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Understanding population differentiation using geographical, morphological and genetic characterization in *Erodium cicunium*

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Abstract

***Erodium cicunium* (Geranaiceae) species are distributed in different habitats of Iran. Some species are of medicinal importance while some are well known weeds and used as forage plants. An investigation was carried out to evaluate 124 randomly collected plants of *E. cicunium* from 15 geographical populations in 5 provinces to study population structure and for morphological and molecular characters. Start Codon Targeted (SCoT) markers were used to analyse molecular diversity. Analysis of molecular variance (AMOVA) revealed significant genetic difference among the studied populations and also revealed that 60% of total genetic variability was due to within population diversity while, 40% genetic differentiation was recorded among population. Principal Coordinates Analysis (PCoA) of populations based on morphological characters was not in agreement with Metric Multidimensional Scaling (MDS) plot of molecular data.**

Key words: *Erodium cicunium*, gene flow, genetic differentiation, SCoT

Introduction

Genetic diversity is a basic component of biodiversity and its conservation is essential for long term survival of any species in changing environments (Mills and Schwartz 2005; Tomasello et al. 2015). Change in environmental conditions often leads to variation in genetic diversity levels among different populations and populations with low variability are generally considered less adapted under adverse circumstances (Falk and Holsinger 1991; Olivieri et al. 2016). In the last decade, experimental and field investigations have demonstrated that habitat fragmentation and population decline reduce the effective population size, which is to the loss of allelic richness and increased population differentiation by genetic drift and inbreeding depression (Frankham 2005). Therefore, knowledge of the genetic variability and diversity within and among different populations is crucial for their conservation and management (Meloni et al. 2015; Peñas et al. 2016; Esfandani-Bozchaloyi et al. 2018a, 2018b, 2018c, 2018d).

Genus *Erodium*, belonging to family Geraniaceae comprises 15 species indifferent parts of Iran (Schonbeck – Temesy, 1970). *Erodium cicunium* is distinguished from other members of its genus by its lobed cotyledons, with sinuses almost reaching the midvein, deeply incised pinnate leaflets, always divided more than halfway to the midrib, mostly actinomorphic flower petals, and dense appressed hairs on the mericarp (Dahlgren1980). The tricolpate pollen grains have a striate-reticulate exine morphology (Verhoeven and Venter 1987; Perveen and Gaiser 1999; Shehata 2008). *Erodium cicunium* is best adapted to Mediterranean climates characteristic of its native habitats, but is found globally in temperate areas with hot summers, most commonly in semi-arid rangelands and prairies of North and South America, South Africa and Australia (Greuter et al. 1986; Hulte´n and Fries 1986). Although the species requires moisture from rainfall or irrigation for optimal germination, established plants are drought tolerant and can survive periodic arid conditions (Blackshaw and Harker 1998a; Busso et al. 1998). In arid and semi-arid regions, *E. cicunium* is also used as a forage plant on ranges in California and Arizona (Anonymous 1939; Busso et al. 1998;

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George et al. 2006) for grazing. It is a good source of protein for ruminants in semideserts and wastelands of the Middle East (Al-Masri 2007). In Turkey, the species is gathered as a food plant in the Aegean region (Bilgir 1982). It is a food plant for the larvae of the brown argus butterfly. The entire plant is edible with a flavor similar to sharp parsley if picked young (Camazine and Bye 1980).

With the progress in plant molecular biology, numerous molecular marker techniques have been developed and used widely in evaluating genetic diversity, population structure and phylogenetic relationships. Start codon targeted (SCoT) polymorphism is one of the novel, simple and reliable gene-targeted marker systems, which offers a simple DNA-based marker alternative and reproducible technique which is based on the short conserved region in the plant genes surrounding the ATG (Collard and Mackill 2009) translation start codon (Collard and Mackill 2009). It also involves a polymerase chain reaction (PCR) based DNA marker with many advantages such as low-cost, high polymorphism and extensive genetic information (Collard and Mackill 2009, Hulte´n and Fries 1986). In the present study, SCoT markers were employed to analyze genetic diversity, population structure and interrelationship in 124 *E. cicunium* accessions belonging to 15 different populations for the first time in the Iran.

Materials and methods

Plant materials and environmental variables

A total of 124 individuals (accessions) were sampled representing 15 natural populations of *E. cicunium* in East Azerbaijan, Lorestan, Kermanshah, Guilan and Ardabil Provinces of Iran during July-Agust 2018 (Supplementary Table S1 and Supplementary Fig. 1). These accessions (four to twelve samples from each populations) belonging to 15 different populations with different eco-geographic characteristics were subjected to morphometric and SCoT analysis and sampled and stored in -20°C till further use.

The data regarding climate variables included elevation, and geographic data (latitude and longitude), and this data was determined at each site using an electronic GPS. The climate variable data of mean annual temperature, mean maximum temperature (°C), mean minimum temperature (°C), annual rainfall (mm), number of frost days were downloaded from http://www.worldclim.org. Soil pH (1:2.5 v/v soil/water mixture; LY/T 1239-1999) for each population was measured using a digital pH meter (PHS-3C, Shanghai Leici Equipment Factory, China).

DNA extraction and SCoT Assay

Fresh leaves were used randomly from four to twelve plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Esfandani-Bozchaloyi et al. 2019). The quality of extracted DNA was examined by running on 0.8% agarose gel. A total of 25 SCoT primers developed by Collard and Mackill (2009), 10 primers with clear, enlarged, and rich polymorphism bands were chosen. PCR reactions were carried in a 25ìl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 ìM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The thermal program was carried out with an initial denaturation for 1 min at 94°C, followed by 40 cycles in three segments: 35 s at 95°C, 40s at 47°C and 55s at 72°C. Final extension was performed at 72°C for 5 min. 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

In total 60 morphological (24 qualitative, 36 quantitative) characters were studied. Four to twelve samples from each population were randomly studied for morphological analyses (Supplementary Table S2 and S3). 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) were used (Podani 2000). PAST version 2.17 (Hammer et al. 2012) was used for multivariate statistical analyses of morphological data.

Molecular studies

The SCoT bands obtained for each sample were scored as binary characters. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% = number of polymorphic loci/number of total loci) were determined (Weising et al. 2005; Freeland et al.

2011).

Shannon's index was calculated by the formula: H' = Σpiln pi. Rp is defined per primer as: Rp = Σ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software (Peakall and Smouse 2006)

Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland et al. 2011; Huson and Bryant 2006). Mantel test checked the correlation between geographical and genetic distances 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'sGst 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= standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jostmeasure of differentiation (Jost 2008).

To assess the population structure of the *Erodium cicunium* accessions, a heuristic method based on Bayesian clustering algorithms were utilized. The clustering method based on the Bayesian-model implemented in the software program STRUCTURE (Pritchard et al. 2000; Falush et al. 2007) was used on the same data set to better detect population substructures. This clustering method is based on an algorithm that assigns genotypes to homogeneous groups, given a number of clusters (K) and assuming Hardy-Weinberg and linkage equilibrium within clusters, the software estimates allele frequencies in each cluster and population memberships for every individual (Pritchard et al. 2000). The number of potential subpopulations varied from two to ten, and their contribution to the genotypes of the accessions was calculated based on 50,000 iteration burn-ins and 100,000 iteration sampling periods. The most probable number (K) of subpopulations was identified following 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 (Nm) which were calculated using POPGENE (version 1.31) program (Yeh et al. 1999). Gene flow was estimated indirectly using the formula: Nm = 0.25(1 - FST)/FST. In order to test for a correlation between pair-wise genetic distances (FST) and geographical distances (in km) between populations, a Mantel test was performed using Tools for Population Genetic Analysis (TFPGA; Miller 1997) (computing 999 permutations). This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwinver 5. (2012).

Results

Populations' genetic diversity

Genetic diversity parameters determined in 15 geographical populations of *E. cicunium* are presented in Table 1. The highest value of percentage polymorphism (66.31%) was observed in Lorestan: Alashtar (population No.2) which shows high value for gene diversity (0.189). and Shanon' information index (0.28). Population Kermanshah (Islamabad No. 10) has the lowest value for percentage of polymorphism (8.34%) and the lowest value for Shanon, information index (0.022), and He (0.040).

Population genetic differentiation

AMOVA (PhiPT = 0.79, P = 0.010), revealed significant difference among the studied populations (Table 2). It also revealed that, 60% of total genetic variability was due to within population diversity and 40% was due to among population genetic differentiation.

The pairwise comparisons of 'Nei genetic identity' among the studied populations *E. cicunium* (Supplementary Table S3) have shown a higher a genetic similarity (0.92) between populations Lorestan: Dorud (pop. No 3) and Lorestan: Choghlevandi (pop. No 4), while the lowest genetic similarity value (0.63) occured between Lorestan: Visian (pop. No.8) and Ardabil: Germi, 20 km from Germi to Pars-Abad populations (pop. No. 12).

Populations' genetic affinity

NJ tree and Neighbor-Net network produced similar results therefore only Neighbor-Net network is presented and discussed (Fig. 1). We have almost

Table 1. Genetic diversity parameters in the studied populations *E. cicunium* (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)

Pop	N	Na	Ne	I	He	UHe	%P
Pop1	10	1.033	1.377	0.225	0.172	0.179	52.35
Pop2	5	1.146	1.337	0.289	0.189	0.241	66.31
Pop3	6	0.747	1.192	0.142	0.103	0.111	27.06
Pop4	4	0.506	1.104	0.090	0.061	0.067	18.47
Pop5	8	0.694	1.131	0.126	0.081	0.087	27.06
Pop6	7	0.482	1.090	0.077	0.052	0.059	15.12
Pop7	5	0.459	1.115	0.089	0.062	0.068	13.29
Pop8	11	0.329	1.036	0.087	0.079	0.021	55.71
Pop9	7	0.388	1.081	0.068	0.046	0.056	20.76
Pop10	6	0.318	1.058	0.040	0.022	0.045	8.34
Pop11	6	0.835	1.206	0.179	0.119	0.132	35.12
Pop12	5	0.541	1.118	0.104	0.070	0.084	18.82
Pop13	12	0.718	1.162	0.147	0.097	0.106	29.41
Pop14	7	0.918	1.265	0.197	0.132	0.159	35.29
Pop15	11	0.376	1.134	0.122	0.073	0.085	28.18

Na = No. of Different Alleles; Ne = No. of Effective Alleles = 1 / (p^2 + q^2); I = Shannon's Information Index = -1* (p* Ln (p) + q* Ln(q)); He = Expected Heterozygosity = 2 * p * q; UHe = Unbiased Expected Heterozygosity = (2N / (2N-1)) * He; Where for Diploid Binary data and assuming Hardy-Weinberg Equilibrium, q = (1 - Band Freq.)^0.5 and p = 1 - q

Table 2. Analysis of molecular variance (AMOVA) of the studied species

Source	df	SS	MS	Est. var.	%	*ΦPT*
Among Pops	12	344.576	29.327	4.072	40%	40%
Within Pops	56	455.767	9.530	8.830	60%	
Total	68	891.342		12.713	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; ÖPT: proportion of the total genetic variance among individuals within an accession, (P < 0.001). Stat Value P(rand >= data) PhiPT 0.793, 0.010; Probability, P(rand>=data), for PhiPT is based on permutation across the full data set; PhiPT = AP / (WP + AP) = AP / TOT; Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

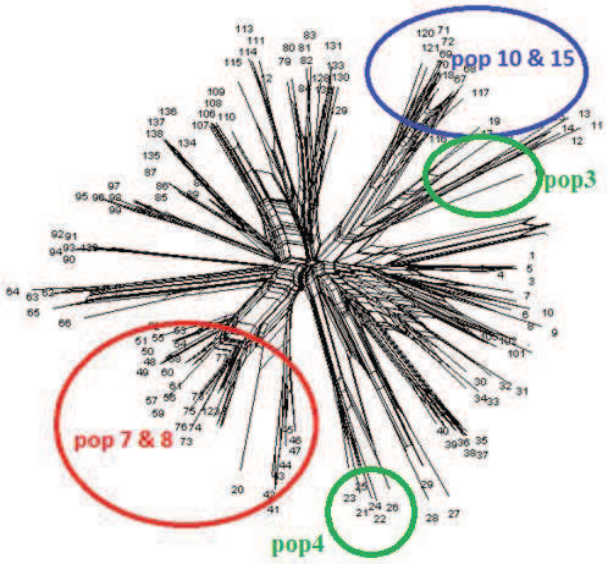

Fig. 1. Neighbor-Net network of populations in *E. cicunium* based on SCoT data

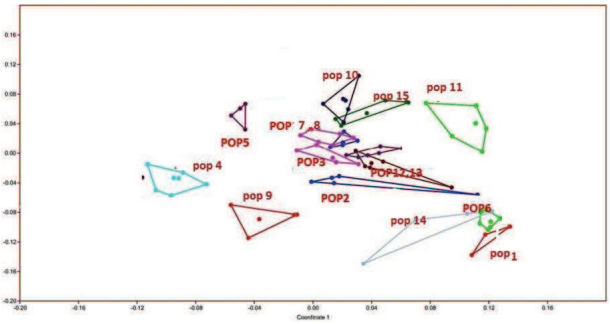

Fig. 2. MDS plot of populations in *E. cicunium* based on SCoT data

complete separation of the studied population in the network, supporting AMOVA result. The populations Lorestan: Dorud (pop. No 3) and Lorestan: Choghlevandi (pop. No 4) are distinct and stand separate from the other populations with great distance. The populations 7 and 8, as well as populations 10 and 15 show closer genetic affinity and are placed close to each other. In general, the description here about Fig. 1 is more or less consistent with Fig. 2, but it is totally in conflict with STRUCTURE. In STRUCTURE, POP7 is not close to POP8, and POP10 is actually closer to POP8 and 9 but not 15. Genetic divergence and separation of populations 1-5, as well as 9 and 11 from the other populations is evident in MDS plot of ISSR data after 1000 permutations (Fig. 3). The other populations showed close genetic affinity. Mantel test after 5000 permutations produced significant correlation between

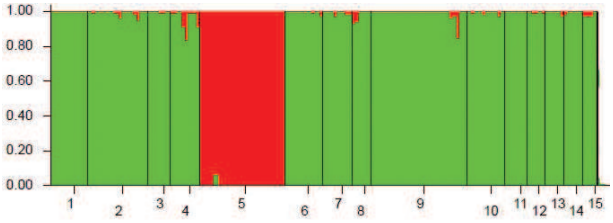

Fig. 3. STRUCTURE plot of *E. cicunium* populations based on k = 2 of SCoT data

genetic distance and geographical distance in these populations (r = 0.32, P = 0.001). Therefore, the populations that are geographically more distant have less amount of gene flow, and we have isolation by distance (IBD) in *E. cicunium*.

Populations genetic structure

K = 2 reveal the presence of 2 genetic group. Similar result was obtained by Evanno test performed on STRUCTURE analysis which produced a major peak at k = 2 (Fig. 5, Table 4). Both these analyses revealed that *E. cicunium* populations show genetic stratification.

STRUCTURE plot based on k = 2 (Fig. 4, Table 3), revealed genetic difference of populations Lorestan: Borujerd (pop. No 5) (differently colored) with other populations. But it showed genetic affinity between populations 1-4 (similarly colored), as well as

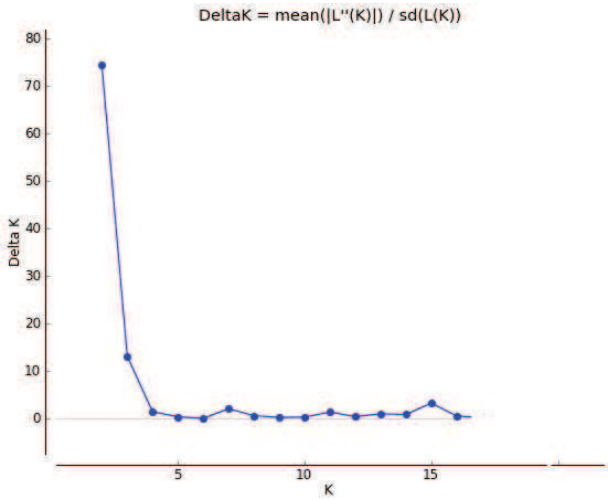

Fig. 4. Delta k plot of Evanno's test based on STRUCTURE analysis

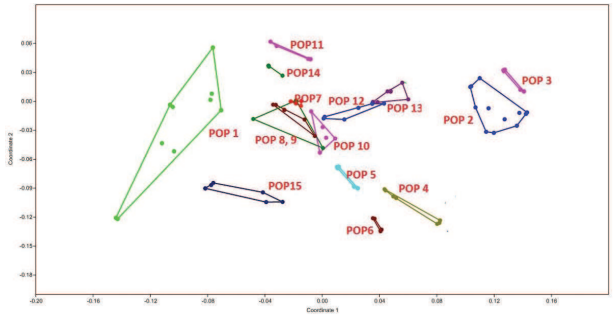

Fig. 5. PCOA plot of *E. cicunium* populations based on morphological characters

Table 3. K-Means clustering result. (* Best clustering according to Calinski and Harabasz' pseudo-F: k = 2. Best clustering according to Bayesian Information Criterion: k = 6)

k	SSD(T)	SSD(AC)	SSD(WC)	r-squared	pseudo-F	AIC	BIC	Rho
1	1119.354	0	0	0	0	216.38	580.088	0
2*	1119.354	114.9	1004	0.103	10.061	209.61	575.617	0.207
3	1119.354	210.5	908.9	0.188	9.147	203.56	571.822	0.263
4	1119.354	292.3	827.1	0.261	9.189	198.04	568.493	0.303
5	1119.354	367.5	751.9	0.328	9.409	192.49	565.084	0.363
6&	1119.354	438.8	680.5	0.392	9.801	186.65	552.895	0.4
7	1119.354	498.3	621	0.445	10.03	181.54	558.22	0.436
8	1119.354	545.8	573.5	0.488	9.467	177.47	556.102	0.472
9	1119.354	581	538.4	0.519	9.846	174.81	555.325	0.496
10	1119.354	612.7	506.7	0.547	9.674	172.43	554.75	0.519
11	1119.354	643.4	475.9	0.575	9.598	169.98	554.029	0.54
12	1119.354	671.7	447.7	0.6	9.548	167.7	553.413	0.559
13	1119.354	697.1	422.3	0.623	9.493	165.74	553.028	0.576
14	1119.354	719.8	399.5	0.643	9.425	164.12	558.895	0.592
15	1119.354	740.3	379	0.661	9.347	162.81	552.989	0.605

* Best clustering according to Calinski&Harabasz' pseudo-F: k = 2; & Best clustering according to Bayesian Information Criterion: k = 6

populations 6-15. The mean Nm = 0.28 was obtained for all SCoT loci, which indicates low amount of gene flow among the populations and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. However, reticulogram obtained based on the least square method, revealed some amount of shared alleles among populations 1 and 5, and between 13 and 6 and 7, also between 8, and 9. This result is in conflict with grouping we obtained with MDS plot, as these populations were placed close to each other. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in these populations and all these results are not in agreement in showing high degree of genetic stratification within *E. cicunium* populations.

Morphometric analyses

PCoA plot of *E. cicunium* populations based on morphological characters produced similar results (Fig. 5). The result showed morphological difference/ divergence among most of the studied populations. This morphological difference was due to quantitative characters only. For example, character (Peduncle length), separated population No. 2, character (Width of basal leaves) separated population No. 6, while character Calyx width, separated populations 14 and 15 from the other populations. A consensus tree was obtained for both SCoT and morphological trees, to reveal the populations that are diverged based on both morphological and molecular features. Interesting enough, it showed divergence of almost all populations at molecular level as well as morphological characteristics. Detailed comparison of the characteristics in these populations revealed that, for example, population No. 2, has the longest peduncle length (10-15 mm), the highest pedicle length (2.56 mm), and the largest ratio of length/width of petal (6-7mm), among the studied populations. Similarly, population No. 6 had, the longest stem-leaf length (45 mm) and the broadest basal-leaf width (57 mm). Population No. 14 had, the narrowest peduncle width (1-3 mm), and the highest ratio of pedicle length/width.

Discussion

Erodium species are found in different parts of Iran (Esfandani-Bozchaloyi et al. 2017a, 2017b). Most of these species are “Irano-Turanian” and Saharo-Sindian elements. The Irano-Turanian floristic region is a major center of endemism in the Holarctic of Eurasia. The Alborz Mountains of northern Iran are a complex and heterogeneous environmental system with rich water resources and great habitat diversity. We have investigated population structure along an altitudinal gradient ranging from approximately 230 m in the Guilan, Loleman to a height of 1474 m at the Kermanshah, Islamabad. The phytogeography of the region changes from omni-Irano-Turanian and Saharo-Sindian transgressive species at lower altitudes to a more limited range of western Irano-Turanian species and local endemics at higher altitudes.

The present study revealed interesting data about its genetic variability, genetic stratification and morphological divergence in north and west part of Iran. The studied populations have a low level of genetic diversity (He = 0.034-0.199). The Genetic diversity is of fundamental importance in the continuity of a species as it is used to bring about the necessary adaptation to the cope with changes in the environment (Warburg 1938; Guittonneau 1972). Degree of genetic variability within a species is highly correlated with its reproductive mode, the higher degree of open pollination/ cross breeding brings about higher level of genetic variability in the studied taxon (Knuth 1908). The flowers of *E. cicunium* subsp*. cicunium* are mostly homogamous or slightly protogynous, so that self-pollination is most likely to occur, but flowers with dark markings serving as guides to the concealed nectar may be protandrous and insect pollinated (Knuth 1908).

Similarly, the lower level of genetic variability occurred in *Geranium* species with limited geographical distribution and probably more selfing capabilities (Esfandani-Bozchaloyi et al. 2017a, 2017b. *G. stepporum* Davisand *G. tuberosum* L. had the lowest level of genetic polymorphism (2.15%). Each of these species have a confined geographical distribution in the country and occur only in one province and low genetic variability may also occur due to small size of the populations 10<, in *G. mascatense* had the lowest level of genetic polymorphism (2.15%) not only have small size of the populations 10<, but also is inbreeding due to annual with small flowers (Warburg, 1938).

Low genetic variability may also occur due to small size of the populations and genetic drift (Dahlgren 1980). These species tend to perform inbreeding as also evidenced by very low Nm value and IBD obtained for the studied species. However, limited gene flow was not solely due to geographical distance among the species, but some of the species, which grew in adjacent areas with overlapping zones, did not form

any hybrids or intermediate forms as evidenced by morphological and ISSR clusters obtained (Webb and Chater 1968). *Erodium cicunium* exhibits great extent of morphological variability, and forms many geographical populations in Iran. These geographical populations have variable eco-geographical features, some of which are in close vicinity, while some others are distributed in distant regions. Considerable morphological and genetic variability has been found within the *E. cicunium* (Webb and Chater 1968; Dahlgren 1980). According to Martin et al. (1997) showed genetic diversity within and among populations of athreatened species: *Erodium paularense* Fern. Gonz. & Izco. They report the use of RAPD markers to gain information about the genetic variability among and within populations of *E. paularense.*

According to Alarcón et al. (2012) AFLP variation suggests that this might have led to their differentiation into groups and speciation during inter glacials, but it probably also provided the basis for recurrent recolonisations and the mixing of neighbouring populations at the last glacial maxima. Their results showed that genetic diversity of the two *Erodium* lineagessuggests two migration episodes took place from southern Iberia towards the north, with one lineage migrating via western Iberia and the other via eastern Iberia. The patterns of genetic diversity observed in populations of 56 European species (27 genera) leads to the hypothesis that disparate proportions of unique polymorphic fragments are the result of the evolutionary histories of their mountain populations irrespective of the currently recognised species. Geography appears to play an important role in isolation by distance, particularly for Mediterranean plants. Reductions in gene flow may lead to the appearance of new species or subspecies, with isolation in glacial refugia as a major promoter of such diversification (Esfandani-Bozchaloyi et al. 2018a, 2018b, 2018d). *E. cicunium*is of wide spread in our country and it has several medicinal applications (Wiesnerova and Wiesner, 2004), however we had no information on its genetic structure and detailed taxonomic information. Our results revealedinteresting data about its genetic variability, genetic stratification and morphological divergence in north and west part of Iran.

Authors' contribution

Conceptualization of research (AAM, YS); Designing of the experiments (XZ, MK, AAM); Contribution of experimental materials (AAM, YS); Execution of field/ lab experiments and data collection (XS, LG, YS); Analysis of data and interpretation (XS, AAM, YS); Preparation of manuscript (AAM, XS, YS).

Declaration

The authors declare no conflict of interest.

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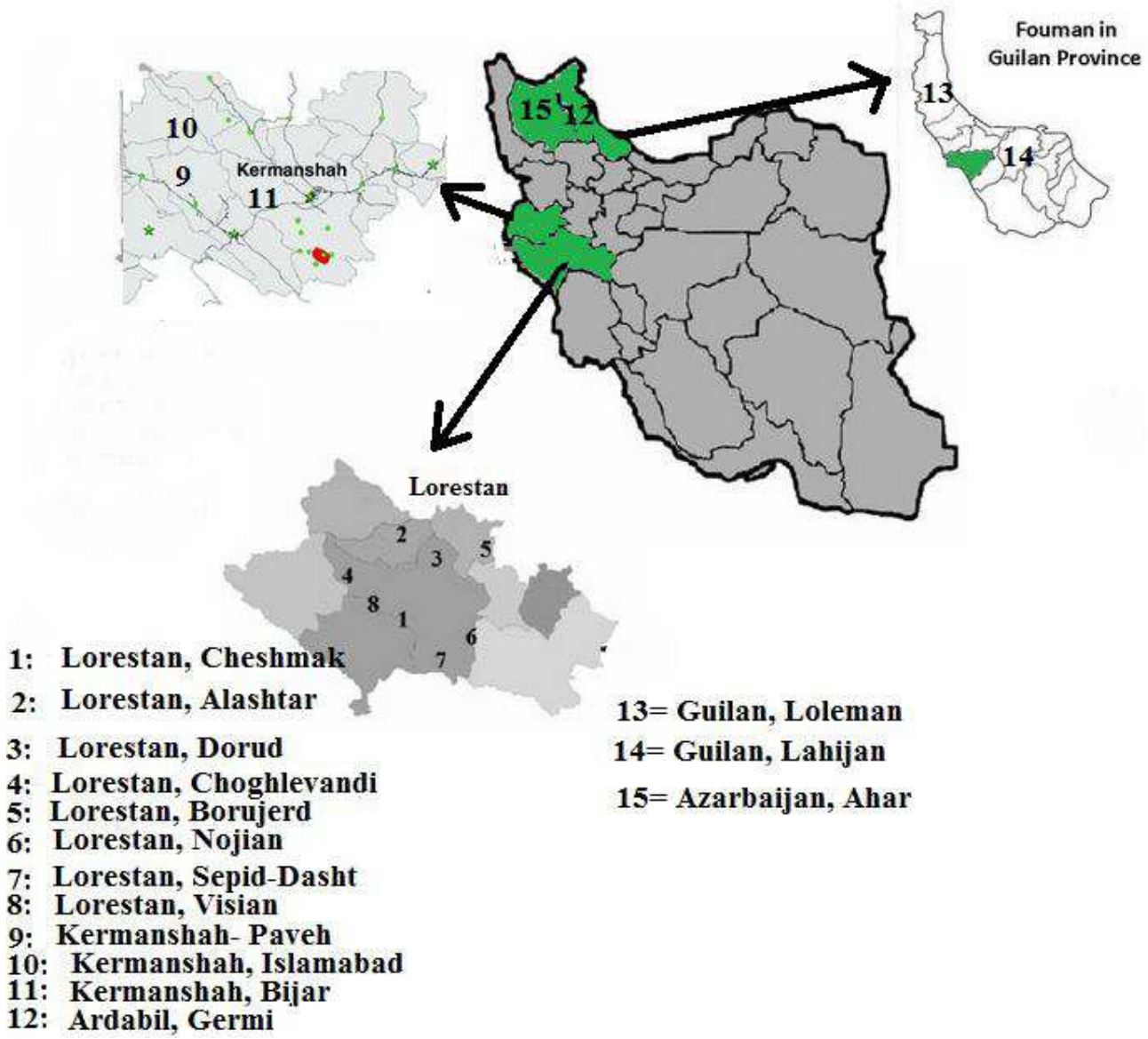
Supplementary Table S1. Populations studied, their locality and ecological features

Pop.no	Locality	No. of collected access-ions	Mean max. temp. (°C)	Mean min. temp. (°C)	Annual temp.	pH	Annual rainfall (mm)	No. of frost days	Elevation (m)	Coordinates	Voucher no.
1	Lorestan: Cheshmak	10	40.12	-18.12	13.4	7.8	325	77	1300	48° 51.778' E; 33° 13.175' N	IAUH-14977
2	Lorestan: Alashtar	5	38.45	-17,66	14.6	7.7	355	86	1280	48° 50.649' E; 33° 13.292' N	IAUH-14978
3	Lorestan: Dorud	6	35.55	-20.34	34	7.4	378	75	1100	48° 10.286' E; 33° 27.407' N	IAUH-14923
4	Lorestan: Choghlevandi	4	41.34	-10.34	15.6	7.7	377	96	1370	48° 15.886' E; 33° 98.327' N	IAUH-14945
5	Lorestan: Borujerd	8	39.14	-17.55	18	7.6	390	73	110	47° 49.748' E; 33° 18.168' N	IAUH-14558
6	Lorestan: Nojian	7	30.34	-19.35	22	7.7	310	54	670	47° 30.663' E; 33° 4.840' N	IAUH-15019
7	Lorestan: Sepid-Dasht	5	36.88	-11.23	16	7.5	320	76	940	47° 57.328' E; 33° 57.121' N	IAUH-14959
8	Lorestan: Visian	11	32.55	-22.45	18	7.8	334	88	800	47° 42.448' E; 33° 6.480' N	IAUH-15004
9	Kermanshah: Paveh	7	30.44	-18.66	15	8	229	120	360	45° 34.376' E; 34° 29.661' N	IAUH-14953
10	Kermanshah: Islamabad	6	32.88	-11.66	12	8.4	210	114	1474	46° 20.252' E; 35° 3.777' N	IAUH-14955
11	Kermanshah: Bijar	6	35.99	-8.44	10	8.3	250	167	1400	46° 20.396' E; 35° 1.812' N	IAUH-14984
12	Ardabil: Germi, 20 km from Germi to Pars-Abad	5	20.44	-25.66	10	8	478	220	380	48° 5.222' E; 39° 10.859' N	IAUH-15029
13	Guilan: Loleman	12	38.77	-5.66	20	7.5	550	30	230	49° 33.188' E; 36° 51.654' N	IAUH-14976
14	Guilan: Lahijan	7	35.87	-2.66	24	7.6	579	31	250	49° 8.158' E; 37° 10.483' N	IAUH-15046
15	Azarbaijan (E): Ahar, 45 Km from Meshkin-Shahr to Ahar	11	15.77	-26.88	5	7.4	467	170	1250	47° 17.038' E; 38° 23.792' N	IAUH-15038



Supplementary Fig. 1. Distribution map of the studied populations

Supplementary Table S2. Evaluated morphological characters

No.	Characters	No	Characters
1	Plant height (mm)	19	Mericarp length (mm)
2	Length of stem leaves petiole (mm)	20	Mericarp width (mm)
3	Length of stem leaves (mm)	21	Mericarp length/width (mm)
4	Width of stem leaves (mm)	22	Seed length (mm)
5	Length / Width of stem leaves (mm)	23	Seed width (mm)
6	Number of segment stem leaves (mm)	24	Seed length/ width (mm)
7	Length of basal leaves petiole (mm)	25	Stipules length (mm)
8	Length of basal leaves (mm)	26	Stipules width (mm)
9	Width of basal leaves (mm)	27	Stipules length/ width (mm)
10	Length / Width of basal leaves (mm)	28	Bract length (mm)
11	Number of segment basal leaves	29	Bract width (mm)
12	Calyx length (mm)	30	Bract length / width (mm)
13	Calyx width (mm)	31	Pedicel length (mm)
14	Calyx length/ width (mm)	32	Peduncle length (mm)
15	Petal length (mm)	33	Rostrum length (mm)
16	Petal width (mm)	34	Style length (mm)
17	Petal length / width (mm)	35	Stamen filament length (mm)
18	Fruit length (mm)	36	Number of flowers per inflorescence
37	Type root: tuberculate (1), not tuberculate (2)	49	Bract outline:linear-lanceolate (1), lanceolate (2),ovate-lanceolate (3)
38	Vegetation-forms::annual (1), annual or biennial (3), perennial rootstock (4)	50	Stipules outline:lanceolate (1),elliptic-obtuse or oblong (2)
39	State of stem strength : erect or decumbent (1), erect-ascending (2)	51	Seed surface ornamentation: 1-micro-reticulate; 2-reticulate; 3- bi- reticulate
40	State of stem branches:bifurcating at middle (1), bifurcating from the collar (2), bifurcating upper than middle of stem (3)	52	Shape of calyx: oval-lanceolate (1), angled (2)
41	Leaf outline: polygonal, cordate (1), sub orbicular to reniform (2),	53	Sepals Apical arista: presence (1), absence (2)
42	Phyllotaxy: alternate (1), opposite (2)	54	Petal shape : obovate (1), spathulate (2)
43	Leaf tips: presence (1), absence (2)	55	State of petal ligule: presence (1), absence (2)
44	Mericarp hair:1-absence2-non-glandular short and medium hairs3-glandular short hairs	56	Petal apex :emarginate or obtuse (1), obtuse or mucronate (2),
45	Seed outline: circular; 2- narrow- elliptical; 3- elliptical	57	State of petal ligulehair: ciliated at base (1), not ciliated at base (2)
46	Seed color: brown; 2-reddish- brown; 3- blackish-brown	58	Stamen filament hair: ciliate (1), not ciliate (2)
47	Stem hair:1-absence2-non-glandular short and medium hairs3-glandular short hairs	59	All organ plant hair density: 1-sparsly hairy2-Glabrous
48	Petioles and Leaf hair: 1-absence2-non-glandular short and medium hairs3-glandular short hairs	60	Mericarp color: 1-yellowish-green; 2- brown;

Supplementary Table S3. Pairwise Population Matrix of Nei Unbiased Genetic Identity

	pop1	pop2	pop3	pop4	pop5	pop6	pop7	pop8	pop9	pop10	pop11	pop12	pop13	pop14	pop15
1.000															pop1
0.736	1.000														pop2
0.694	0.807	1.000													pop3
0.691	0.776	0.921	1.000												pop4
0.728	0.817	0.798	0.752	1.000											pop5
0.727	0.776	0.746	0.715	0.902	1.000										pop6
0.719	0.802	0.785	0.736	0.781	0.772	1.000									pop7
0.760	0.708	0.720	0.694	0.739	0.755	0.831	1.000								pop8
0.736	0.792	0.788	0.691	0.822	0.750	0.826	0.797	1.000							pop9
0.760	0.820	0.787	0.728	0.769	0.727	0.772	0.732	0.873	1.000						pop10
0.773	0.821	0.804	0.727	0.824	0.787	0.824	0.791	0.854	0.860	1.000					pop11
0.790	0.826	0.806	0.719	0.806	0.747	0.757	0.632	0.797	0.810	0.879	1.000				pop12
0.768	0.863	0.800	0.760	0.836	0.778	0.837	0.772	0.804	0.787	0.806	0.811	1.000			pop13
0.765	0.769	0.812	0.736	0.783	0.771	0.731	0.716	0.781	0.730	0.762	0.743	0.804	1.000		pop14
0.855	0.849	0.823	0.744	0.781	0.729	0.756	0.725	0.768	0.830	0.820	0.785	0.854	0.884	1.000	pop15