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SPECIES RELATIONSHIP AND POPULATION STRUCTURE ANALYSIS IN *GERANIUM* SUBG. *ROBERTIUM* WITH THE USE OF ISSR MOLECULAR MARKERS

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Species delimitation is essential since species is regarded as the basic unit of analysis in nearly all biological disciplines, such as ecology, biogeography, conservation biology, and macroevolution. The genus *Geranium* (Geraniaceae) comprises about 350 species distributed throughout most parts of the world. The subg. *Robertium* comprises 30 species which are arranged in 8 sections. This subgenus is represented in Iran by 10 species. These species are grouped into 5 sections. In spite vast distribution of many *Geranium* species that grow in Iran, there are not any available report on their genetic diversity, mode of divergence and patterns of dispersal. Therefore molecular (ISSR markers) and morphological studies of 147 accessions from 10 species of *Geranium* (subg. *Robertium*), that were collected from different habitats in Iran were performed. The aims of the present study are: 1) to find the diagnostic value of ISSR markers in delimitation of *Geranium* species, 2) to find the genetic structure of these taxa in Iran, and 3) to investigate the species inter-relationship. The present study revealed that combination of morphological and ISSR data can delimit the species. AMOVA and STRUCTURE analysis revealed that the species of subg. *Robertium* are genetically differentiated but have some degree of shared common alleles.

Key words: *Geranium*, ISSR, morphology, species delimitation, STRUCTURE

INTRODUCTION

Species delimitation is important in different biological disciplines, like ecology, biogeography, and plant conservation (Mayr 1982, Wiens 2007). Species delimitation is done by tree-based and non-tree-based approaches (Sites and Marshall 2003). In the first method, species form distinguishing clades (phylogenetic species concept), whereas in non-tree-based method, the species are recognised on the basis of gene flow assessments (biological species concept; Pérez-Losada *et al.* 2005).

Wiens and Penkrot (2002), proposed to use DNA data, morphological data and character data for species delimitation while, Knowles and Carstens

(2007) addressed how molecular data (i.e., gene trees from DNA sequence data) can be used in species delimitation. The latter authors used coalescent simulations to test the species limits and incorporated data from multiple loci. They showed the importance of population genetics in species delimitation. Similarly, Medrano *et al.* (2014) applied population genetics methods to the species delimitation problem in *Narcissus* Linnaeus (1753: 289) (Amaryllidaceae J. St.-Hil., nom. cons.) by the help of amplified fragment length polymorphism (AFLP) molecular markers.

The genus *Geranium* Linnaeus (1753: 676) (Geraniaceae Juss.) with about 350 species is distributed through most of the world habitats (Aedo 2014, Aedo *et al*. 1998*b*). A brief history of the generic delimitation and infra-generic classification, as well as a description of the genus, can be found in Aedo (1996). According to the currently accepted classification (Yeo 1984), *Geranium* is divided into three subgenera: subg. *Geranium* Linnaeus (1753: 676), subg. *Erodioidea* (Picard) Yeo (1984: 89), and subg. *Robertium* (Picard) Rouy (1897: 94). According to Yeo's (1984) sectional classification, the subg. *Robertium* comprises 30 species in 8 sections, some of which have also been revised (Aedo *et al*. 1998*a*, Yeo 1973, 1992). Section *Polyantha* Reiche (1890: 8) (with 7 species) is endemic to the Eastern Himalayas and southern China, and section *Trilopha* Yeo (1984: 89) (with 5 species) is restricted to the mountains of tropical Africa, western Asia, and the eastern Himalayas. The distributions of the remaining 5 sections as *Lucida* R. Knuth (1912: 60), *Ruberta* Dumort. (1827: 112), *Divaricata* Rouy (1897: 88), *Batrachioidea* W. D. J. (1835: 139) and *Unguiculata* (Boiss.) (1890: 8) are in the Mediterranean area and western Asia (Aedo 2005, Aedo and Estrella 2006, Aedo *et al.* 1998*a*, *b*, Yeo 2004). Controversy exists on the number of species in this genus, for example, there is occurring 22 annual and perennial species for this genus in Iran according to Flora Iranica (Schönbeck-Temesy 1970), but in Flora of Iran (Janighorban 2009). The genus is represented by 25 species but there are not clarified any sections for it (Onsori *et al.* 2010). Diagnostic features in infrageneric classification are related to fruit discharge methods, mericarp margin and leave shape. In Iran, there are *Geranium* species with carpel projection or seed ejection. Some species of the genus *Geranium* (cranesbill) are utilised as an anti-diabetic, hemostatic, anti-hemorrhoid, and anti-diarrhea, and as a remedy for tonsillitis, cough, whooping cough, urticaria, dysentery, pain, fevers, and gastrointestinal ailments in some folk medicines. (Bate-Smith 1973, Baytop 1999). *Geranium* is both cross-pollinated and self-pollinated (Stebbins 1957, 1970), and interspecific hybrids and intermediate forms do occur in few *Geranium* species in the area of species overlap. Hybridisation experiments in *Geranium* subg. *Robertium* have involved species of section *Anemonifolia* R. Knuth (1912: 98), *Batrachioidea*, *Lucida*, *Ruberta* and *Unguiculata* (Van Loon 1984, Widler-Kiefer and Yeo 1987). There are no available data for sections as *Divaricata*, *Polyantha* and *Trilopha*.

Previous study on species delimitation and species relationship performed in this genus (Salimi Moghadam *et al.* 2015) revealed that fruit characters are important for separating taxa at infrageneric rank and their results show that the species can be separated into subgenera and sections based on fruit morphology, while seed micromorphological features generally do not support the sectional taxonomy, but provide valuable characters for the delimitation at species groups, species, and infraspecific levels (Salimi Moghadam *et al.* 2015). Studies on *Geranium* species are mainly dealing with taxonomy, seed and pollen morphology, stem and leaf anatomy (Esfandani Bozchaloyi *et al*. 2017*a*, *b*, *c, d*, Keshavarzi *et al*. 2015, Onsori *et al*. 2010, Salimi Moghadam *et al.* 2015, Salimpour *et al.* 2009), but there are no attempt to study genetic diversity, ecological adaptation and intra- and interspecific differentiation along with morphometric studies on *Geranium* of Iran. Therefore, we performed morphological and molecular study of 10 collected species of 5 sections in the subg. *Robertium*. The project try to answer the following questions: 1) Is there infra- and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa? 4) Is there any gene exchange between *Geranium* species in Iran? Therefore it is important to delimit the identified species for performing further detailed molecular studies.

MATERIALS AND METHODS

Plant materials

In present study, 147 plant samples were collected from 21 geographical populations belong to 10 species in the subg. *Robertium*. The species studied are *G. pyrenaicum* Burm. f. (1759: 27), *G. pusillum* L. (1759: 1144), *G. molle* L. (1753: 682) (sec. *Batrachioidea*); *G. robertianum* L. (1753: 681), *G. purpureum* Vill. (1786: 272) (sec. *Ruberta*); *G. lucidum* L. (1753: 682) (sec. *Lucida*); *G. divaricatum* Ehrh. (1792: 164), *G. albanum* M. Bieb. (1808: 137) (sec. *Divaricata*); *G. trilophum* Boiss. (1846: 30), *G. mascatense* Boiss. (1843: 59) (sec. *Trilopha*). Different references were used for the correct identification of species (Aedo *et al.* 1998*a*, Janighorban 2009, Schönbeck-Temesy 1970, Zohary 1972). Details of sampling sites are mentioned (Table 1, Fig. 1). Voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU).

For morphological studies five samples from each species were used for morphometry. In total 80 morphological (42 qualitative, 38 quantitative) characters were studied (Table 2). Data obtained were standardised (mean= 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000).

Table 1
Geranium species and populations, their localities and voucher numbers

Sp.	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
G. molle	East Azerbaijan, kaleybar, Shojabad	38° 52′ 39.3″	47° 25′ 92″	1,133	HSBU 201677
	East Azerbaijan, kaleybar, cheshme ali akbar	38° 52′ 35.3″	47° 27′ 92″	1,143	HSBU 201678
G. pyrenacium	East Azerbaijan, kaleybar, road side	38° 52′ 37.3″	47° 23′ 92″	1,144	HSBU 201679
	East Azerbaijan, kaleybar cheshme ali akbar	38° 52′ 35.3″	47° 27′ 92″	1,143	HSBU 201680
	East Azerbaijan, kaleybar, Shojabad	38° 52′ 39.3″	47° 25′ 92″	1,137	HSBU 201681
	East Azerbaijan, Babak fort	38° 51′ 51″	47° 02′ 28″	1,155	HSBU 201682
G. pussilum	East Azerbaijan, kaleybar, road side	38° 52′ 37.3″	47° 23′ 92″	1,144	HSBU 201683
	East Azerbaijan, kaleybar cheshme ali akbar	38° 52′ 35.3″	47° 27′ 92″	1,143	HSBU 201684
	East Azerbaijan, kaleybar, Shojabad	38° 52′ 39.3″	47° 25′ 92″	1,137	HSBU 201685
G. purpureum	East Azerbaijan, kaleybar, cheshme ali akbar	38° 52′ 35.3″	47° 27′ 92″	1,143	HSBU 201686
	Guilan, Gole rodbar	37° 09′ 55″	49° 55′ 49″	27	HSBU 201687
	Guilan, Gole rodbar, road side	37° 09′ 45″	49° 55′ 39″	15	HSBU 201688
G. robertianum	Guilan, Gole rodbar	37° 09′ 55″	49° 55′ 49″	32	HSBU 201689
G. albanum	Guilan, Sangar, road side	37° 07′ 02.32″	49° 44′ 32.6″	48	HSBU 201690
	Guilan, Lahijan	37° 12′ 04.81″	50° 03′ 11.98″	9	HSBU 201691
	Guilan, Jirandeh	36° 41′ 58.62″	49° 47′ 30.34″	1,335	HSBU 201692
	Mazandaran, Siah bisheh to Chalus	36° 14′ 14.32″	51° 18′ 07.09″	1,807	HSBU 201693
	Golestan, Ramian	37° 08′ 0.23″	55° 85′ 07.03″	1,320	HSBU 201694
G. divaricatum	East Azerbaijan kaleybar	38° 52′ 37.3″	47° 23′ 92″	1,144	HSBU 201695
	Tehran, Darband	35° 50′ 03.36″	51° 24′ 28.62″	1,700	HSBU 201696
G. lucidum	East Azerbaijan, kaleybar cheshme ali akbar	38° 52′ 37.3″	47° 23′ 92″	1,144	HSBU 201697
G. mascatense	Khuzestan, Shushtar-Masjed solyman	32° 11′ 40.18″	48° 16′ 11.47″	88	HSBU 201698
G. trilophum	Hormozgan, Amani village, Kushk-e Nar Rural	27° 15′ 20.83″	52° 57′ 50.79″	36	HSBU 201699

Table 2
Morphological characters in studied species

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

DNA extraction and ISSR assay

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 (Sheidai *et al.* 2013). The quality

of extracted DNA was examined by running on 0.8% agarose gel. Ten ISSR primers: (AGC) 5GT, (CA) 7GT, (AGC) 5GG, UBC 810, (CA) 7AT, (GA) 9C, UBC 807, UBC 811, (GA) 9T and (GT) 7CA commercialised 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 standardised (mean = 0, variance = 1) and used to establish Euclidean distance among pairs of taxa (Podani 2000). For grouping of the plant specimens, UPGMA (unweighted paired group using average) and Ward (minimum spherical characters), as well as ordination methods of MDS (multidimensional scaling)

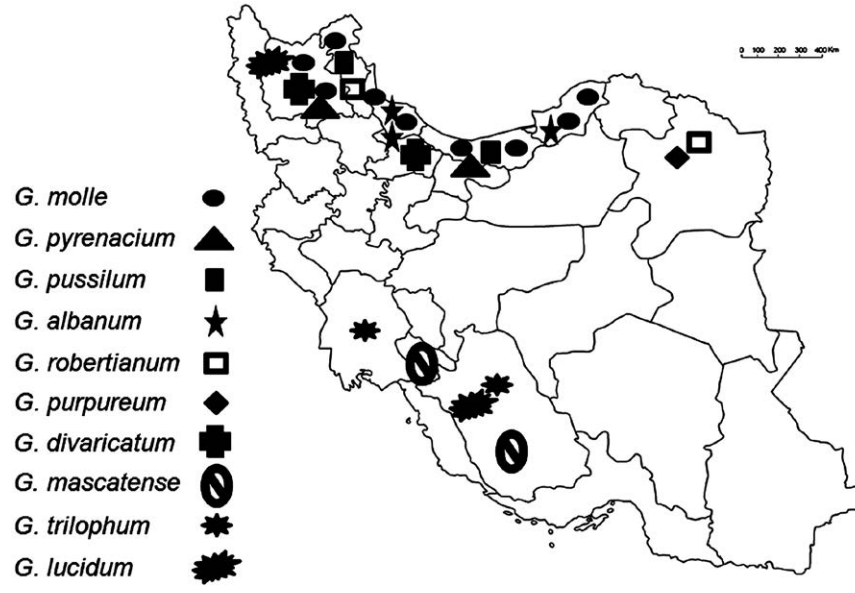

Fig. 1. Distribution map of studied species

Table 3
Genetic diversity parameters in the studied *Geranium* species. (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, populations)

Pop	N	Na	Ne	I	He	UHe	P%
sp1	12.000	1.247	1.304	0.281	0.184	0.192	55.91
sp2	8.000	0.419	1.097	0.084	0.056	0.060	16.13
sp3	6.000	0.258	1.029	0.028	0.018	0.020	5.38
sp4	12.000	0.925	1.259	0.233	0.155	0.162	44.09
sp5	11.000	0.774	1.171	0.162	0.104	0.109	36.56
sp6	14.000	0.344	1.069	0.064	0.041	0.043	13.98
sp7	14.000	0.570	1.106	0.098	0.064	0.066	21.51
sp8	10.000	0.441	1.036	0.033	0.022	0.023	7.53
sp9	5.000	0.301	1.031	0.027	0.018	0.020	5.38
sp10	5.000	0.312	1.029	0.028	0.018	0.020	5.38

and PCoA (principal coordinate analysis), were used (Podani 2000). 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 (Podani 2000). PAST version 2.17 (Hammer *et al*. 2012) was used for multivariate statistical analyses of morphological data.

Molecular analyses – ISSR bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. A parameter like Nei's gene diversity (H), Shannon information index (I), the number of effective alleles, and percentage of polymorphism were determined (Freeland *et al.* 2011, Weising *et al.* 2005). Nei's genetic distance among populations was used for neighbour joining (NJ) clustering and neighbour-net networking (Freeland *et al.* 2011, Huson and Bryant 2006). Mantel test checked the correlation between geographical and genetic distance of the studied populations (Podani 2000). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software. AMOVA (analysis of molecular variance) test (with 1,000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006), and Nei's Gst analysis as implemented in GenoDive ver. 2 (2013) (Meirmans and Van Tienderen 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardised measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008). The genetic structure of populations was studied by Bayes-

ian based model STRUCTURE analysis (Pritchard *et al.* 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2 (2013). For STRUCTURE analysis, data were scored as dominant markers (Falush *et al*. 2007). We used the admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K after a burn-in period of 10^5. The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (Evanno *et al*. 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC) provide the best fit for k (Meirmans 2012).

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 the 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

Species delimitation and inter-relationship

Morphometry – ANOVA showed significant differences ($P < 0.01$) in quantitative morphological characters among the species studied. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 64% of the total variation. In the first PCA axis with 32% of total variation, such characters as shape of petals, shape of sepals, peduncles and pedicels hair, stem hair, petioles hair, mericarp hair density have shown the highest correlation (> 0.7), length of bract and pedicel, length and width of the petal, length and width of stem leaves, width of mericarp were characters influencing PCA axis 2 and 3, respectively.

Different clustering and ordination methods produced similar results therefore, UPGMA clustering and MDS plot of morphological characters are presented here (Figs 2–3). In general, plant samples of each species belong to a distinct section, were grouped together and formed separate cluster. This result show that morphological characters studied can delimit *Geranium* species in two different major clusters or groups. In the studied specimens we did not encounter intermediate forms. In general, two major clusters were formed in UPGMA tree (Fig. 2), Populations of *G. robertianum*, *G. purpureum* (sec. *Ruberta*) and *G. lucidum* (sec. *Lucida*) were placed in the first major cluster and were

placed with great distance from the other species. The second major cluster included two subclusters. Plants of *G. divaricatum*, *G. albanum* (sec. *Divaricata*) and *G. trilophum*, *G. mascatense* (sec. *Trilopha*) comprised the first subcluster, while plants of *G. pyrenaicum*, *G. pusillum*, *G. molle* (sec. *Batrachioidea*) formed the second subcluster.

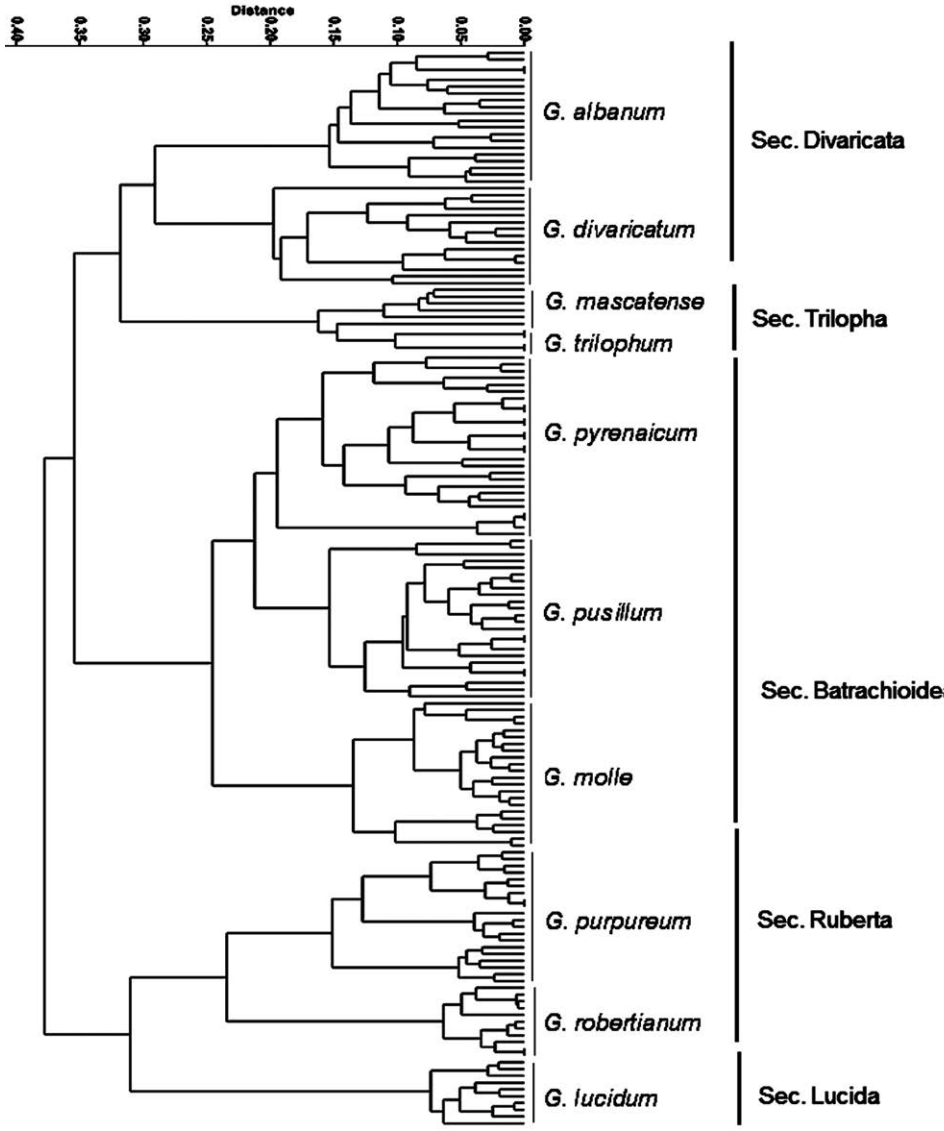

Fig. 2. UPGMA clustering of morphological characters revealing species delimitation in subg. *Robertium*

The MDS plot of morphological characters (Fig. 3) separated the species into distinct groups with no intermixing. This is in agreement with UPGMA tree presented before. The plants of *G. lucidum* (sec. *Lucida*) showed more similarity to *G. robertianum*, *G. purpureum* (sec. *Ruberta*) and were placed close to each other. Similarly, plants of *G. pyrenaicum*, *G. pusillum*, *G. molle* (sec. *Batrachioidea*) were placed close to each other due to morphological similarity and were separated from the other species.

Species delimitation and genetic diversity

All ISSR primers produced polymorphic bands. Genetic diversity parameters determined in the studied species (Table 3) revealed that *G. molle* (sp1) had the highest level of genetic polymorphism (55.91%), while the lowest level of genetic polymorphism (5.38%) occurred in *G. pusillum*, *G. trilophum*, *G. mascatense* (sp3, sp9, sp10). *G. molle* also had the highest values for effective number of alleles (Ne = 1.30) and Shannon information index (I = 0.28).

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 65% of total variation was among species and 35% was within species. Pair-wise FST values showed significant difference among all studied species (Table 4). Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.51, P = 0.01) and D_est values (0.189, P = 0.01).

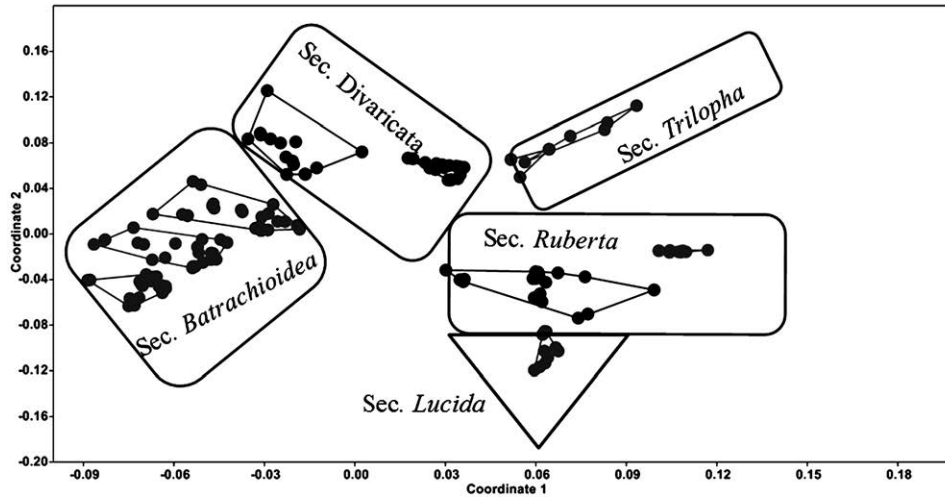

Fig. 3. Multidimentional scaling plots of morphological characters revealing species delimitation in subg. *Robertium*

Table 4
Pair-wise FST values among the studied *Geranium* species (above diagonal = FST value, below diagonal = P value)

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10
sp1	–	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
sp2	0.582	–	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
sp3	0.597	0.743	–	0.020	0.010	0.010	0.010	0.010	0.010	0.010
sp4	0.429	0.513	0.515	–	0.010	0.010	0.010	0.010	0.010	0.010
sp5	0.520	0.500	0.593	0.459	–	0.010	0.010	0.010	0.010	0.010
sp6	0.636	0.699	0.775	0.576	0.598	–	0.010	0.010	0.020	0.010
sp7	0.628	0.568	0.709	0.554	0.631	0.720	–	0.010	0.010	0.010
sp8	0.654	0.855	0.923	0.665	0.713	0.891	0.816	–	0.010	0.010
sp9	0.579	0.724	0.845	0.543	0.596	0.759	0.697	0.926	–	0.060
sp10	0.565	0.737	0.848	0.551	0.597	0.758	0.717	0.923	0.244	–

Non-metric MDS plots of ISSR data (Fig. 4) showed higher within species genetic diversity in the species number 1 (*G. molle*), supporting genetic diversity parameters obtained (Table 3).

The MDS plot separated the species into distinct groups. This indicates that ISSR molecular markers can be used in *Geranium* species delimitation. This is in agreement with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other. The

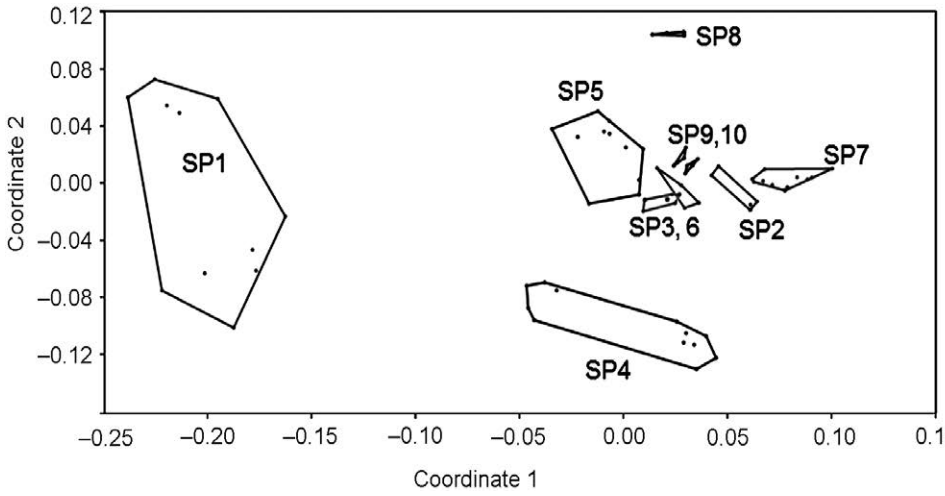

Fig. 4. MDS plot of *Geranium* species based on ISSR data. SP1 = *G. molle*, SP2 = *G. pyrenaicum*, SP3 = *G. pusillum*, SP4 = *G. purpureum*, SP5 = *G. robertianum*, SP6 = *G. albanum*, SP7 = *G. divaricatum*, SP8 = *G. lucidum*, Sp9 = *G. mascatense,* and SP10 = *G. trilophum*

Nm analysis by Popgene software also produced mean Nm = 0.21, that is considered very low value of gene flow among the studied species.

Mantel test with 5,000 permutations showed a significant correlation (r = 0.3, p = 0.0002) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Geranium* species studied.

Nei's genetic identity and the genetic distance determined among the studied species (Table is not included). The results showed that the highest degree of genetic similarity (0.98) occurred between *G. trilophum* and *G. mascatense* (sec. *Trilopha*). The lowest degree of genetic similarity occurred between *G. molle* and *G. lucidum* (0.71).

NJ tree based on Nei's genetic distance (Fig. 5), showed that *G. lucidum* differed genetically from the other studied species, as it stands far from them. This dendrogram showed close genetic affinity between *G. trilophum* and *G. mascatense* (sec. *Trilopha*) supporting our morphological result presented before (Fig. 2). The other *Geranium* species were placed closer to each other based on ISSR data, but their genetic affinity is not evident as is in morphology tree.

The species genetic STRUCTURE

We performed STRUCTURE analysis followed by the Evanno test to identify the optimal number of genetic groups. We used the admixture model to illustrate interspecific gene flow and/or ancestrally shared alleles in the species studied.

STRUCTURE analysis followed by Evanno test produced $\Delta K = 7$. The STRUCTURE plot (Fig. 6) produced more detailed information about the ge-

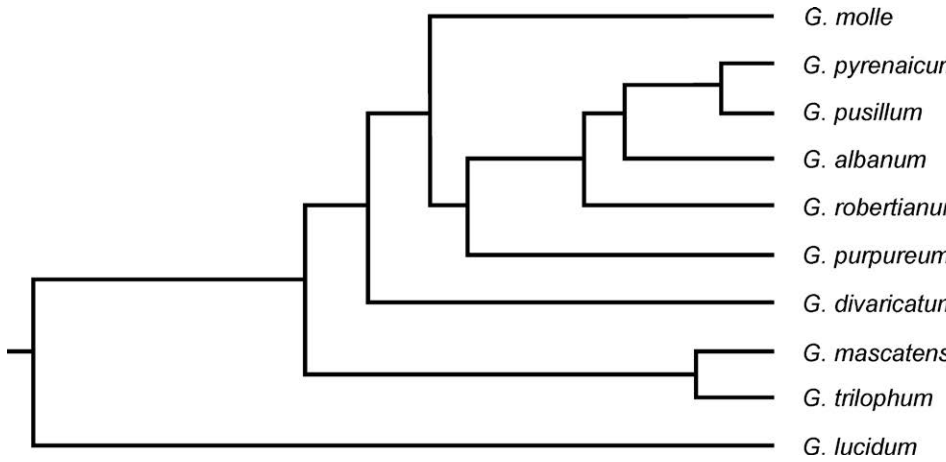

Fig. 5. Neighbor joining tree of inter simple sequence repeats data in the studied *Geranium* species

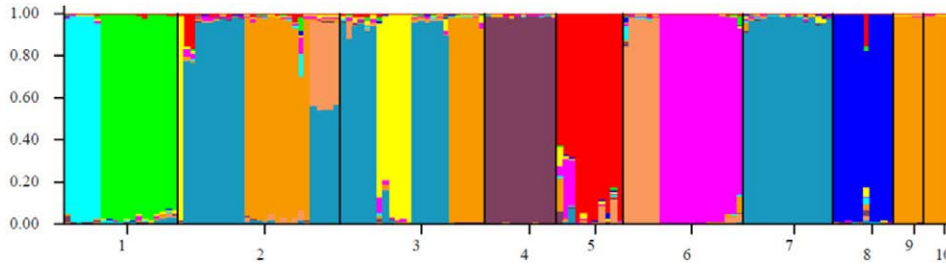

Fig. 6. STRUCTURE plot of *Geranium* species based on ISSR data. SP1 = *G. molle*, SP2 = *G. pyrenaicum*, SP3 = *G. pusillum*, SP4 = *G. purpureum*, SP5 = *G. robertianum*, SP6 = *G. albanum*, SP7 = *G. divaricatum*, SP8 = *G. lucidum*, Sp9 = *G. mascatense,* and SP10 = *G. trilophum*

netic structure of the species studied as well as shared ancestral alleles and/or gene flow among *Geranium* species. This plot revealed that Genetic affinity between *G. pyrenaicum*, *G. pusillum* and *G. divaricatum* (similarly colored, No. 2, 3, 7), as well as *G. trilophum* and *G. mascatense* (sec. *Trilopha,* No. 9, 10) due to shared common alleles. This is in agreement with neighbour joining dendrogram presented before. The other species are distinct in their allele composition.

The low Nm value (0.2) indicates limited gene flow or ancestrally shared alleles between the species studied and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among members of the studied species. However, reticulogram obtained based on the least square method (Fig. 7), revealed some amount of shared alleles between species 6 and 5, 1 and between 7 and 4 also between 4 and 1, 2, 5, 6, 7, 8.

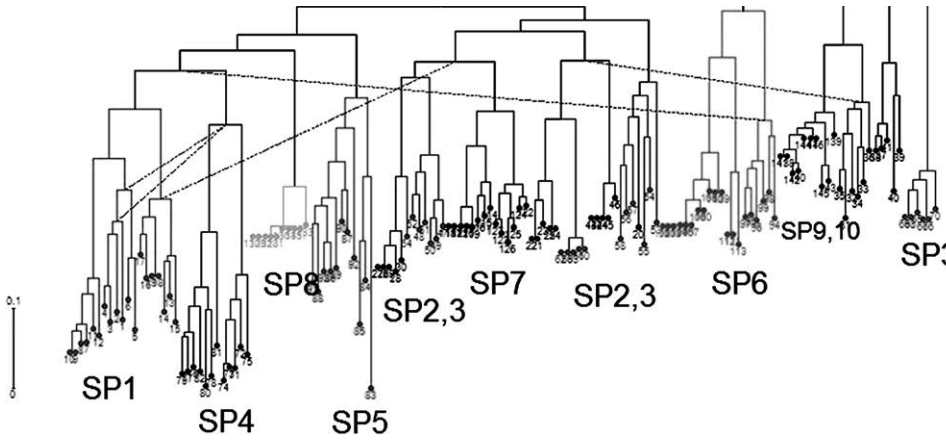

Fig. 7. Reticulogram of *Geranium* species. SP1 = *G. molle*, SP2 = *G. pyrenaicum*, SP3 = *G. pusillum*, SP4 = *G. purpureum*, SP5 = *G. robertianum*, SP6 = *G. albanum*, SP7 = *G. divaricatum*, SP8 = *G. lucidum*, Sp9 = *G. mascatense,* and SP10 = *G. trilophum*

As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in species studied and all these results are in agreement in showing a high degree of genetic stratification in species studied.

DISCUSSION

Species delimitation and taxonomic consideration

In the present study, subg. *Robertium* that is characterised by the "carpel-projection" type fruit discharge, comprises 10 species in 5 sections in Iran: 1) sec. *Ruberta* is characterised by the leaves divided to the base, 2) sec. *Divaricata* characterised by the fruit discharge mechanism inoperative, 3) sec. *Batrachioidea* characterised by the fruit discharge mechanism operative and pollen blue, 4) sec. *Lucida* characterised by the calyx longitudinally carinate, 5) sec. *Trilopha* characterised by annual and petals shape (Yeo 1984).

Morphological analyses of the studied *Geranium* species showed that they are well differentiated from each other both in quantitative measures (ANOVA test result) and qualitative characters (MDS plot result). In addition, PCA suggests that characters like pedicel length, bract length, seed shape, calyx shape, petal shape, length and width of stem-leaf, length and width of petal, peduncle and pedicel hair, mericarp hair density, mericarp surface, fruit discharge type, fruit discharge mechanism, pollen colour, habit and petal claw could be used in species groups delimitation. This morphological difference was due to quantitative and qualitative characters, for example, *G. robertianum* has longest stem-leaf (4–5 cm), the broadest stem-leaf (6–7 cm) and the longest petal (10–13 mm) among the studied species. Similarly, *G. trilophum* and *G. mascatense* had the broadest petal (6–7 mm) and the broadest mericarp (3.2 mm). *G. albanum* has the longest pedicel (20–30 mm) among the studied species. The genus *Geranium* contains a number of apparently suitable small-flowered annual species, some of which hybridise together, but the greatest range of variation appears to be provided by *G. robertianum* and its close relative *G. purpureum.* The hybrid *G. robertianum* × *G. purpureum* might occur where both parents are present. It is possible that *G. purpureum* subsp. *forsteri* (Wilmott) comb. nov. H. G. Baker arose from this cross (Stace 1997). The present study revealed that *G. robertianum* and *G. purpureum* are clearly separated from each other based on few morphological characters as shape of petal lobes, anthers colour, mericarp colour, petal shape and mericarp surface. No intermediate forms were observed throughout the area studied. These species, together with *G. lucidum* were placed by Knuth (1903) in the section *Robertiana*, which was originally created by Boissier (1867). Later, the same

author (Knuth 1912) made *G. lucidum* the type species of another section. Morphologically, however, the differences between the members of the otherwise isolated sections *Lucida*, *Anemonefolia* and *Robertiana* are not extreme and *G. lucidum*, in particular, shares a number of characters with *G. purpureum. Geranium lucidum,* however, is extremely uniform in morphology and ecology. Morphological analysis also separated *G. lucidum* from the other species studied. It differs in characters like outer sepals winged and with transverse keels, bright green (with shorter petioles than in *G. robertianum* and *G. purpureum*), lamina of the rosette up to 6 cm wide, bright green, sometimes red-edged.

Genetic structure and gene flow

AMOVA and STRUCTURE analysis revealed that the species of this subg. *Robertium* are genetically differentiated but have some degree of shared common alleles. Several trends in 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 an increased selfing and a reduction in flower size. Selfing is here related to annual or coloniser strategies, which occur in many other taxa (Ambruster 1993, Baker 1955, 1967, Stebbins 1957, 1970). Annual or biennial species with small flowers, such as *G. lucidum*, *G. pusillum*, *G. molle*, *G. dissectum*, *G. rotundifolium* are expected to be automatically self-pollinated. This has been proved for *G. molle*, *G. dissectum*. Usually large flowered perennial species rely on insects for pollination. The flowers of *G. pratense* are pollinated by bees, honeybees and bumblebees. The methods we used are indirect estimation of gene flow and if it is identified to occur among species may be either due to ancestral shared alleles or ongoing gene flow. The Nm value obtained based on ISSR data, revealed very limited amount of gene flow among the studied species that was also supported by STRUCTURE analysis as *Geranium* species mostly had distinct genetic structure. Reticulation analysis also showed some degree of gene flow for ISSR. We did not observe any intermediate forms in our extensive plant collection, but morphological variability within each species did occur to some extent. Therefore, the low degree of gene flow identified by indirect methods applied may be due to a low degree of gene flow both ancestral shared alleles and ongoing gene.

To conclude, the present study revealed the use of ISSR molecular markers along with morphological characters in *Geranium* species delimitation. Some degrees of interspecific genetic admixture occur in *Geranium*, but the studied species are strongly differentiated during the speciation process and invasion in new habitats. Genetic drift, strong inbreeding, and local adapta-

tion are effective evolutionary forces operating in *Geranium* species and population divergence and adaptation.

Plant species delimitation is of central importance in phylogenetic systematics, evolution, biogeography and biodiversity. It is significant to infer patterns and mechanisms of speciation and hybridisation, the evolutionary process by which new biological species arise and gene flow between closely related phylogenetic species can occur (Duminil and Di Michele 2009, Schluter 2001). Isolation by distance, local adaptation and gene flow are different mechanisms responsible for species differentiation and genetic diversity (Freeland *et al*. 2011, Frichot *et al*. 2013).

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