

SPECIES DELIMITATION IN *GERANIUM* SECT. *BATRACHIOIDEA*: MORPHOLOGICAL AND MOLECULAR

S. ESFANDANI BOZCHALOVI*¹, M. SHEIDAI¹
M. KESHAVARZI² and Z. NOORMOHAMMADI³

¹Department of Plant Sciences, Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran; E-mails: *somayehsfand@yahoo.com, msheidai@yahoo.com, msheidai@sbu.ac.ir

²Department of Plant Sciences, Faculty of Biological Science, Alzahra University, Tehran, Iran; E-mail: neshat112000@yahoo.com

³Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran; E-mail: marjannm@yahoo.com

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Geranium subg. *Robertium* (Geraniaceae) comprises eight sections, of which sect. *Batrachioidea* contains four species centred in Eurasia, Mediterranean region and the Himalaya Mountains. Three species of *Geranium pusillum*, *G. molle* and *G. pyrenaicum* occur in Iran show some degree of morphological overlaps that make the species delimitation difficult. Moreover, hybrids are known to be formed between these species elsewhere. Till present time, there has been no detailed information available on molecular phylogeny and genetic structure of these species in the country. Therefore, the present study was conducted with the aim to investigate species delimitation by both morphological and molecular data and to reveal genetic diversity and population structure in these three *Geranium* species. For this study, 216 randomly collected plants from 30 geographical populations in three *Geranium* species were used. We encountered extensive within species genetic and morphological diversity. ISSR molecular markers could not delimit the studied species. STRUCTURE analysis revealed the occurrence gene flow between these species. The Mantel test showed no correlation between genetic distance and geographical distance of the populations studied. Statistical analysis showed no significant difference between ITS and *rbcL* sequences and phylogenetic tree was constructed based on combined data set which separated outgroups from the studied species. Genetic affinity of the studied species has been discussed.

Key words: *Geranium*, ISSR, network, population structure, species delimitation

INTRODUCTION

Geranium L. is a cosmopolitan genus, with about 420 species being recognized in three subgenera: *Eroideae* (Picard) Yeo, *Robertium* (Picard) Bouy and *Geranium*, distinguished by fruit dispersal and eighteen sections (Aedo *et al.* 1998a). A brief history of the generic delimitation and infra-generic classifica-

tion, and a complete description of the genus are provided by Aedo (1996). Latter on Aedo *et al.* (1998a, b), provided an identification key for subgenera and their relative sections.

Some of the most interesting health benefits of *Geranium* include its ability to reduce inflammation, improve kidney health, relieve pain, lower stress levels, strengthen the immune system, and ease digestion. The essential oil of *Geranium* flowers is also highly sought after for its health benefits. The aerial parts of some *Geranium* species have been used as a tonic, diuretic, antidiabetic, antidiarrheal and antihemorrhoidal and also as a remedy to cure gastric disorders and wounds (Baytop 1999).

Geranium subg. *Robertium* (Geraniaceae) comprises eight sections, of which sect. *Batrachioidea* W. D. J. Kotch contains four species that are centred in Eurasia, Mediterranean region and the Himalayas.

Most *Geranium* species are herbaceous perennial plants with highly divided palmately lobed or cleft leaves, horizontal rhizomes. However, some species in subg. *Robertium* and *Geranium* are annuals, while sect. *Batrachioidea* has one perennials and three annual species. *G. pyrenaicum* Burm. f. is a perennial species with vertical, napiform rhizome. All species of this section have deeply palmatifid leaves. The most distinctive characters for *Geranium* sect. *Batrachioidea* are its obdeltate leaf segments, short petal claws, and chromosome number of $2n = 26$. The chromosome number is especially relevant as a derived character, as it has not been found in any other section of subg. *Robertium* (Aedo *et al.* 1998b).

There have been reports on the occurrence of interspecific hybridisation within *Geranium*. For example, hybrids do occur between the species of sect. *Anemonifolia*, *Batrachioidea*, *Lucida*, *Ruberta* and *Unguiculata* (Van Loon 1984, Yeo 1987), but no data are available for sect. *Divaricata*, *Polyantha*, and *Trilopha*.

In sect. *Batrachioidea*, three hybrids have been described as *G. × oenense* (*G. molle* × *G. pusillum*); *G. × luganense* (*G. molle* × *G. pyrenacium*); and *G. × hybridum* (*G. pusillum* × *G. pyrenacium*) (Van Loon 1984). As the studied species in this work show geographical distribution overlaps, there is a possibility of encountering hybrid zone and inter-specific hybrids.

Three species of *Geranium pusillum* L., *G. molle* L., and *G. pyrenaicum* occur in Iran and show some degree of morphological overlaps. Therefore, their taxonomic delimitation becomes somewhat problematic particularly in areas that may grow in vicinity.

Plant species delimitation and interspecific genetic diversity are two important areas of investigation in phylogenetic systematics, evolution, biogeography and biodiversity studies. Data obtained can help to understand the patterns and mechanisms of speciation and hybridisation (Minaeifar *et al.* 2016, Safaei *et al.* 2016). They can reveal the pattern of gene flow between

closely related phylogenetic species versus isolation by distance and identify the evolutionary process by which new biological species arise (Freeland *et al.* 2011, Sheidai *et al.* 2016). Species delimitation is a difficult task particularly in the species with cross-pollination breeding system that tend to form frequent interspecific hybrids (Sheidai *et al.* 2016).

Different molecular markers have been used in species delimitation and genetic diversity studies. These include both neutral multilocus markers (e.g., SSRs (simple sequence repeats), ISSRs (inter-simple sequence repeats) and AFLPs (amplified fragments length polymorphism)), etc. as well as DNA sequences (e.g., ITS (internal transcribed sequences) of ribosomal DNA and chloroplast DNA) (see for example, Sheidai *et al.* 2012, 2013, Koohdar *et al.* 2015, Mosaferi *et al.* 2015, 2016, Minaeifar *et al.* 2016, Safaei *et al.* 2016).

We used both ISSRs as well as ITS and *rbcL* gene of cp-DNA to carry out species delimitation. Similarly, we used ISSRs to investigate population genetic diversity and structure. For this purpose we collected plants of *Geranium pusillum*, *G. molle* and *G. pyrenaicum* from the areas they grow and the areas of overlap and delimit these three species and identify potential hybrids if at all occurring.

MATERIALS AND METHODS

Plant materials

In present study 216 plant samples were collected from 30 geographical populations. Different references were used for the correct identification of species (*G. molle*, *G. pyrenaicum* and *G. pusillum*) (Aedo *et al.* 1998b, Davis 1965, Janighorban 2009, Schönbeck-Temesy 1970, Zohary 1972). Details of sampling sites are mentioned (Table 1 and Fig. 1). Voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU).

Morphological studies – In total 80 morphological characters (42 qualitative, 38 quantitative) were studied. Five plant specimens were randomly studied or morphological analyses (Table 2).

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 MgCl₂; 0.2 mM of each dNTP (Bioron, Ger-

Table 1

Location and herbarium accession numbers of the studied populations of *G. molle* L., *G. pyrenaicum* Burm. f. and *G. pusillum* L. collected by Esfandani in Iran. Po = population, Alt. = altitude

Po	Locality	Latitude	Longitude	Alt. (m)	Voucher number
<i>G. molle</i> L.					
1	Mazandaran, Siahbisheh to Chalus	36° 14' 14"	51° 18' 07"	1807	HSBU 201648
2	Guilan, Bandar Anzali, road side	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, Khey-rudkenar Forest	36° 38' 05"	51° 29' 05"	-16	HSBU 201652
6	Mazandaran, Babol	36° 20' 53"	52° 30' 20"	606	HSBU 201653
7	Guilan, Golerodbar	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, Sang Deh 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, Qaemshahr	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	38° 52.393'	47° 25.92'	1133	HSBU 201662
16	East Azerbaijan kaleybar	38° 52.393'	47° 25.92'	1133	HSBU 201663
17	Mazandaran, Tonekabon, Shirod	37° 0' 8.23"	55° 8' 50.73"	-16	HSBU 201664
18	Mazandaran, Tuska Cheshmeh	36° 38.952'	53° 48.569'	1427	HSBU 201665
<i>G. pyrenaicum</i> Burm. f.					
19	Mazandaran, Sang Deh Forest	36° 01' 11"	53° 13' 26"	1720	HSBU 201666
20	Mazandaran, Veresk Bridge	35° 54' 12"	52° 59' 27"	1579	HSBU 201667
21	E Azerbaijan kaleybar	38° 52.393'	47° 25.92'	1144	HSBU 201668
22	E Azerbaijan kaleybar, Chesh-mealiakbar	38° 52.393'	47° 25.92'	1133	HSBU 201669
23	E Azerbaijan kaleybar, Shojobad	38° 52.393'	47° 25.92'	1137	HSBU 201670
24	E Azerbaijan kaleybar, road side	38° 52.393'	47° 25.92'	1122	HSBU 201671
25	E Azerbaijan, Babak fort	38° 51' 51"	47° 02' 28"	1155	HSBU 201672

Table 1 (continued)

Po	Locality	Latitude	Longitude	Alt. (m)	Voucher number
<i>G. pusillum</i> L.					
26	E Azerbaijan kaleybar	38° 52.393'	47° 23.92'	1144	HSBU 201673
27	Mazandaran, Sang Deh Forest	36° 01' 11"	53° 13' 26"	1720	HSBU 201674
28	E Azerbaijan kaleybar, Chesh-mealiakbar	38° 52.393'	47° 25.92'	1133	HSBU 201675
29	E Azerbaijan kaleybar, Shojabad	38° 52.393'	47° 25.92'	1137	HSBU 201676
30	E Azerbaijan kaleybar, road side	38° 52.393'	47° 25.92'	1122	HSBU 201677

many); 0.2 μ M 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

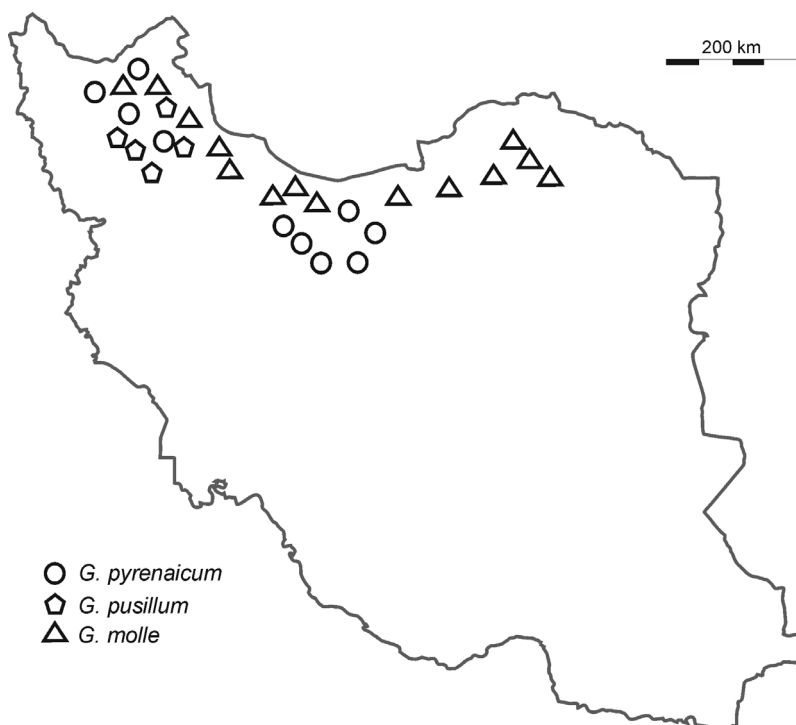


Fig. 1. Distribution map of the populations studied

Table 2
Evaluated morphological characters

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

Table 2 (continued)

No	Character	No	Character
65	Shape of calyx	73	Mericaip colour
66	Calyx apex	74	Seed colour
67	Petal shape	75	Seed shape
68	State of petal ligule	76	Seed surface ornamentation
69	Shape of petal lobes	77	Peduncle and pedicel hair density
70	State of petal ligule hair	78	Petioles hair
71	Stamen filament hair	79	Petioles hair density
72	Mericaip hair density	80	Leaf hair density

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, the UPGMA (unweighted paired group using average) and MDS (multidimensional scaling) were used (Podani 2000). 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. Parameter like Nei's gene diversity (H), Shannon information index (I), 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, while TCS network (Clement *et al.* 2002) was used for networking by Popart program (<http://popart.otago.ac.nz>).

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 (Hamer *et al.* 2012), DARwin ver. 5 (2012) software.

AMOVA (analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006), was used to show genetic difference of the populations.

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al.* 2000). 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). Gene flow was determined by (i) calculating N_m an estimate of gene flow from G_{st} by PopGene ver. 1.32 (1997) as: $N_m = 0.5(1-G_{st})/G_{st}$.

ITS and *rbcL* data of the species studied were obtained from NCBI (The National Centre for Biotechnology Information) and used for further analyses. Accession numbers for species retrieved from GenBank are presented in the Appendix.

ITS and *rbcL* DNA sequences were first aligned and cured by MUSCLE, and then used for constructing phylogenetic trees and TCS networking. MEGA 7 (2017) was used for drawing phylogenetic trees and Popart program (2017) was used for networking.

Incongruence between ITS and *rbcL* sequences was checked by two different methods. First by performing Mantel test (Podani 2000) as implemented in PAST ver. 5. (2000) program and second by estimating the Robinson–Foulds distance as implemented in PHYLIP ver. 3.69 (2013). We found no difference between ITS and *rbcL* sequences, and therefore phylogenetic tree was constructed based on a combined data set. Two species, namely *Geranium purpureum* and *G. robertianum* of the sect. *Ruberta* were used as outgroup taxa.

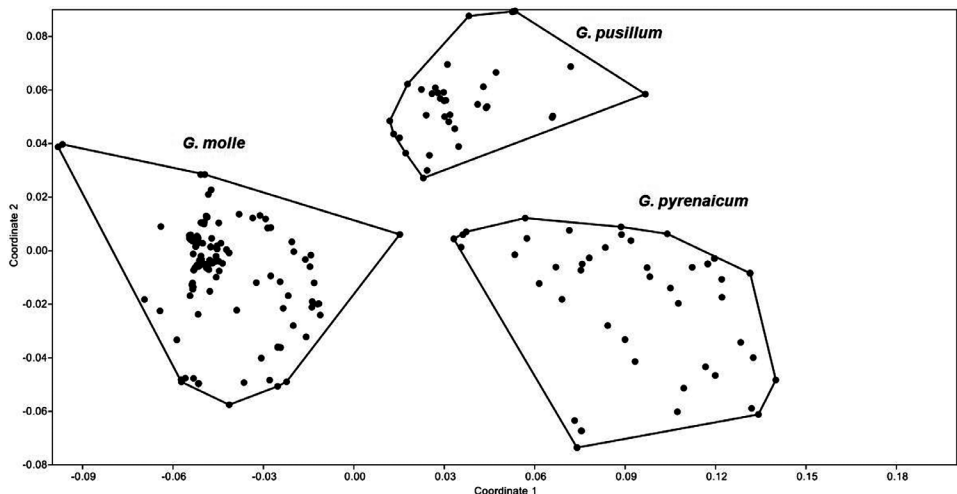


Fig. 2. Multidimensional scaling plot based on both quantitative and qualitative morphological characters delimiting the studied species in sect. *Batrachioidea*

RESULTS

Species delimitation and inter-relationship

Morphometry – Different clustering and ordination methods produced similar results therefore, only MDS plot of morphological characters are presented here (Fig. 2). In general, plant samples of each species were grouped together and formed a separate group. This result shows that morphological characters studied can delimit *Geranium* species. In the studied specimens we did not encounter intermediate forms.

PCA analysis of morphological traits revealed that the first three PCA components comprised about 71.5% of total variation. Morphological characters like, bract width, mericarp surface, mericarp hair, seed shape, sepal hair, positively correlated with these components and are the most variable morphological characters among the studied species and therefore, they may be used in taxonomy of these taxa.

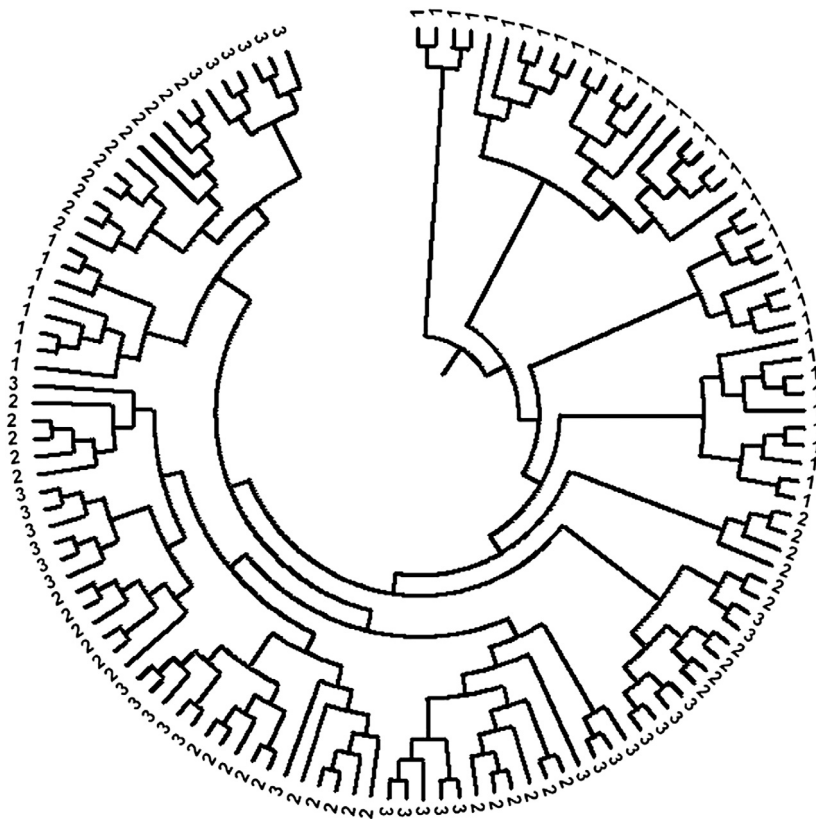


Fig. 3. UPGMA dendrogram of the studied populations based on ISSR markers. Abbreviations: 1–3 are *G. molle* (1); *G. pyrenaicum* (2); *G. pusillum* (3)

Molecular analysis – UPGMA clustering and TCS networking (Figs 3–4), of the studied populations did not entirely delimit the studied species and revealed that plants in these species are intermixed. In UPGMA circular dendrogram, a higher degree of intermixture occurred between *G. pusillum* and *G. pyrenaicum*.

TCS network (Fig. 4) revealed that although species 1 (*G. molle*) is more distinct than the other two species, but its accessions showed a high degree of intraspecific genetic variability as they are positioned in different places of the network.

Incongruence tests between ITS and *rbcL* sequences by Mantel test and estimating the Robinson–Foulds distance revealed no difference between ITS and *rbcL* sequences, and therefore phylogenetic tree was constructed based on a combined data set. The phylogenetic tree separated two outgroup species namely *Geranium purpureum* and *G. robertianum* of the sect. *Ruberta* in a single clade, while the in-group species were distributed in two separate clades.

UPGMA trees of combined data set of ITS and *rbcL* supported separation of the three species as their accessions formed separate clusters with high bootstrap value (> 0.98) (Fig. 5). In general UPGMA trees of cp-DNA and ITS

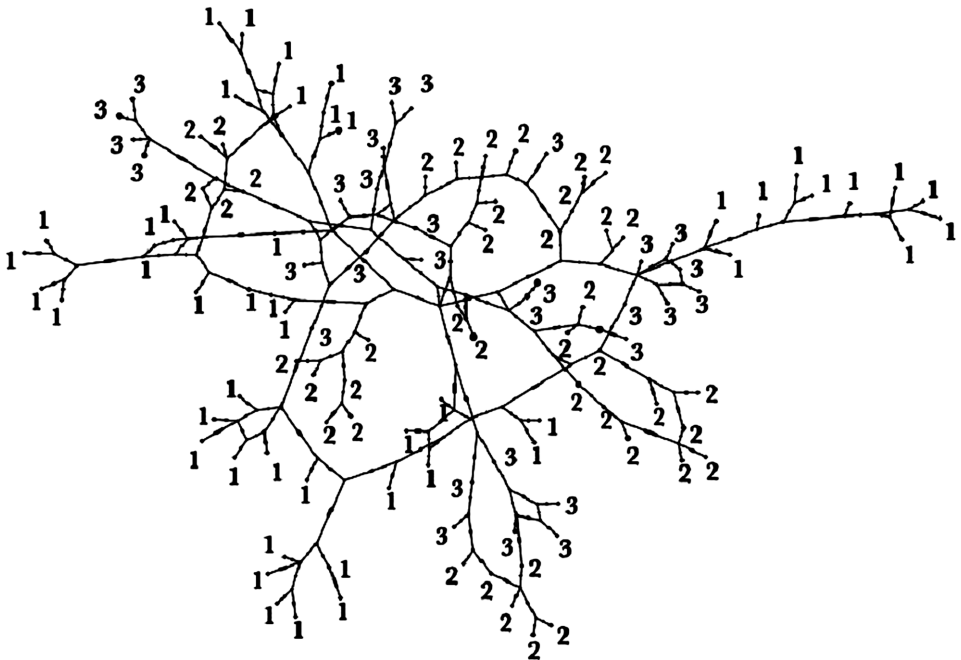


Fig. 4. TCS network of the studied populations based on ISSR markers. Abbreviations: 1–3 are *G. molle* (1); *G. pyrenaicum* (2); *G. pusillum* (3)

Table 3

Genetic diversity parameters based on ISSR data in the studied *Geranium* species (N = number of samples, Na = number of different alleles, Ne = number of effective alleles, I = Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P% = percentage of polymorphism, populations)

Species	N	Na	Ne	I	He	UHe	P%
<i>G. molle</i>	132.000	1.935	1.348	0.364	0.226	0.227	96.74
<i>G. pyrenaicum</i>	49.000	1.402	1.236	0.243	0.150	0.151	69.57
<i>G. pusillum</i>	35.000	1.250	1.234	0.233	0.146	0.148	61.96

trees is in agreement with morphology data. It showed affinity between *G. pusillum* and *G. pyrenaicum*. It also separated *G. molle* from the other species.

Population genetic analysis (ISSR)

Genetic diversity parameters determined in three studied species (Table 3) revealed that *G. molle* had the highest level of genetic polymorphism (96.74%), while the lowest level of the same occurred in *G. pusillum* (61.96%). In general these species have moderate to high degree of genetic variability which may be due to cross-pollination mode of reproduction in these plants.

The AMOVA test did not produce significant molecular differences ($P = 0.07$) among the studied species. This is in agreement with high Nm value ($Nm = 11.64$) and gene flow obtained in them. AMOVA revealed that 8% of total genetic variability occurred among the studied populations while 92% occurred within these species.

Nei's genetic identity and the genetic distance determined among the studied species are presented in Table 4. The results showed that the highest

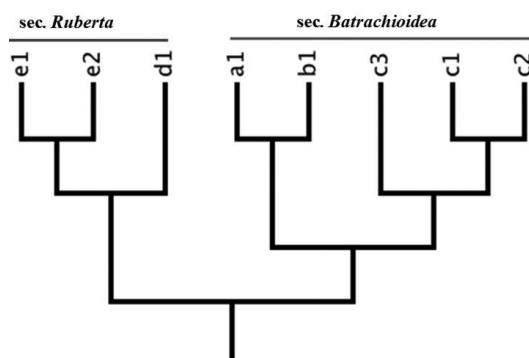


Fig. 5. Phylogenetic tree of the species studied based on combined ITS and *rbcL* sequences. Species abbreviations: a1 = *G. pyrenaicum*; b1 = *G. pusillum*; c1–c3 = *G. molle*; d1 = *G. purpureum*; e1–e2 = *G. robertianum*

Table 4

Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the study population ID (1 = *G. molle*, 2 = *G. pyrenaicum*, 3 = *G. pusillum*)

Population ID	1	2	3
1	–	0.9821	0.9850
2	0.0181	–	0.9941
3	0.0151	0.0059	–

degree of genetic similarity (0.99) occurred between *G. pusillum* and *G. pyrenaicum* and then between *G. molle* and *G. pusillum*, *G. pyrenaicum* (0.985). As we did not encounter any intermediate plants particularly in the areas of overlap, we consider these species to have high degree of ancestral shared alleles.

Mantel test did not produce significant correlation ($r = 0.01$, $p = 0.73$) between geographical distance and genetic distance of these species and therefore, no isolation by distance (IBD) exists between them.

STRUCTURE analysis followed by Evanno test also produced delta $K = 2$. Therefore, we do have at least 2 genetic groups in the studied species: 1) – specimens with green and red segments. These are accessions of *Geranium molle*; and 2) – specimens having green coloured segments as dominant colour. These are accessions of *G. pyrenaicum* and *G. pusillum*. Admixture ancestry model used in STRUCTURE revealed genetic affinity between *G. pyrenaicum* and *G. pusillum*, due to shared common alleles (Fig. 6). This is in agreement with TCS network presented before.

Populations of *Geranium molle* revealed two types of coloured segments, 1) – mostly green (similar to the other two studied species, e.g. populations 1, 3, 6, 12–14, 17–18), and 2) – mostly red coloured segments. This is in agreement with UPGMA circular dendrogram presented before that revealed within species genetic variability among populations. As stated before we did not

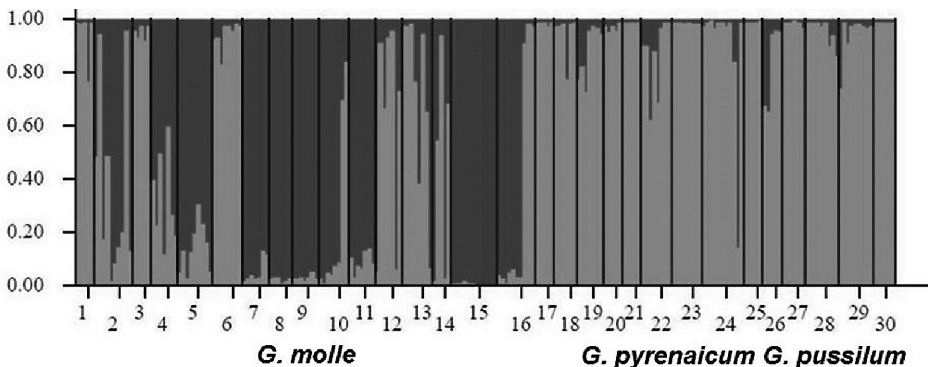


Fig. 6. STRUCTURE plot of *Geranium* species showing interspecific genetic variability and admixture

encounter plants with intermediate morphological characters or combination of characters from two species, therefore, we may suggest that these plants may differ in their genetic content possibly due to local adaptation. The genetic variability observed did not affect morphological characters of the plants and all were identified as *G. molle*.

DISCUSSION

Species delimitation and taxonomic consideration

The species delimitation in complex groups and in those that the species have different degree of morphological overlap is a tedious and difficult task. In these situations, it is suggested to use different and combined approaches like morphological, molecular, cytological, etc. to determine the species boundaries (Carstens *et al.* 2013). In the last few decades the use of molecular markers as tools for species and subspecies delimitation has drastically increased (Sheidai *et al.* 2013, 2016). The basic premise for the use of molecular markers for species delimitation is that the “species tree” should be inferred from a “gene tree”.

The sect. *Batrachioidea* is known to have interspecific hybrids (Van Loon 1984c). Successful interspecific cross was obtained between *Geranium molle* and *G. brutium*. Some of the previously identified interspecific hybrids were latter on considered not to be hybrid. For example, according to (Aedo *et al.* 1998b) *Geranium luganense*, *G. oenense* and *G. hybridum* are probably not hybrid, but are the synonyms of *Geranium molle* (the first two) or *G. pusillum*.

The present study did not identify intermediate plants in either of *G. pusillum* and *G. molle*.

Aedo *et al.* (1998b) suggested that sect. *Divaricata* and *Batrachioidea* could constitute a monophyletic group, and that the taxonomic problems on sect. *Batrachioidea* is due to morphological overlaps in two species, i.e. *G. pyrenaicum* and *G. molle*.

Variability within the species in sect. *Batrachioidea* is the source of morphological complexity, as evidenced in *G. pyrenaicum* (Ortiz 1989). The present study revealed these species may be delimited by morphological characters as well as combination of ITS and *rbcL* sequences. The species relationship obtained also is in agreement with morphological analysis and supports taxonomic treatment of Flora Iranica (Schönbeck-Temesy 1970).

PCA analysis suggests that characters like bract width, mericarp surface, mericarp hair, seed shape, sepal hair, length and width of mericarp, seed length, shape of petal lobes, length and width of corolla, and length and width of leaves may be used in species delimitation.

Interspecific morphological and genetic variability

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. (Sheidai *et al.* 2016). These information have different applications, and from like pure understanding of biology of the species to conservation of endangered species, choosing of proper parents for hybridisation and breeding and phylogeography and mechanism of invasion (Freeland *et al.* 2011). *Geranium pyrenaicum* is widespread in our country and it has several medicinal applications (Baytop 1999), however, we had no information on its population genetic structure. The present study revealed the presence of high degree of genetic variability within this species which is highly correlated with its reproductive mode. 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.

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APPENDIX

GenBank accession numbers for nrDNA ITS and cpDNA *rbcL* sequence data, and voucher information with herbarium where specimen is deposited.

Geranium pyrenaicum – HE795061.1 (Palazzesi *et al.* 2012), KJ204349.1 (Wyler and Litman 2015)

Geranium pusillum – DQ525070.1 (Fiz-Palacios and Aldasoro 2008), JX913454.1 (Park *et al.* 2017)

Geranium molle – KX167627.1 (de Vere and Ford 2016), JQ412370.1 (Bezeng *et al.* 2013)

Geranium purpureum – KX167629.1 (de Vere 2016), JN893649.1 (de Vere *et al.* 2012)

Geranium robertianum – AB689056.1 (Nishida and Azuma 2012), HQ590118.1 (Burgess *et al.* 2016)