal of these populations might be constrained by distance and gene flow is most likely to occur between the neighboring populations. As a result, the closer situated populations tend to be more similar genetically to each other (Slatkin 1993; Hutchison & Templeton 1999; Medrano & Herrera 2008).

Population divergence may be accompanied by local adaptation. The use of multilocus molecular markers (such as SSR, AFLP, RAPD, ISSR, etc.) in the population genetic studies has shown that these are neutral molecular markers (they are not acting directly as adaptive genes), but they may be linked to a gene or a genetic region with adaptive value (Freeland & al. 2011). Therefore, the combination of genetic divergence, limited gene flow and local adaptation has played a role in diversification of *G. robertianum* populations in the country.

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