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Species identification and population structure analysis in *Geranium* subg. *Geranium* (Geraniaceae)

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Ključne besede: ISSR, morfologija, določitev vrst.

Abstract

Species identification is fundamentally important within the fields of biology, biogeography, ecology and conservation. The genus *Geranium* L. (Geraniaceae) comprises about 430 species distributed throughout most parts of the world. According to the most recent treatments, subg. *Geranium* is the largest subgenus with over 370 species classified in ten sections. The subg. *Geranium* is represented in Iran by 13 species. These species are grouped 3 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, we performed molecular (ISSR markers) and morphological studies of 102 accessions from 13 species of *Geranium* (subg. *Geranium*) that were collected from different habitats in Iran. The aims of present study are: 1) can ISSR markers identify *Geranium* species, 2) what is 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 identify the species.

Izvleček

Določitev vrst je pomembna v biologiji, biogeografiji, ekologiji in naravovarstvu. V rod *Geranium* L. (Geraniaceae) uvrščamo okoli 430 vrst razširjenih po večini sveta. V skladu z najnovejšimi objavami je subg. *Geranium* najštevilčnejši podrod z več kot 370 vrstami, ki jih naprej delimo v deset sekcij. V Iranu v podrod *Geranium* uvrščamo 13 vrst in jih nadalje združujemo v tri sekcije. Navkljub številnim splošno razširjenim vrstam rodu *Geranium*, ki uspevajo v Iranu ne obstaja nobena raziskava o njihovi genetski raznolikosti, načinih divergence in vzorcih razširjenosti.

Zato smo izvedli molekularno (ISSR markerji) in morfološko raziskavo 102 primerkov 13 vrst rodu *Geranium* (subg. *Geranium*), ki smo jih nabrali v različnih rastiščih v Iranu. V raziskavi smo ugotavljali: 1) ali lahko z ISSR markerji ločimo vrste Geranium, 2) kakšna je genetska struktura the taksonov v Iranu in 3) kakšni so medsebojni odnosi med temi vrstami. Ugotovili smo, da s kombinacijo morofoloških in ISSR podatkov uspešno določimo vrste.

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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 & Marshall 2003). In the first method, species form distinguishing clades (phylogenetic species concept), whereas in non-tree-based method, the species are recognized on the basis of gene flow assessments (biological species concept; Pérez-Losada et al. 2005).

Wiens & Penkrot (2002), proposed to use DNA data, morphological data and character data for species delimitation while, Knowles & 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* L. (Geraniaceae) comprises about 430 species distributed through out most of the world (Aedo et al. 1998b). 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*, subg. *Erodioidea* (Picard) Yeo, and subg. *Robertium* (Picard) Rouy. Most recent treatments revealed that *G.* subgen. *Geranium* is the largest subgenus with over 370 species (in 10 sections) (Aedo et al. 2003, 2005a, 2005b, 2007, Aedo & Estrella 2006). Some of these sections have already been revised (Davis 1970, Carlquist & Bissing 1976), but further studies should be done to attain a satisfactorily knowledge of subg. *Geranium*. The diversity of fruit-types in *Geranium* is greatest in the Mediterranean region (Yeo 1984, 2004). Within *G.* subgen. *Geranium*, sect. *Geranium* is a widespread and heterogeneous group with wide distribution patterns except tropical lowlands, deserts and polar regions, whereas *G.* sect. *Dissecta* Yeo is widely distributed in Eurasia, between the Mediterranean region and the Himalaya mountains and *G.* sect. *Tuberosa* (Boiss.) Reiche is present in the Mediterranean area and central parts of Asia, Western Europe and northwestern Africa. *Geranium* sect. *Tuberosa* was subdivided by Yeo (1984) into the subsections *Tuberosa* (Boiss.) Yeo and *Mediterranea* R. Knuth based on the vegetative traits. Yeo (1984) indicated that *G.* subsect. *Tuberosa* was characterized by tuberose rootstock and palmatisect leaves and the highest diversity of the group is found at regions between Turkey and Iran (Aedo & Estrella 2006, Aedo et al. 2007).

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 Iran Flora (Janighorban 2009), the genus is represented by 25 species but there are not clarified sections for it (Onsori et al. 2010). Diagnostic features in infrageneric classification are related to fruit discharge methods, mericarp margin and leaves shape. In Iran there are *Geranium* species with carpel projection or seed ejection.

Geranium is both cross-pollinated and self-pollinated (Stebbins, 1957, 1970), and inter-specific hybrids and intermediate forms do occur in few *Geranium* species in the area of species overlap. Yeo (2002: 214) indicated that artificial hybrids between many species of subsect. *Mediterranea* (including *G. ibericum* and *G. platypetalum*) have been produced by Bremner. However, no names for these hybrids are available except *Geranium* × *magnificum* which is usually considered as a hybrid between *G. ibericum* (without glandular hairs) and *G. platypetalum* (with glandular hairs).

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 infra-generic rank and their results show that the species can be separated into subgenera and sections based on fruit morphology while seed micro-morphological features generally do not support the sectional taxonomy, but provide valuable characters for the delimitation at species groups, species, and infra-specific levels (Salimi Moghadam et al. 2015). Literature revealed that studies are mainly dealing with taxonomy, seed and pollen morphology, stem and leaf anatomy (Salimpour et al. 2009, Onsori et al. 2010, Salimi Moghadam et al. 2015, Keshavarzi et al. 2015, 2016, Esfandani-Bozchaloyi et al. 2017a, 2017b, 2017c, 2017d) of *Geranium* species but there are no attempt to study genetic diversity, ecological adaptation and intra- and inter-specific differentiation along with morphometric studies on *Geranium* of Iran. Therefore, we performed morphological and molecular study of 159 collected specimens of 3 section in the subg. *Geranium.* We 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?

● *G. dissectum*, ● *G. collinum*, ● *G. columbinum*, ● *G. rotundifolium*, ● *G. persicum*, ● *G. tuberosum*, ● *G. kotschyi*, ● *G. pratense*, ● *G. stepporum*, ● *G. sylvaticum*, ● *G. platypetalum*, ● *G. gracile*, ● *G. ibericum*

Figure 1: Distribution map in studied species.
Slika 1: Karta razširjenosti preučevanih vrst.

Table 1: *Geranium* species and populations, their localities and voucher numbers.
Tabela 1: Vrste in populacije rodu *Geranium,* lokalitete in številke vavčerjev.

Sp.	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
1. *G. dissectum*	Guilan, Siahkal, Ezbaram	37° 07' 48"	49° 54' 04"	165	HSBU 201658
	Guilan, Lahijan	37° 07' 08"	49° 54' 11"	159	HSBU 201659
2. *G. columbinum*	East Azerbaijan kaleybar cheshme ali akbar	38° 52' 93"	47° 25' 92"	1133	HSBU 201660
	East Azerbaijan kaleybar, Shojabad	38° 52' 93"	47° 25' 92"	1139	HSBU 201661
3. *G. rotundifolium*	Tehran, Tuchal	35° 50' 36"	51° 24' 28"	2383	HSBU 201662
4. *G. collinum*	Tehran, Damavand	35° 42' 29"	52° 20' 51"	2421	HSBU 201663
5. *G. platypetalum*	East Azerbaijan kaleybar	38° 52' 39"	47° 23' 92"	1144	HSBU 201668
6. *G. sylvaticum*	East Azerbaijan kaleybar cheshme ali akbar	38° 52' 39"	47° 25' 92"	1133	HSBU 201669
7. *G. pratense*	East Azerbaijan kaleybar, Shojabad	38° 52' 39"	47° 25' 92"	1137	HSBU 201670
8. *G. ibericum*	Mazandaran, Tonekabon-jannat rudbar	36° 48' 47"	50° 53'68"	1600	HSBU 201671
9. *G. gracile*	Mazandaran, Noshahr, Kheyrud kenar Forest	36° 38' 05"	51° 29' 05"	1250	HSBU 201672
10. *G. persicum*	Tehran, Firuz kuh	35° 43' 15"	52° 04' 12"	1975	HSBU 201673
11. *G. kotschyi*	Alborz, Karaj- Qazvin	35° 49' 23"	51° 00' 04"	1365	HSBU 201674
12. *G. tuberosum*	East Azerbaijan kaleybar cheshme ali akbar	38° 52' 39"	47° 25' 92"	1133	HSBU 201675
13. *G. stepporum*	Tehran, Tuchal	35° 50' 03"	51° 24' 28"	2383	HSBU 201676

Materials and methods

Plant materials

We performed morphological and molecular analysis of 13 *Geranium* species growing in Iran (Table 1). For morphometric studies we used 159 plant specimens (7–35 samples from each species) (Figure 2), and for ISSR analysis, we used 102 (Figure 5). The species studied are: *G. columbinum* L., *G. rotundifolium* L., *G. collinum* Stephan ex Willd, *G. sylvaticum* L., *G. pratense* (sec. *Geranium*); *G. dissectum* L. (sec. *Dissecta*); *G. persicum* Schönb.-Tem., *G. tuberosum* L., *G. kotschyi* Boiss., *G. stepporum* P.H.Davis (sec. *Tuberosa* subsect. *Tuberosa* (Boiss.) Yeo); *G. platypetalum* Fisch. & C. A. Mey., *G. gracile* Ledeb. ex Nordm., *G. ibericum* Cav. (sec. *Tuberosa* subsect. *Mediterranea* R. Knuth). Different references were used for the correct identification of species (Davis 1967, Schonbeck-Temesy 1970, Zohary 1972, Aedo et al. 1998b, Janighorban 2009). Details of sampling sites are mentioned (Table 1, Figure 1). Voucher specimens are deposited in Herbarium of Shahid Beheshti University (HSBU).

Morphological studies

In total 80 morphological (42 qualitative, 38 quantitative) characters were studied (supplementary Table 2). Data obtained were standardized (Mean = 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000).

DNA extraction and issr assay

Fresh leaves were used randomly from 5–11 plants in each of the studied species. 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. 10 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 in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94 °C, followed by 40 cycles of 1 min at 94 °C; 1 min at 52–57 °C and 2 min at 72 °C. The reaction was completed by final extension step of 7–10 min at 72 °C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Morphological studies

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa (Podani 2000). For grouping of the plant specimens, the UPGMA (Unweighted paired group using average) and Ward (Minimum spherical characters) as well as ordination methods of MDS (Multidimensional scaling) and PCoA (Principal coordinate analysis) were used (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. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were determined (Weising et al. 2005, Freeland et al. 2011). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland et al. 2011, Huson & 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 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (Meirmans & Van Tienderen 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008).

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard et al. 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2. (2013). For

Table 2: Morphological characters in studied species.

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
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 color
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 petale ligule
69	Shape of petal lobes
70	State of petale ligule hair
71	Stamen filament hair
72	Mericarp hair density
73	Mericarp color
74	Seed color
75	Seed shape
76	Seed surface ornamentation
77	Peduncle and pedicel hair density
78	Petioles hair
79	Petioles hair density
80	Leaf hair density

Table 2: Morfološke značilnosti preučevanih vrst.

STRUCTURE analysis, data were scored as dominant markers (Falush et al. 2007). 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 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

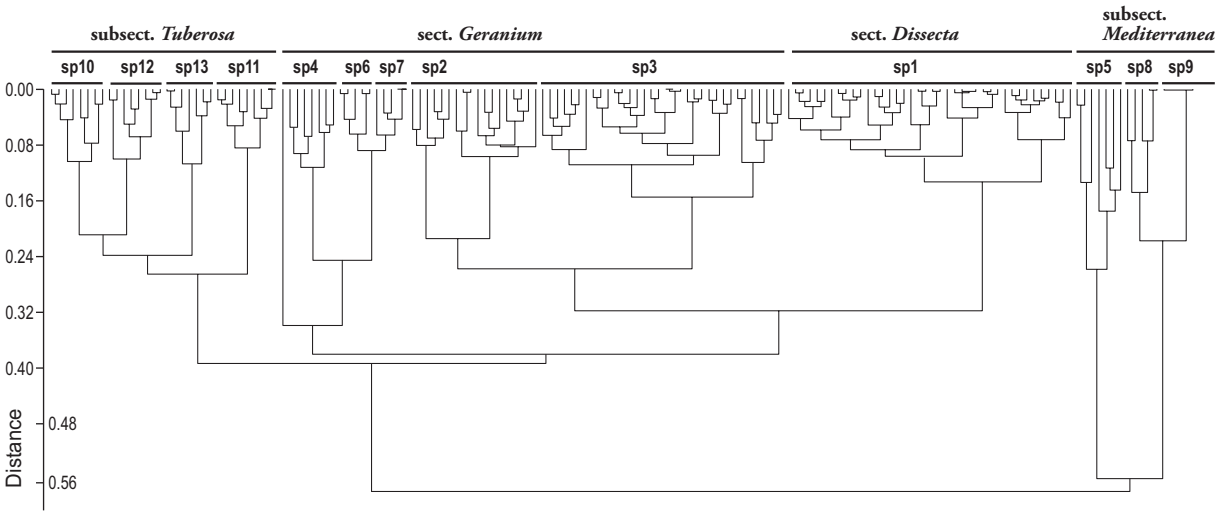
Species identification and inter-relationship

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 62% of the total variation. In the first PCA axis with 32% of total variation, such characters as shape of bract, peduncle and pedicel hair, stem hair, bract and leaf hair, petiole hair, mericarp hair density have shown the highest correlation (>0.7), length of bract and peduncle, width of petal, sepal hair, number of flowers per inflorescence were characters influencing PCA axis 2 and 3 respectively.

Different clustering and ordination methods produced similar results therefore, UPGMA clustering and PCA plot of morphological characters are presented here (Figures 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 differntiate the *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 (Figure 2), Populations of *G. platypetalum*, *G. gracile* and *G. ibericum* (sect. *Tuberosa* subsect. *Mediterranea*) were placed in the first major cluster and were placed with great distance from the other species. The second major cluster included two sub-clusters. Plants of *G. persicum*, *G. tuberosum*, *G. kotschyi, G.stepporum* (sect. *Tuberosa* subsect. *Tuberosa)* comprised the first sub-cluster due to morphological similarity, while plants of *G. rotundifolium*, *G. collinum*, *G. sylvaticum*, *G. pratense, G. columbinum* (sect. *Geranium*) and *G. dissectum* (sect. *Dissecta*) formed the second sub-cluster.

The PCA plot of morphological characters (Figure 3) separated the species into distinct groups with no intermixing. This is in agreement with UPGMA tree presented before.



SP1: G. dissectum, SP2: G. columbinum, SP3: G. rotundifolium, SP4: G. collinum, SP5: G. platypetalum, SP6: G. sylvaticum, SP7: G. pratense, SP8: G. ibericum, SP9: G. gracile, SP10: G. persicum, SP11: G. kotschyi, SP12: G. tuberosum, SP13: G. stepporum

Figure 2: UPGMA clustering of morphological characters revealing species delimitation in subg. *Geranium.*
Slika 2: Klasifikacija morofloških značilnosti z metodo UPGMA kaže na ločitev posameznih vrst v subg. *Geranium.*

Species identification and genetic diversity

All ISSR primers produced polymorphic bands. Genetic diversity parameters determined in the studied species (Table 3) revealed that *G. dissectum* (sp1) had the highest level of genetic polymorphism (47.31%), while the lowest level of genetic polymorphism (2.15%) occurred in *G. gracile* and *G. tuberosum* (sp9, sp13). *G. dissectum* also had the highest values for effective number of alleles (Ne = 1.30) and Shannon information index (I = 0.25).

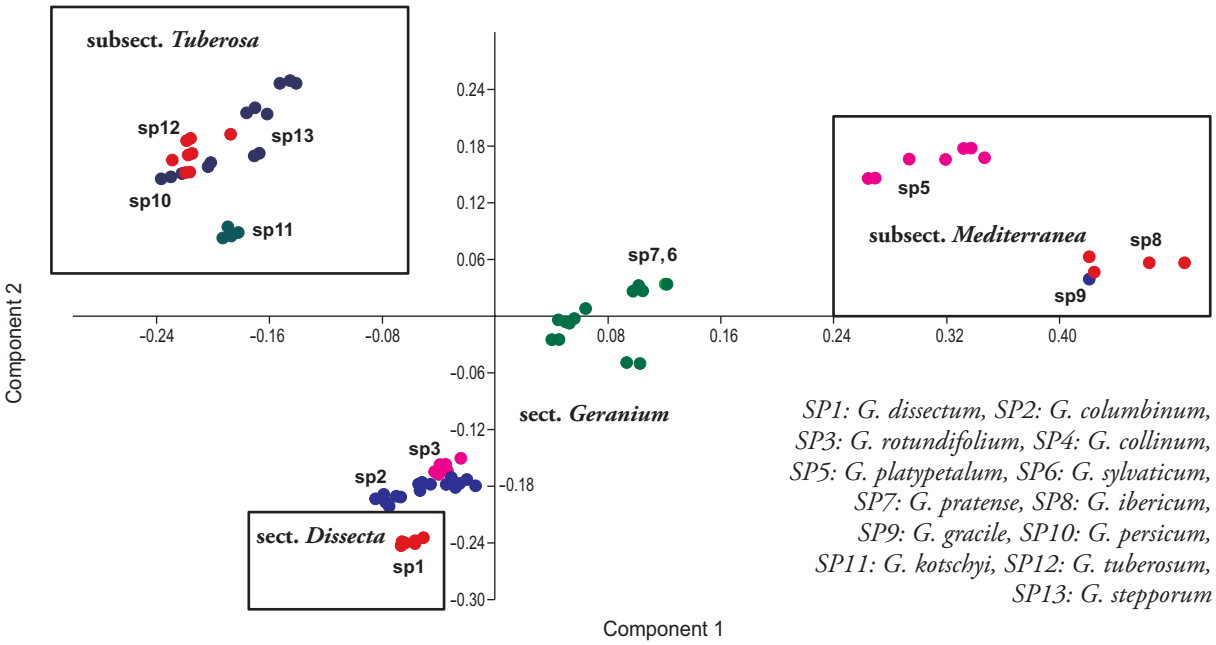


Figure 3: PCA plots of morphological characters revealing species delimitation in subg. *Geranium*.
Slika 3: Diagram PCA morfoloških značilnosti kaže na ločitev posameznih vrst v subg. *Geranium*.

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 79% of total variation was among species and 21% 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).

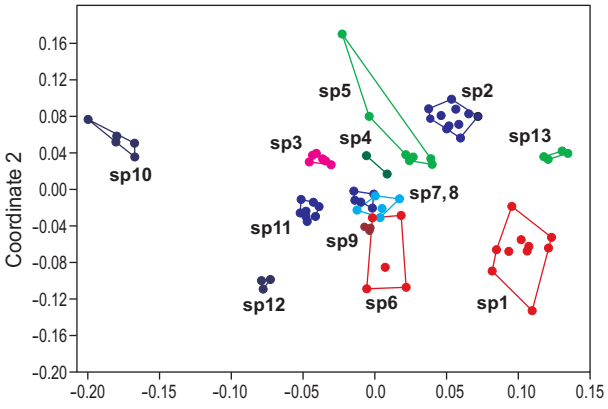


Figure 4: MDS plot of *Geranium* species based on ISSR data.
Slika 4: Diagram MDS vrst rodu *Geranium* na podlagi podatkov ISSR.

Non-metric MDS plots of ISSR data (Figure 4) showed higher within species genetic diversity in *G. dissectum* (sp1), supporting genetic diversity parameters obtained (Table 3).

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).
Tabela 3: Genetska diverziteta spremenljivk preučevanih vrst rodu *Geranium*. (N = število vzorcev, Ne = efektivno število alelov, I= Shannonov informacijski index, He = genetska diverziteta, UHe = nepristranska genetska diverziteta, P%= delež polimorfizma, populacij).

Pop	N	Na	Ne	I	He	UHe	%P
sp1	10.000	0.978	1.302	0.256	0.173	0.182	47.31%
sp2	12.000	0.376	1.061	0.053	0.036	0.037	9.68%
sp3	7.000	0.355	1.029	0.031	0.019	0.021	7.53%
sp4	8.000	0.301	1.004	0.008	0.004	0.004	3.23%
sp5	7.000	0.677	1.087	0.093	0.057	0.062	23.66%
sp6	5.000	0.699	1.156	0.143	0.094	0.105	27.96%
sp7	5.000	0.376	1.054	0.055	0.035	0.039	11.83%
sp8	5.000	0.452	1.064	0.061	0.039	0.044	12.90%
sp9	5.000	0.269	1.021	0.015	0.011	0.012	2.15%
sp10	8.000	0.548	1.013	0.023	0.012	0.012	9.68%
sp11	9.000	0.452	1.089	0.078	0.052	0.055	15.05%
sp12	8.000	0.333	1.006	0.009	0.005	0.005	3.23%
sp13	7.000	0.323	1.010	0.011	0.007	0.007	2.15%

Table 4: Pair-wise FST values among the studied *Geranium* species. (Above diagonal = FST value, bellow diagonal =P value).
Tabela 4: Primerjava parov vrednosti FST med preučevanimi vrstami rodu *Geranium* (Vrednosti nad diagonalo-FST, vrednosti pod diagonalo-P).

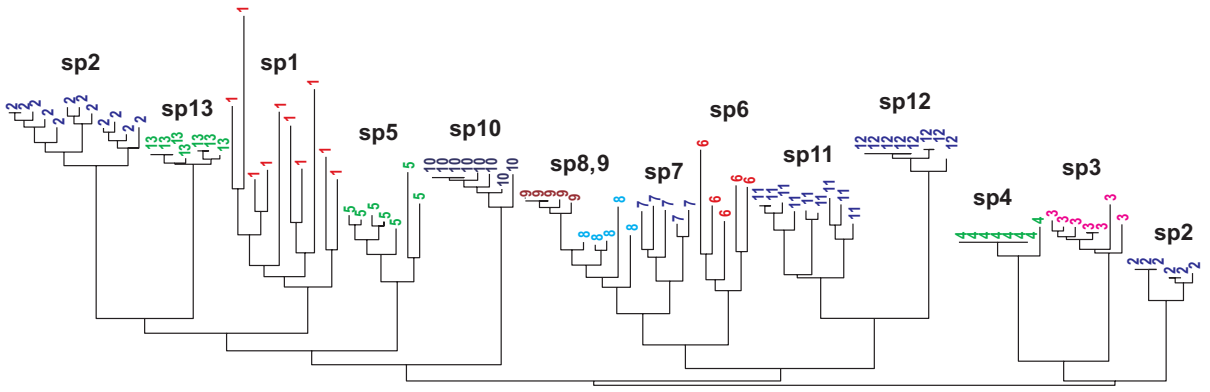
	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	sp11	sp12	sp13
sp1	-	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
sp2	0.593	-	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
sp3	0.590	0.856	-	0.010	0.010	0.020	0.010	0.010	0.010	0.010	0.010	0.010	0.010
sp4	0.593	0.870	0.897	-	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
sp5	0.507	0.752	0.774	0.803	-	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
sp6	0.414	0.766	0.710	0.773	0.580	-	0.030	0.010	0.010	0.010	0.010	0.010	0.020
sp7	0.477	0.810	0.793	0.853	0.661	0.478	-	0.010	0.010	0.010	0.010	0.010	0.010
sp8	0.474	0.831	0.831	0.860	0.667	0.507	0.479	-	0.030	0.010	0.010	0.010	0.010
sp9	0.513	0.878	0.925	0.963	0.794	0.667	0.775	0.577	-	0.010	0.010	0.010	0.010
sp10	0.682	0.908	0.920	0.953	0.844	0.831	0.880	0.883	0.944	-	0.010	0.010	0.010
sp11	0.565	0.795	0.780	0.834	0.741	0.622	0.630	0.730	0.790	0.851	-	0.010	0.010
sp12	0.634	0.912	0.938	0.970	0.874	0.785	0.891	0.911	0.966	0.954	0.788	-	0.010
sp13	0.597	0.878	0.945	0.965	0.819	0.791	0.884	0.895	0.970	0.951	0.861	0.971	-

The MDS plot separated the species into distinct groups. This indicates that ISSR molecular markers can be used in *Geranium* species differentiation. This is in agreement with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other. The Nm analysis by Popgene software also produced mean Nm= 0.10, that is considered very low value of gene flow among the studied species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.16, 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 (0.94) occurred between *G. ibericum* and *G. gracile* (sect. *Tuberosa* subsect. *Mediterranea*). The lowest degree of genetic similarity occurred between *G. persicum* and *G. columbinum* (0.61).

NJ tree based on Nei,s genetic distance (Figure 5), showed that *G. kotschyi*, *G. tuberosum* (sect. *Tuberosa* subsect. *Tuberosa*) are separated from the other studied species and join the others with a great distance. This dendrogram showed close genetic affinity between *G. columbinum*, *G. rotundifolium*, *G. collinum* (sect. *Geranium*). Similarly, *G. gracile* and *G. ibericum* (subsect. *Mediterranea)* were placed close to each other, to which, *G. platypetalum* was joined with some distance. In general, species relationships obtained from ISSR data agrees well with species relationship obtained from morphological characters.



SP1: G. dissectum, SP2: G. columbinum, SP3: G. rotundifolium, SP4: G. collinum, SP5: G. platypetalum, SP6: G. sylvaticum, SP7: G. pratense, SP8: G. ibericum, SP9: G. gracile, SP10: G. persicum, SP11: G. kotschyi, SP12: G. tuberosum, SP13: G. stepporum

Figure 5: Neighbor joining tree of inter simple sequence repeats data in the studied *Geranium* species.
Slika 5: Dendrogram, narejen z združevanjem najbližjega soseda podatkov ISSR preučevanih vrst rodu *Geranium*.

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 or / and ancestrally shared alleles in the species studied.

STRUCTURE analysis followed by Evanno test produced ΔK = 10. The STRUCTURE plot (Figure 6) produced more detailed information about the genetic 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. rotundifolium* and *G. collinum* (similarly colored), as well as *G. ibericum* and *G. gracile* (similarly colored) due to shared common alleles. This is in agreement with Neighbor joining dendrogram presented before. The other species are distinct in their allele composition and differed genetically from each other.

The low Nm value (0.10) 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 (Figure 7), revealed some amount of shared alleles between species 10 and 11 and between 1 and 8

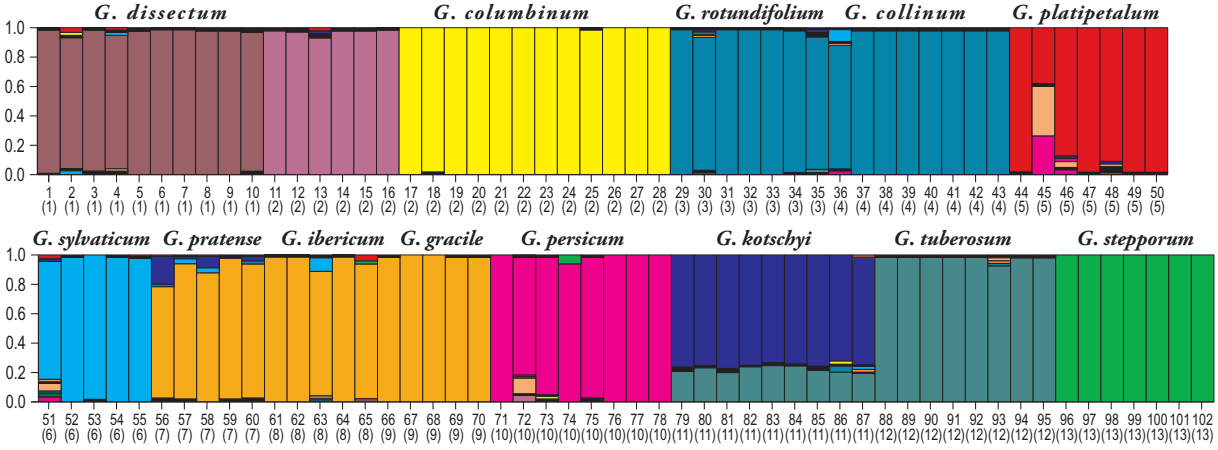
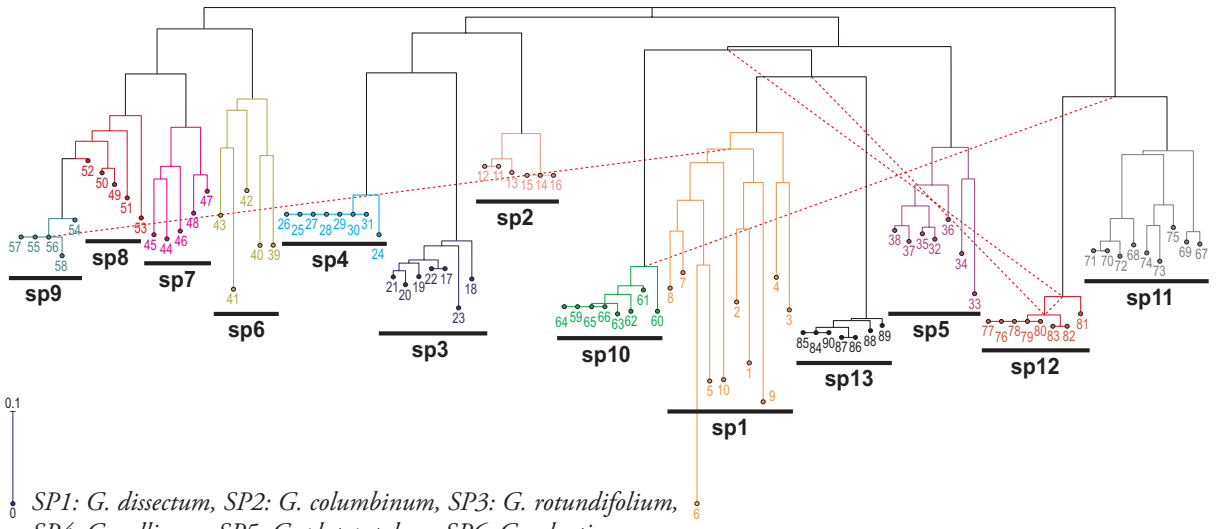


Figure 6: STRUCTURE plot of *Geranium* species based on ISSR data.
Slika 6: Diagram STRUCTURE vrst rodu *Geranium* na osnovi podatkov ISSR.



SP1: G. dissectum, SP2: G. columbinum, SP3: G. rotundifolium, SP4: G. collinum, SP5: G. platypetalum, SP6: G. sylvaticum, SP7: G. pratense, SP8: G. ibericum, SP9: G. gracile, SP10: G. persicum, SP11: G. kotschyi, SP12: G. tuberosum, SP13: G. stepporum

Figure 7: Reticulogram of *Geranium* species.
Slika 7: Retikulogram vrst rodu *Geranium*.

also between 12 and 1, 10, 13. 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 high degree of genetic stratification in species studied

Discussion

Species identification and taxonomic consideration

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 Iran Flora (Janighorban 2009), the genus is represented by 25 species but there are not clarified sections for it (Onsori et al. 2010). Moreover, the present study showed that the subg. *Geranium* is characterized by a fruit of seed-ejection type, the comprises 13 species in three sections in Iran: 1) Sect. *Tuberosa* indicating that it differs from the general model of subgen. *Geranium* in two ways: the awn, with the mericarps attached, falls away from the columella, and there is no structure for retaining the seed in the pre-explosive interval. These important fruit characters seem to support sect. *Tuberosa* as a natural group, in which two subgroups can be differentiated: a) subsect. *Tuberosa* with tuberose rootstock and ± palmatisect leaves, and b) subsect. *Mediterranea* without tuberose rootstock and palmatifid leaves (Yeo 1984). All species of *Geranium* subsect. *Mediterranea* except *G. bohemicum* and *G. lanuginosum* are perennial herbaceous plants. The leaves are polygonal in outline, cordate, palmatifid, with (3-) 5–7 segments. The inflorescence is dichasial, with dichotomous branching and a long pedunculate cymule at the primary branch (Yeo 1984).

The leaves in all species of subsect. *Tuberosa* are polygonal in outline, cordate, with 5–7(13) segments, They are usually palmatisect, The inflorescence is dichasial, with dichotomous branches and a long pedunculate cymule at the main fork (except in *G. kotschyi*), 2) sect. *Dissecta* characterized by the mericarp with the margin at the base drawn out into a prong lacking setae. A multivariate morphometric study showed that some quantitative characters such as deeply divided leaves, palmatifid, with 5–7 segments, shorter and narrower petals and shorter filaments clearly distinguished the annual *G. dissectum*, 3) sect. *Geranium* characterized by the mericarp with the margin at the base drawn out into a horny setiferous tubercle (Yeo 1984).

Morphological analyses of the studied *Geranium* species showed that they are well differentiated from each other both in quantitative measures (the ANOVA test result) and qualitative characters (The PCA plot result). In addition, PCA analysis suggests that characters like peduncle length, bract length, stipule length, bract shape, number of flowers per inflorescence, width of petal, peduncle and pedicel hair, leaf and petiole hair, stem hair, stipule and bract hair, habit and petal claw could be used in species groups delimitation. This morphological difference was due to quantitative and qualitative characters, for example, *G. platypetalum* has the longest bract length (13 mm), the longest stipule length (13–14 mm),the longest peduncle length (70–100 mm) and the broadest petal width (16 mm) among the studied species. Similarly, *G. dissectum* and *G. rotundifolium* had the narrowest petal length (4–4.5 mm) and the narrowest petal width (1.5–2.5 mm) among the studied species. Yeo (2002: 214) indicated that artificial hybrids between many species of subsect. *Mediterranea* (including *G. ibericum* and *G. platypetalum*) have been produced by A. Bremner. However, no names for these hybrids are available except *Geranium* × *magnificum*. We did not encounter any intermediate forms throughout the studied area.

Genetic structure and gene flow

AMOVA and STRUCTURE analysis revealed that the species of this subg. *Geranium* 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 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* L., *G. pusillum* L., *G. molle* L., *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.

To conclude, the present study revealed the use of ISSR molecular markers along with morphological characters in *Geranium* species identification. 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 adaptation are effective evolutionary forces operating in *Geranium* species and population divergence and adaptation.

Plant species identification is of central importance in phylogenetic systematics, evolution, biogeography and biodiversity. It is significant to infer patterns and mechanisms of speciation and hybridization, the evolutionary process by which new biological species arise and gene flow between closely related phylogenetic species can occur (Schluter 2001, Duminil & Di Michele 2009). 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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