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Original scientific article

RAPD PROFILING IN DETECTING GENETIC VARIATION IN *Stellaria* L. (Caryophyllaceae)

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Stellaria species are common herbs, preferred humid mountainously slopes, but some grew in desert. Main center of diversification for *Stellaria* is Eurasia, with a center of distribution in the mountains of central Asia. Some species are also cosmopolitan. It is represented by 9 species in Iran. The genus has high medicinal value. To determine the genetic diversity and understand the species' limits within the Iranian *Stellaria*, we produced molecular data using 139 randomly collected plants representing 8 species from five provinces of Iran. A total of 122 reproducible bands were generated by 10 of 25 random amplified polymorphic DNA (RAPD) primers, with an average of 12.2 bands/primer and 33% polymorphism. Largest number of effective alleles (Ne), genetic diversity (H), and Shannon Index (I) were shown by *S. media.* Our data depicted highest similarity between *S. media* and *S. pallida* and lowest between *S. media* and *S. graminea*. *S. pallida* showed relatively low level of genetic variation. Finally, the Neighbor Joining (NJ) trees based on RAPD markers data divided the populations into two different clusters, indicating their genetic difference which is discussed in details.

Keywords: Endemism, Gene flow, Random Amplified Polymorphic DNA (RAPD).

INTRODUCTION

One of the most important aspect of biological diversity for conservation strategies is the genetic diversity, particularly in rare, and narrow endemic species (MILLS and SCHWARTZ, 2005;

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TOMASELLO *et al*. 2015). Most authors agree that longstanding evolutionary potential of a species necessitates maintenance of genetic diversity (FALK and HOLSINGER, 1991; ESFANDANI-BOZCHALOYI *et al*., 2017a; 2017b; 2017c; 2017d). Similarly, most geneticists regard population size as a significant factor in preserving genetic diversity (ELLEGREN and GALTIER, 2016; TURCHETTO *et al.* 2016).

Stellaria L. (Caryophyllaceae, Alsinoideae) comprises *ca*. 150–200 species across the world (BITTRICH, 1993). This genus has nine species grouped in two sections. *S. blatteri* Mattf., *S. scaturiginella* Rech.f. and *S. sarcophylla* Rech.f. have an uncertain section (RECHINGER, 1988). According to *Flora Iranica*, *Stellaria* sections in Iran include: *Stellaria* with two annual species [*S. media* (L.) Vill. and *S. pallida* (Dumort.) Pire] and four perennial species which grow in the mountain areas, including *S. holostea* L., *S. persica* Boiss., *S. graminea* L., and *S. nemorum* L. Section *Pseudalsine* Boiss. consists of only one annual species (*S. alsinoides* Boiss. & Buhse) growing in the mountains of Iran. *Stellaria* species are common herbs, preferred humid mountainously slopes, but some grew in desert. Main center of diversification for *Stellaria* is Eurasia, with a center of distribution in the mountains of E. central Asia. Some species are also cosmopolitan (BITTRICH, 1993). There are limited chromosome records for *Stellaria* in the world. Basic Chromosome numbers of x=10, 11, 12 and 13 have been reported for the genus. The genus is characterized by the presence of five sepals and petals which are usually bifid; however, in some species the petals are markedly reduced or absent (FIOR *et al.*, 2006; HARBAUGH *et al*., 2010).

Previous study on species delimitation and species relationship performed in this genus. VERKLEIJ *et al.,* (1980) obtain some more information about the genetic differences among and between the two species and within S. *media* between the two local sub-populations, by means of the electrophoretically detectable variation in isoenzymes.

Literature revealed that studies are mainly dealing with taxonomy, seed and pollen morphology, stem and leaf anatomy (MAHDAVI *et al.,* 2012; KESHAVARZI and ESFANDANI-BOZCHALOYI 2014a, 2014b; ESFANDANI-BOZCHALOYI and KESHAVARZI, 2014) of *Stellaria* species but there are no attempt to study genetic diversity, ecological adaptation and intra- and inter-specific differentiation along with morphometric studies on *Stellaria* of Iran. Therefore, we performed molecular study of 139 collected specimens of 2 sections in *Stellaria*.

Molecular markers are commonly used in genetic analysis, fingerprinting, linkage mapping, germplasm characterization, and molecular breeding. RAPD analysis using PCR along with short arbitrary sequence primers has been reported sensitive to detecting variation at level of individuals. The benefits of this method are: a) a large number of samples are tested easily and efficiently using less quantity of material; b) the DNA amplicons are independent of ontogenetic expression; c) several genomic regions may be sampled with likely infinite numbers of markers (SONIYA *et al.* 2001; ESFANDANI-BOZCHALOYI *et al.,* 2018 a; 2018b; 2018c; 2018d).

This study has been carried out to evaluate the genetic diversity and relationships among the Iranian *Stellaria* species based on RAPD data. This is the first step towards using RAPD markers on a broader sampling of Iranian *Stellaria* and aims at answering the following questions: 1) is there infra- and interspecific genetic diversity among *Stellaria* species? 2) Is genetic distance correlated with distribution of these species? 3) What is the populations' genetic structure? 4) Is there any genetic exchange within *Stellaria* species?

MATERIALS AND METHODS

Plant materials

A total of 139 individuals were collected from 15 geographical populations belong 6 *Stellaria* species of *Pseudalsine* (*S. alsinoides*), *Stellaria* (*S. media*, *S. pallida*, *S. holostea*, *S. persica* and *S. graminea*) sections in East Azerbaijan, Guilan, Mazandaran, Golestan and Tehran Provinces of Iran during July–August 2017–2019 (Table 1). All of these samples were used during RAPD analysis and stored for further use in -20°C.

Table 1. Voucher details of Stellaria species and relative genera examined in this study from Iran.

Sp.	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
1. *Stellaria media* (L.) VILL.	East Azerbaijan, Kaleybar, Shojabad	38 ˚ 52393"	47 ˚ 25 92"	1133	HIAU 201677
	East Azerbaijan Kaleybar, Cheshme ali akbar	38 ˚ 52353"	47 ˚ 27 92"	1143	HIAU 201678
2. *S. pallida* (Dumort.) pire	East Azerbaijan, Kaleybar Cheshme ali akbar	38 ˚ 52'353	47 ˚ 27' 92"	1143	HIAU 201680
	East Azerbaijan, Kaleybar, road side	38 ˚ 52'373	47 ˚ 23' 92"	1144	HIAU 201683
3. *S. holostea* L.	East Azerbaijan, Kaleybar cheshme ali akbar	38 ˚ 52'353"	47 ˚ 27' 92"	1143	HIAU 201684
	East Azerbaijan, Kaleybar, Shojabad	38 ˚ 52'393"	47 ˚ 25' 92"	1137	HIAU 201685
	East Azerbaijan Kaleybar,Cheshme Ali akbar	38 ˚ 52'353	47 ˚ 27' 92"	1143	HIAU 201686
4. *S. persica* Boiss.	Guilan,Gole rodbar	37 ˚ 09 55"	49 ˚ 55 49 "	27	HIAU 201687
	Guilan,Gole rodbar, Road sid	37 ˚ 09 45"	49 ˚ 55 39 "	15	HIAU 201688
5. *S. graminea* L.	Guilan,Gole rodbar	37 ˚ 09 55"	49 ˚ 55 49 "	32	HIAU 201689
	Guilan, Sangar, Road sid	370702.32	494432.6	48	HIAU 201690
6. *S. alsinoides* Boiss & Buhse	Guilan, Lahijan	371204.81	500311.98	9	HIAU 201691
	Guilan, Jirandeh	364158.62	494730.34	1335	HIAU 201692
	Mazandaran: Haraz road, Emam Zad-e-Hashem	361414.32	511807.09	1807	HIAU 201693
	Golestan, Ramian	37 080.23	55 8507.03	1320	HIAU 201694
7. *Mesostemma kotschyanum* (Fenzl in Boiss) Vved. Subsp. *kotschyanum*	East Azerbaijan kaleybar	38 ˚ 52'373	47 ˚ 23' 92"	1144	HIAU 201695
	Tehran, Darband	355003.36	512428.62	1700	HIAU 201696
8. *Myosoton aquaticum* (L.) Moench	East Azerbaijan Kaleybar Cheshme ali akbar	38 ˚ 52'373	47 ˚ 23' 92"	1144	HIAU 201697

DNA extraction and RAPD Assay

In each of the populations studied, fresh leaves from one to twelve plants were used randomly. Leaves were dried with silica gel prior to DNA extraction. In order to obtain genomic DNA, the CTAB-activated charcoal protocol was used (ESFANDANI-BOZCHALOYI *et al.*, 2019). By running on 0.8 percent agarose gel, the quality of extracted DNA was examined. A total of 25 Operon Technology Decamer RAPD Primers (Alameda, Canada) belonging to OPA, OPB, OPC, OPD sets were used. Among them, ten primers were selected with simple, enlarged and rich bands of polymorphism PCR reactions were performed in a 25µl volume mixture containing the following component: Tris-HCl buffer (10 mM) at pH 8; KCl (50 mM); $MgCl_2$ (1.5 mM); dNTPs (0.2 mM); primer (0.2 µM); genomic DNA (20 ng) and of *Taq* DNA polymerase (3 U). In Techne thermocycler (Germany), the amplification reactions were carried out with the following PCR settings: 5 min initial denaturation at 94°C; 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 7–10 min extension at 72°C. The PCR amplified products were detected by running on 1% agarose gel, preceded by staining with ethidium bromide. The size of fragments was measured using a ladder with a molecular size of 100 bp (Fermentas, Germany).

Data analyses

Molecular analyses

RAPD bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. A parameter like Nei's gene diversity (H), Shannon information index (I), the number of effective alleles, and percentage of polymorphism were determined (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 and BRYANT, 2006). Mantel test checked the correlation between geographical and genetic distance of the studied populations (PODANI, 2000). These analyses were done by PAST ver. 2.17 (HAMMER *et al.*, 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) software. AMOVA (Analysis of molecular variance) test (with 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 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 = 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 STRUCTURE analysis, data were scored as dominant markers (FALUSH *et al.*, 2007). We used the admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K after a burn-in period of 10^5. The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (EVANNO *et al.*, 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC) provide the best fit for k (MEIRMANS, 2012).

Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers the equal amount

of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

RESULTS

Species Identification and Genetic Diversity

All RAPD primers produced polymorphic bands. Genetic diversity parameters determined in the studied species (Table 2) revealed that *S. media* had the highest level of genetic polymorphism (67.50%), while the lowest level of genetic polymorphism (33.50%) occurred in *S. persica*. *S. holostea* also had the highest values for effective number of alleles (Ne = 1.18) and Shannon information index (I =0.37).

Table 2. Genetic diversity parameters in the studied Stellaria species.

Pop	N	Na	Ne	I	He	UHe	%P
Stellaria media	10.000	0.431	1.088	0.35	0.38	0.33	67.50%
S. pallida	9.000	0.261	1.014	0.24	0.23	0.23	47.15%
S. holostea	6.000	0.555	1.021	0.29	0.25	0.28	41.53%
S. persica	10.000	0.431	1.088	0.19	0.11	0.13	33.50%
S. graminea	3.000	0.255	1.021	0.25	0.18	0.22	43.15%
S. alsinoides	3.000	0.288	1.024	0.29	0.15	0.27	64.30%
Mesostemma kotschyanum	9.000	0.352	1.083	0.23	0.22	0.24	45.05%
Myosoton aquaticum	12.000	1.244	1.322	0.28	0.28	0.192	50.91%

(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).

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 75% of total variation was among species and 25% was within species (Table 3). Pairwise AMOVA produced significant difference among the studied populations. Moreover, we got high values for Hedrick standardized fixation index after 999 permutation (G'st = 0.745, P = 0.001) and Jost, differentiation index (D-est = 0.956, P = 0.001). These results indicate that the geographical populations of *Stellaria* are genetically differentiated from each other.

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	28	1801.384	75.789	13.154	75%	75%
Within Pops	129	374.449	3.905	2.888	25%	
Total	144	1855.807		15.060	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; *Φ*PT: proportion of the total genetic variance among individuals within an accession, (P < 0.001).

Species identification and inter-relationship

Different clustering and ordination methods produced similar results therefore, UPGMA clustering and PCA and PCoA plot are presented here (Fig. 1-3). In general, plant samples of each species belong to a distinct section, were grouped together and formed separate cluster. These results show that RAPD primers can differentiate the *Stellaria* species. In the studied specimens we did not encounter intermediate forms.

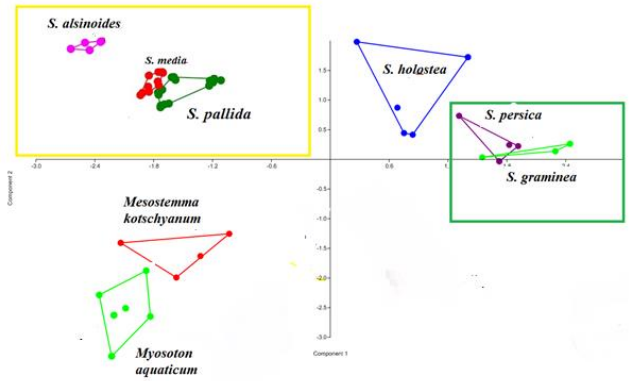

Fig. 1. PCA plots of RAPD data revealing species delimitation in the *Stellaria*

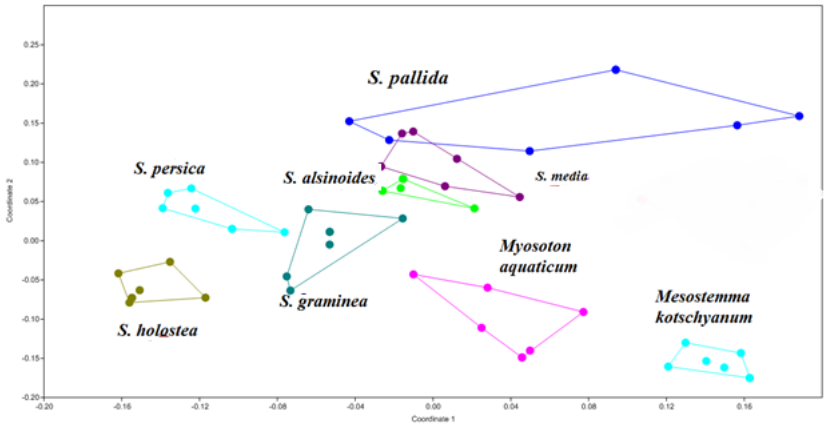

Figure 2. PCOA plots of RAPD data revealing species delimitation in the *Stellaria*

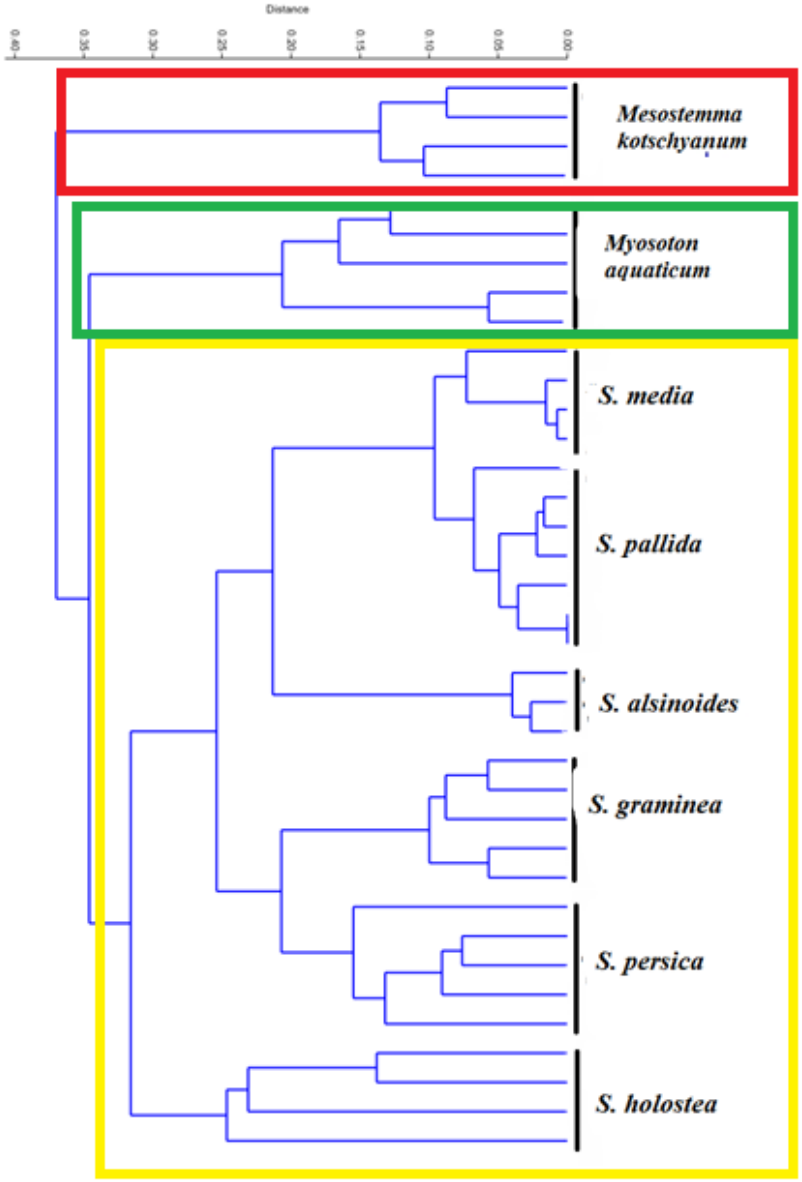

Figure 3. UPGMA tree of RAPD data in the studied *Stellaria* species.

In general, two major clusters were formed in UPGMA tree (Fig. 3). In the first cluster, the *Mesostemma* taxa and *Myosoton aquaticum* are separated from the other studied species and join the others with a great distance. The second major cluster included two sub-clusters. Plants of *S. media* and *S. pallida* from the *Stellaria* section and *S. alsinoides* (*Pseudalsine* section) comprised the first sub-cluster due to morphological similarity, while plants of *S. persica, S. graminea* and *S. holostea* (*Stellaria* section) were located in the second sub-cluster. The PCA plot (Fig. 1) separated the species into distinct groups with no inter-mixing.

In general, relationships obtained from RAPD data agrees well with species relationship obtained from PCA plot. 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= 0.654, that is considered very low value of gene flow among the studied species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.43, p=0.0001) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Stellaria* species studied. Nei's genetic identity and the genetic distance determined among the studied species (Table 4). The results showed that the highest degree of genetic similarity (0.95) occurred between *S. media* and *S. pallida*. The lowest degree of genetic similarity occurred between *S. media* and *S. graminea* (0.70).

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Pop ID	1	2	3	4	5	6	7	8
1	****	0.9568	0.8583	0.8316	0.7094	0.7198	0.7520	0.7546
2	0.1781	****	0.9105	0.8758	0.8892	0.7961	0.8139	0.8098
3	0.1528	0.0937	****	0.9195	0.9356	0.8539	0.8709	0.8522
4	0.1843	0.1327	0.0839	****	0.9076	0.8116	0.8173	0.8293
5	0.1632	0.1175	0.0666	0.0434	****	0.8044	0.8411	0.8258
6	0.3288	0.2280	0.1579	0.2087	0.2176	****	0.8993	0.8553
7	0.2851	0.2059	0.1383	0.2018	0.1731	0.1061	****	0.8703
8	0.2816	0.2110	0.1599	0.1872	0.1915	0.1563	0.1389	****

Populations, genetic affinity

NJ tree and Neighbor-Net network produced similar results therefore only Neighbor-Net network is presented and discussed (Fig. 4). We have almost complete separation of the studied population in the network, supporting AMOVA result. The *S. media* and *S. pallida* are distinct and stand separate from the other populations with great distance. The *S. persica, S. graminea* and *S. holostea*, as well as *S. alsinoides* (*Pseudalsine* section) closer genetic affinity and are laced close to each other.

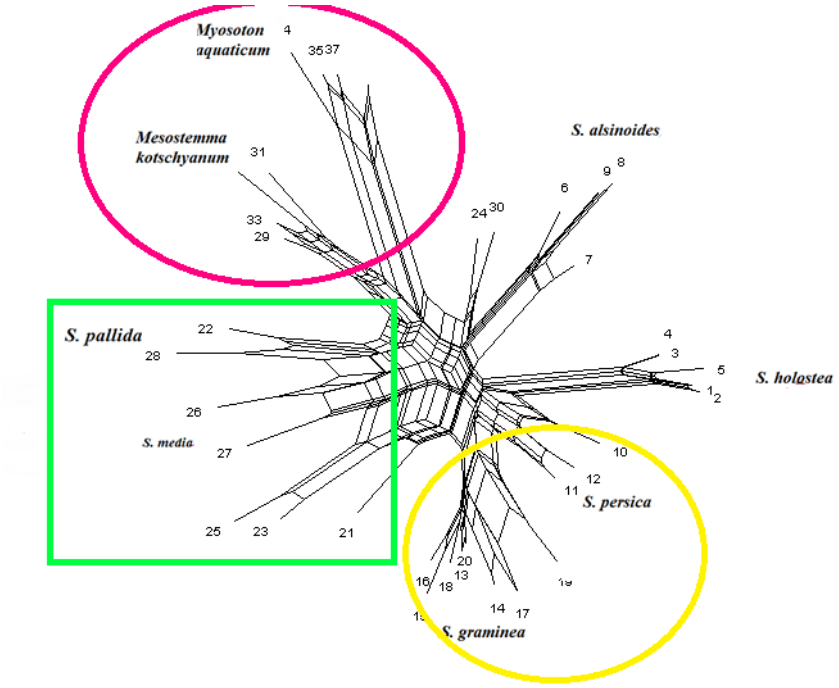

Fig 4. NeighberNet diagram of the studied *Stellaria* species based on Scot data

DISCUSSION

Genetic diversity is one aspect of biological diversity that is extremely important for conservation strategies (KALJUND and JAASKA, 2010; GORDON *et al.*, 2012). Population size is considered an important factor for maintaining genetic variation. Small populations are more vulnerable than large ones to extinction because of environmental stochasticity, genetic drift and inbreeding. Genetic drift decreases heterozygosity and eventual fixation of alleles, and inbreeding increases homozygosity within populations (FRANKHAM, 2005). In general, a drop in population size may cause the decline of genetic diversity by genetic drift and inbreeding. In the longer term, diminished genetic diversity may cause a loss of fitness and evolutionary capacity to adapt to environmental changes (LANDE, 1993; KALJUND and JAASKA, 2010). Therefore, quantifying patterns of genetic variability and diversity within and among different populations is very important for small population species conservation and management planning.

In the present study, genetic diversity within *Stellaria* was detected using RAPD markers. Our study showed that *S. persica* showed a lower level of genetic diversity (P: 33.50 %, He: 0.11, I: 0.19). In general, biological traits, reproductive mode and breeding system have often been regarded as important factors that affect genetic diversity levels. Outcrossing species usually have considerably higher levels of genetic diversity than selfing species (HAMRICK and

GODT, 1989; NYBOM, 2004). Previous studies suggested that the mating system of *Stellaria* may be predominantly selfing (PETERSON, 1936).

Stellaria media and *Stellaria pallida* are mainly self-fertile and between these species there exists a crossing barrier (PETERSON, 1936), perhaps mainly due to the diploidy of *S. pallida* (2n = 22) and the hypotetraploidy of *S. media* (2n=40-44) (SCHOLTE, 1978). According to CHINNAPPA and MORTON (1984) the genetic variation and phenotypic plasticity contributing to the population differentiation within the *S. longipes* complex was investigated using isozyme, RFLP, and RAPD analyses, and comparative morphological studies. Two aspects are of particular importance in the success of this species: (1) genetic variability due to polyploidy, facultative outbreeding, and interspecific gene flow; and (2) development of phenotypic plasticity due to environmentally induced changes in the physiology and morphological expression of the genotypes. All genotypes were self–compatible, but protandrous, gynodioecy, and partial gynodioecy are common in the species (PHILIPP, 1975; CHINNAPPA, 1985). CHINNAPPA and MORTON (1984) found no correlation with chromosome number and morphology or reproductive biology, which agreed with PHILIPP (1972) and CHINNAPPA and MORTON (1974, 1976). Based on earlier studies (CHINNAPPA and MORTON, 1974, 1976, 1984; MACDONALD *et al.*, 1987), CHINNAPPA and MORTON (1991) proposed the *Stellaria* taxa in question be grouped into a *Stellaria longipes* complex with two subspecies: *Stellaria longipes* Goldie subsp. *longipes* and *Stellaria longipes* Goldie subsp. *arenicola* (Raup). The evolution of the *arenicola* subspecies was hypothesized to have originated by colonization of a sand dune habitat with a possible shift in breeding system to self-pollination. The subspecies *arenicola* is interfertile with other populations of *S. longipes* and intergrades with them in its natural habitat, but field studies indicated that *arenicola* is primarily a self-pollinator (MACDONALD *et al.*, 1987). *Stellaria longipes* is, otherwise, a single polymorphic species without well-differentiated infraspecific taxa (CHINNAPPA and MORTON, 1991). The present population divergence may be under influence of isolation-by distance across the distribution range of the studied *Stellaria* populations. The dispersal of these populations might be constrained by distance and gene flow is most likely to occur between neighboring populations. As a result, more closely situated populations tend to be more genetically similar to one another (MACDONALD *et al.*, 1987).

The populations, divergence may be accompanied by local adaptation. When we use multilocus molecular markers (such as SSR, AFLP, RAPD, ISSR, etc.) for population genetic studies we understand that these are neutral molecular markers (they are not directly acting as adaptive genes), but they may be linked to a gene or a genetic region with adaptive value (FREELAND *et al*., 2011). The LFMM analysis in present study revealed that some of the genetic loci were significantly correlated with the studied environmental features and possibly is adaptive and may be used by local populations to adapt to their environment.

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RAPD PROFIL U DETEKCIJI GENETIČKIH VARIJACIJA KOD *Stellaria* L. (Caryophyllaceae)

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Izvod

Stellaria vrste su biljke koje preferiraju vlažne planinske padine, ali neke rastu i u pustinji. Glavni centar diverzifikacije za *Stellaria* je Evroazija, sa centrom distribucije u planinama centralne Azije. Neke vrste su takođe kosmopolitske. U Iranu ovaj rod predstavlja 9 vrsta. Rod ima visoku lekovitu vrednost. Da bismo utvrdili genetsku raznolikost i razumeli ograničenja vrste unutar *Stellaria* u Iranu, uradili smo molekularnu analizu koristeći 139 nasumično sakuplje biljke koje predstavljaju 8 vrsta iz pet provincija Irana. Generisane su ukupno 122 ponovljive trake pomoću 10 od 25 prajmera sa slučajno amplifikovanom polimorfnom DNK (RAPD), sa prosečno 12,2 trake / prajmeru i 33% polimorfizma. Najveći broj efikasnih alela (Ne), genetska raznolikost (H) i Šenonov indeks (I) pokazala je vrsta *S. media.* Naši podaci pokazuju najveću sličnost između *S. media* i *S. pallida*, a najmanju između *S. media* i *S. graminea*. *S. pallida* je pokazala relativno nizak nivo genetičkih varijacija. Konačno, na osnovu podataka dobijenih RAPD markerima, populacije su podeljene u dva različita klastera, ukazujući na njihovu genetsku razliku koja je detaljno analizirana.

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