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Original scientific article

GENETIC DIVERSITY IN *Stellaria* L. (Caryophyllaceae) USING SEQUENCE RELATED AMPLIFIED POLYMORPHISM

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Li J., X. Yang, S.Mehri (2021). *Genetic diversity in Stellaria L. (Caryophyllaceae) using sequence related amplified polymorphism*. - Genetika, Vol 53, No.3, 1369 - 1377.
Nearly 120 species exist in the genus *Stellaria* L. (Caryophyllaceae, Alsinoideae) with a general distribution in the temperate zones of Asia and Europe. Totally, *Stellaria* exhibits 9 taxa in Iran and sequence-related amplified polymorphism was used to assess their genetic diversity. Therefore, 72 cases of 5 *Stellaria* were collected in seven provinces. Then, polymerase chain reaction amplifications (PCR) amplification of 5 species of *Stellaria* was employed for producing 78 (Number of total loci) (NTL) DNA bands. In addition, five selective primers were combined to produce the above bands. Results showed the total number of amplified fragments in ranges between 8 and 15 and variations of the predicted unbiased heterozygosity (H) between 0.22 (*S. persica*) and 0.39 (*S. holostea*). Moreover, estimations indicated the genetic similarity between 5 species from 0.73-0.92 and two key clusters were obtained by the clustering results. Considering the analysis of the SRAP markers, the minimum similarity was observed in *S. pallida* and *S. holostea*. Furthermore, distance (Mantel test results) showed a considerable signature of isolation. According to the findings, SRAP can detect and decipher genetic affinities in the *Stellaria* species. Hence, these findings could be used in conservation and biodiversity programs and also provide the ground for choosing appropriate ecotypes for pasture and forage purposes in Iran in the future.

Key words: Gene Flow, Genetic Diversity, Sequence-related amplified polymorphism, *Stellaria.*

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INTRODUCTION

According to the studies, *Stellaria* L. (Caryophyllaceae, Alsinoideae) includes ca. 150–200 species worldwide (BITTRICH, 1993), with 9 species classified in 2 sections. *S. sarcophylla* Rech.f., *S. scaturiginella* Rech.f., and *S. blatteri* Mattf. have an unknown section (RECHINGER, 1988). Based on the *Flora Iranica*, *Stellaria* sections in our country, Iran consists of *Stellaria* with two annual species [*S. pallida* (Dumort.) Pire & *S. media* (L.) Vill.] and 4 perennial species (*S. persica* Boiss., *S. holostea* L., *S. nemorum* L., & *S. graminea* L.) growing in the mountain regions. Section *Pseudalsine* Boiss. consists of only one annual species (*S. alsinoides* Boiss. & Buhse) growing in the mountains of Iran. *In fact*, *Stellaria* species have been considered to be common herbs that prefer humid mountainously slopes; however, a number of them grow in deserts. Experts in the field found that Eurasia is the focus of diversification for *Stellaria*, so that it mainly distributes in the mountains of E. central Asia. Several species of this genus are also cosmopolitan (BITTRICH, 1993) with the characteristics of the presence of five sepals and petals that are commonly bifid. Nonetheless, petals are remarkably reduced or missing in several species (HARBAUGH *et al*., 2010; FIOR *et al.*, 2006). Review of the related research indicated their main focus on the pollen and seed morphology, leaf and seed anatomy, and taxonomy (MAHDAVI *et al.*, 2012; KESHAVARZI & ESFANDANI-BOZCHALOYI, 2014a; 2014b) of *Stellaria* species; however, ecological adaptation, inter- and intera-specific differentiation, morphometric studies on *Stellaria* of Iran, and genetic diversity have been not investigated. Hence, the present research addressed the molecular study of 72 specimens of 2 section in *Stellaria.*

Finally, molecular variation of five species in Iran was studied in the research, with two major objectives: 1. Estimating genetic diversity of this genus and 2. Evaluating the population relationship by adopting WARD approaches. The obtained results can be followed by good implications in conservation and breeding plans.

MATERIALS AND METHODS

Plants collection

In this study, we sampled 72 individuals from May to August 2015 to 2020. According to the research design, 5 species of *Stellaria* in Mazandaran, west Azerbaijan, Kurdistan, Hamadan, Semnan, Esfahan, and Khorasan Provinces, Iran, were chosen (see Table 1). Then, 72 plant accessions were analyzed by SRAP. In the next step, we chose 5-12 samples from all populations of 5 various species according to other eco-geographic properties and stored them at the temperature of – 20 °C for additional uses. Table 1 presents the complete data of the samples' location and geographical distribution.

Sequence-related amplified polymorphism method

In this section, we applied fresh leaves of one to 12 plants and used silica gel powder to dry them. Moreover, the protocol proposed by ESFANDANI-BOZCHALOYI *et al.*, 2019 was followed to extract the Genomic DNA and LI and QUIROS's (2001) approach was used for SRAP assay. Finally, we utilized 5 SRAP in different primer combinations (see Table 2).

Table 1. List of the investigated taxa including origin of voucher specimens.

Taxa	Locality	Latitude	Longitude	Altitude(m)
S. pallida (Dumort.) pire	Kurdestan, Sanandaj	37° 07' 48 "	49° 54' 04"	165
	Hamedan, 20km s of Nahavand			
S. holostea L.	West-Azarbaijan, Urumieh, Silvana	37° 07' 08"	49°54' 11"	159
S. media (L.) VILL.	Kurdestan, Sanandaj	38 ˚ 52' 93"	47 ˚25' 92"	1133
	Esfahan, ardestan on road to taleghan			
S. persica Boiss.	Bojnord, Ghorkhod protected area	38˚52' 93"	47 ˚25' 92"	1139
	Semnan, 20km NW of shahrud			
S. graminea L.	Mazandaran, 40 km Tonekabon to janat abad	35 ˚50' 36"	51˚ 24' 28"	2383
	Mazandaran, Nowshahr			

RESULTS

Species identification and genetic diversity

Among from 10 primer combinations (PCs), 5 proper PCs were screened in our study and 70 amplified polymorphic bands (number of the polymorphic loci) were created, with wide ranges between 150 and 3000 bp. Minimum and maximum number of the polymorphic bands equaled 8 Em5-Me1 and 15 Em3-Me1. On average, all primers individually produced 11 polymorphic bands and Polymorphic information content (PIC) was in ranges between 0.29 (Em3-Me1) and 0.50 (Em5-Me1) for the 5 SRAP primers, with an average of 0.40 per primer. In addition, the primers′ *RP* was in ranges between 26.55 (Em3-Me1) and 52.11 (Em3-Me4) with an average of 39.55 in each primer (Table 2). Table 3 reports the genetic parameters calculated for *Stellaria* species. As seen in the table, unbiased heterozygosity (H) varies from 0.22 (*S. persica*) to 0.39 (*S. holostea*) with a mean of 0.29. Shannon's information index (I), which is maximum in *S. holostea* (0.471) and minimum Shannon's information index in *S. persica* (0.201). Furthermore, the alleles (*N*a) observed were in ranges between 0.172 in *S. graminea* and 1.145 in *S. holostea* so that the significant number of alleles (*N*e) was observed between 1.031 (*S. persica)* and 1.940 (*S. media*) in the study.

Table 2. SRAP primer information and results

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em3-Me4	10	10	100.00%	0.33	52.11
Em3-Me1	20	15	79.00%	0.29	26.55
Em4-Me1	13	13	100.00%	0.43	44.23
Em5-Me1	8	8	100.00%	0.50	38.55
Em5-Me2	12	9	90.00%	0.42	37.65
Mean	14	11	91.00%	0.40	39.55
Total	78	70			322.99

a) Number of total loci (NTL), b) Number of polymorphic loci (NPL), c) Polymorphic ratio(P %), d) Polymorphic information content (PIC), e) Resolving power (Rp)

Table 3. Genetic diversity parameters in the studied Stellaria species. Abbreviations:

SP	N	Na	Ne	I	He	UHe	%P
S. pallida (Dumort.) pire	10.000	0.223	1.099	0.292	0.27	0.32	40.23%
S. holostea L.	15.000	1.145	1.190	0.471	0.484	0.392	59.11%
S. media (L.) VILL.	16.000	0.228	1.940	0.324	0.40	0.33	36.50%
S. persica Boiss.	18.000	0.193	1.031	0.201	0.21	0.22	24.38%
S. graminea L.	17.000	0.172	1.095	0.288	0.35	0.27	42.05%

(N = number of samples, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Molecular Variance was analyzed and significant genetic differences (p=0.01) were observed among *Stellaria* species; however, a high level of differences were observed among the species. According to AMOVA, 60% of the total variations occurred between the species, with the less genetic variations at the species level (Table 4). We used genetic statistics (Nei's G_{ST}) for showing the genetic differences between different species of *Stellaria* so that significant *p*-values of Nei's G_{ST} were 0.395, p = 0.01 and D_est were 0.139, p = 0.01.

The same findings were obtained by ordination and clustering techniques. Figures 1 and 2 depict PCoA plot and NJ clustering. It should be mentioned that we classified the plant samples of each species belonging to a different section together and thus created an independent cluster. Therefore, it was concluded that that molecular characters could delimit the species of *Stellaria* in 2 distinct groups or clusters. However, intermediate forms were not observed in the specimens examined here. Generally, two main clusters were produced in NJ tree (see Figure 2) and *S. media* population was put in the first major cluster with a great distance from the other species but the second major cluster consisted of two subclusters. Plants of *S. pallida*; *S. graminea* and *S. persica* produced the second sub-cluster whereas the plants of *S. holostea* created the first sub-cluster.

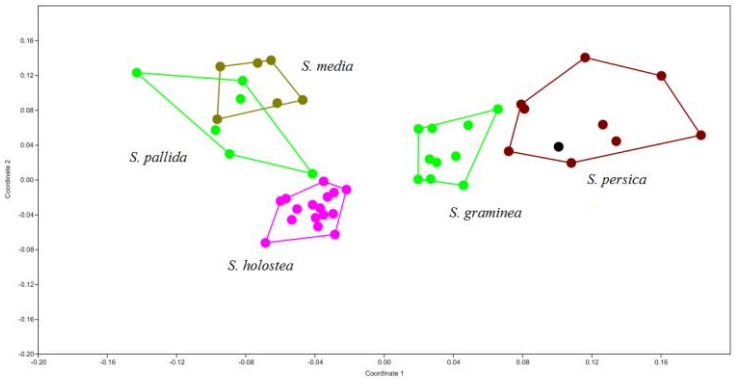

Fig 1. PCoA plot of SRAP data in the studied *Stellaria*

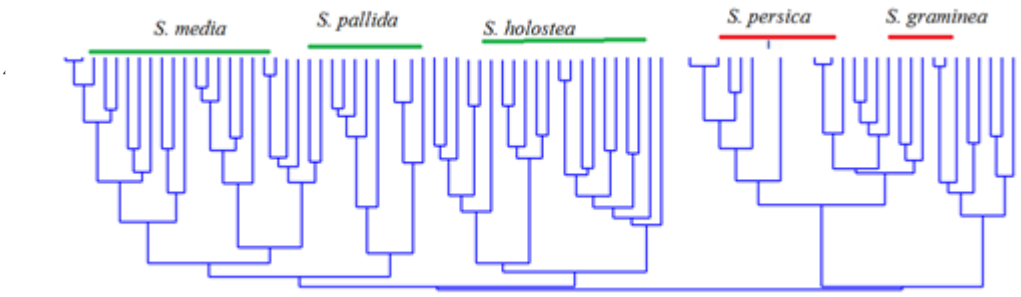

Fig 2. Neighbor joining dendrogram of SRAP data in the studied *Stellaria*

Table 4. Molecular variance analysis

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	19	1000.364	55.789	14.164	60%	
Within Pops	120	100.443	16.88	15.238	40%	60%
Total	139	1100.807		29.060	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, ($P < 0.001$).

According to the results, genetic and geographical distances (r=0.567, p=0.0002) strongly correlated to each other and gene flow ($N_{m)}$ score of the species equaled 0.754. A supplementary Table presents data of the genetic identity and distances (Nei's). As shown by the table, maximum degree of genetic similarity (0.92) was observed in *S. graminea* and *S. persica* but the minimum genetic similarity (0.73) was found in *S. holostea* and *S. pallida.*

In addition, STRUCTURE analysis and Evanno test were employed for identifying the best number of the genetic groups. Then, ancestrally shared alleles and/or interspecific gene flow in the species studied were illustrated by the admixture model. Results of K-Means clustering revealed k=5 according to pseudo-F and k=2 according to BIC. Moreover, K=5 matched with the AMOVA analysis and NJ grouping. In fact, K=5 reveals the presence of five genetic groups. Of course, the same results were observed by Evanno test, which was conducted on the STRUCTURE analysis, producing a major peak at k=5. The STRUCTURE plot (its figure is not shown) presented more complete data of the species' genetic structure and their common ancestral alleles and/or gene flow among the species of *Stellaria*. According to this plot, genetic differences occurred between Species 1 and 2 (that is shown by different colors) and also Species 3 and 4, 5, which is consistent with the Neighbor joining dendrogram mentioned previously. However, other species exhibited different allele compositions and genetic properties.

Based on the results, the low Nm value (0.754) represents the low gene flow or alleles with common ancestors between the species, and supporting the genetic stratification that was

shown by STRUCTURE analyses and K-Means. Finally, population assignment test showed consistency with the Nm result but could not detect any significant gene flows among the species' members.

DISCUSSION

As mentioned earlier, species relationships in *Stellaria* was evaluated by molecular (SRAP) data. Results obtained in the study demonstrated the importance of molecular information for identifying and studying the genetic diversity of the species. Generally, we found consistency of the genetic relationships from SRAP data with the morphometric outputs that matches with the results obtained for genetic diversity and AMOVA parameters. In addition, SRAP molecular markers implied the specific genetic differences among the studied specie, reflecting the SRAP potential for studying the taxonomy and systematics in the *Stellaria* members.

With regard to the unpleasant impacts of over-exploitation as well as biodiversity threats of *Stellaria* plant species in Iran, genetic diversity must be studied on *Stellaria* species to enhance knowledge on the development of the conservation approaches (JI *et al*., 2020a; 2020b). On the other hand, suitable primers should be chosen for research on the genetic diversity; therefore, indexes such as marker index (MI) and Polymorphic information content (PIC) could be of high importance to measure genetic variations in the species (SIVAPRAKASH *et al.*, 2004). It is widely accepted that various makers are differently capable of assessing genetic diversity, and genetic diversity is generally associated with polymorphism (SIVAPRAKASH *et al*., 2004). Therefore, the present study found the PIC values of SRAP primers to be in ranges of 0.29-0.50, with a mean value of 0.40. According to the results, PIC values showed high and low genetic diversity among the genotypes, with the values between 0 and 0.25 showing the low genetic diversity and between 0.25 and 0.50 highlighting the mid-level of genetic diversity. Moreover, those values >0.5 were shown to be linked with the higher genetic diversity (TAMS *et al*., 2005; BI *et al.*, 2021; CHENG *et al*., 2021). Thus, efficiency of the SRAP markers was confirmed for estimating genetic diversity in the *Stellaria* species. Additionally, SRAP markers showed the average percentage of polymorphism (91%), with the average RP (resolving power) values to be 39.55 of SRAP markers and average PIC values of SRAP makers to be 0.40 that are greater than the earlier values observed in the markers on *Stellaria* species (e.g., MARIA *et al*., 2007; DANA *et al*., 2007; YIN *et al*., 2021; JI *et al*., 2020, 2021). It should be noted that *Stellaria* species exhibited low gene flow (N_m). Finally, geographical and genetic distances significantly correlated to each other. According to the obtained results, isolation by distance (IBD) was observed among the *Stellaria* species (Mantet test results). However, population/species differentiation is shaped by numerous mechanisms like local adaptation, genetic drift, and isolation (ZHENG *et al*., 2021; ZHU *et al.*, 2021; KARASAKAL, 2020a, 2020b). Furthermore, degree of variability in the *N*e, *N*a, *I*, and *H* indexes reflects the higher levels of genetic diversity among *this genus*. Finally, results obtained by the Dendrogram and principal component analysis indicated certain differences among the reported *Stellaria* species, representing the higher uses of the SRAP approach for identifying the genus. Hence, these findings can detect appropriate ecotypes for pasture and forage (JIA *et al*., 2021; ESFANDANI-BOZCHALOYI *et al*., 2018a, b,c).

As stated in different studies, two hypotheses have been considered for the similarities between the isolated populations. According to the first hypothesis, genetic diversity between

and within populations represents the gene flow processes, resulting in the fragmentation of bigger populations (DOSTÁLEK *et al.*, 2010, MA *et al.*, 2021; PENG *et al.*, 2021; SI *et al.*, 2021). Considering the second hypothesis, gene flow connects the geographically proximate populations with higher efficiency than the populations separated by greater distance.

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GENETIČKI DIVERZITET *Stellaria* L. (Caryophyllaceae) OCENJEN POMOĆU SRAP

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Izvod

Rod *Stellaria* L. (Caryophyllaceae, Alsinoideae) obuhvata oko 120 vrsta rasprostranjenih u umerenim zonama Evrope i Azije. *Stellaria* je predstavljena sa ukupno 9 taksona u Iranu. Genetički diverzitet je procenjen pomoću SRAP-a. U 7 provincija prikupljena su 72 uzorka iz 5 *Stellaria*. Ukupno 78 (broj ukupnih lokusa) (NTL) DNK traka je proizvedeno PCR-om kod pet vrsta *Stellaria*. Ove trake su proizvedene sa kombinacijama pet selektivnih prajmera. Ukupan broj amplifikovanih fragmenata kretao se od 8 do 15. Predviđena heterozigotnost (H) varirala je između 0,22 (*S. persica*) i 0,39 (*S. holostea*). Genetske sličnosti između pet vrsta se procenjuju od 0,73 do 0,92. Rezultati grupisanja su pokazali dva glavna klastera. Prema analizi markera SRAP (*Sequence-related amplified polymorphism*), *S. holostea* i *S. pallida* su imali najmanju sličnost. Ovi rezultati su pokazali da pojačani polimorfizam povezan sa sekvencom ima potencijal da identifikuje i dešifruje genetski afinitet kod vrsta *Stellaria*. Trenutni rezultati imaju implikacije na biodiverzitet i programe konzervacije. Osim toga, ovi rezultati bi mogli otvoriti put za odabir odgovarajućih ekotipova za ishranu i ispašu u Iranu.

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