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SPECIES RELATIONSHIP AND POPULATION DIFFERENTIATION IN *STELLARIA* L. (CARYOPHYLLACEAE) OF IRAN USING SCOT MOLECULAR MARKER AND MORPHOLOGICAL DATA

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Abstract

Recognition of species is essential in a variety of domains, most remarkably biology, biogeography, ecology, as well as conservation. The genus *Stellaria* L. (Caryophyllaceae) has over 120 species spread across Europe and Asia's temperate zones. According to the most remarkable current treatments, nine species recognize *Stellaria* in Iran. These species are categorized into two types. Despite the broad distribution of several *Stellaria* species in Iran, no research on their genetic variability, method of divergence, or dispersion trends is accessible. As a result, we conducted genetic and morphological research on six *Stellaria* species and two of their closest relatives gathered from various habitats in Iran. This research aims to 1) Can SCoT markers be utilized to recognize *Stellaria* species? 2) What are the genetic characteristics of the mentioned taxa in Iran? and 3) To examine the interrelation of the species. In this research, ten SCoT markers were employed for molecular analysis, and 112 accessions were utilized for morphological study. The genetic distances were calculated using the Jaccard similarity coefficient, and descriptive data on the populations were used to estimate genetic parameters. There were 98 polymorphic bands all over. The integration of morphological and SCoT data demonstrated that the *Stellaria* species of Iran could be delimited and recognized. The *Stellaria* species are genetically unique; however, they share some similar alleles, according to AMOVA and STRUCTURE analyses.

Introduction

The delimitation of species is significant in various biological fields, including ecology, biogeography, and plant preservation. Species delimitation is accomplished using both tree-based and non-tree-based methods. In the first technique, species are classified into distinct clades depending on synapomorphic traits (phylogenetic species concept); however, in the second method, species could be identified using any taxonomic feature and gene flow evaluations. Wiens and Penkrot (2002) recommended using DNA as well as morphological data for species delimitation. In contrast, Knowles and Carstens (2007) focused on how molecular data (for example, DNA sequence data is utilized to generate gene trees) could be utilized to delimitate species. The former writers utilized coalescent simulations for evaluating the species limitations and combined information from numerous loci. They demonstrated the relevance of population genetics in determining the boundaries of a species.

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Stellaria L. (Caryophyllaceae, Alsinoideae) has around 150–200 species worldwide (Bittrich, 1993). In Iran, nine species in this genus are classified into *Stellaria* and *Pseudalsine.* An unclear part exists in *S. blatteri* Matt., *S. scaturiginella* Rech.f., and *S. sarcophylla* Rech. F (Rechinger, 1988). The *Stellaria* section includes two annual species, *S. medium* (L.) Vill in Iran.

Stellaria species are widespread plants that favor humid mountainous slopes; however, some have been discovered growing in deserts. The genus is defined by the existence of five sepals and bifid petals; however, certain species have petals that are much reduced or nonexistent (Fior *et al.*, 2006; Harbaugh *et al.,* 2010). Eurasia is the primary breeding ground for *Stellaria,* with the eastern central Asian highlands acting as a major distribution hub. Additionally, several species are cosmopolitan in their distribution (Bittrich, 1993). There are just a few *Stellaria* chromosomal records throughout the globe. The genus has been reported to contain basic chromosome numbers x=10, 11, 12, and 13. Earlier, investigations on the delimitation of species and the linkages between species in this genus have been conducted.

The primary focus of these researches was on the taxonomy, seed, as well as pollen morphology, stem, and leaf anatomy of *Stellaria* species (Mahdavi *et al.,* 2012; 2014b; Esfandani-Bozchaloyi and Keshavarzi, 2014). Verkleij *et al.* (1980) utilize electrophoretically observable variation in isoenzymes to gain insight into the genetic variations between and within the two species and two local subpopulations of *S. media.*

They demonstrated a variation in the isoenzyme pattern between the species *S. medium* and *S. pallida* for five enzymes. Two of these five enzymes showed interpopulation variation in *S. media,* and no variability in the isoenzyme structure may be described by the species' persistent autogamous (cleistogamous) condition. There was no indication of polyploidy influencing the isoenzyme pattern of activity. However, no effort has been made to investigate the genetic variation, ecological adaptability, intra-, and inter-specific delineation, and morphometric variation of *Stellaria* of Iran. There were 112 specimens of two sections of *Stellaria* collected for morphological and genetic analysis. We point to reply to the taking after questions: 1) Are there infrared and interspecific hereditary differing qualities among the *Stellaria* species examined? 2) Is there a linkage between genetic and geographical distances among these species? 3) How are populations and taxa genetically structured 4) Does gene exchange occur across *Stellaria* species in Iran?

Materials and Methods

Plant materials

As mentioned in table one, during 2015-2018, 112 plant samples from six *Stellaria* species of *Pseudalsine* (*S. alsinoides*) and *Stellaria* (*S. media, S. pallida, S. holostea, S. persica, and S. graminea*) were gathered from natural habitats of Fifteen geographical populations. Several sources were examined to ensure that species were correctly identified (Rechinger, 1988). The sampling sites details are listed (Table 1, Fig. 1). The Herbarium of Azad Islamic University (HAIU) has voucher specimens.

Morphological studies

Morphometry was conducted on five samples of each species. 24 morphological features (9 qualitative, 15 quantitative) were investigated (Table 2). According to Podani (2000), a Euclidean distance estimate was made for clustering and ordination analysis using the given data, which was standardized (Mean=0, variance=1).

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

Species	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
1. *S. media* (L.) VILL.	East Azerbaijan, kaleybar, Shojabad	38 ° 52'393"	47 ° 25' 92"	1133	HIAU 201677
2. *S. pallida* (Dumort.) pire	East Azerbaijan kaleybar, cheshme ali akbar	38 ° 52'353"	47 ° 27 92"	1143	HIAU 201678
	East Azerbaijan, kaleybar cheshme ali akbar	38 ° 52'353	47 ° 27' 92"	1143	HIAU 201680
3. *S. holostea* L.	East Azerbaijan, kaleybar, road side	38 ° 52'373	47 ° 23' 92"	1144	HIAU 201683
	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
4. *S. persica* Boiss.	East Azerbaijan kaleybar, cheshme ali akbar	38 ° 52'353	47 ° 27' 92"	1143	HIAU 201686
	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
6. *S. alsinoides* Boiss & Buhse	Guilan, Sangar, Road sid	370702.32	494432.6	48	HIAU 201690
	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

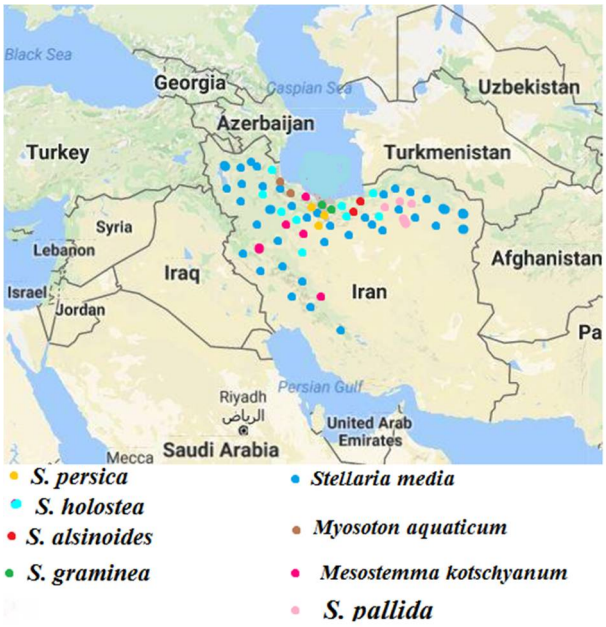

Fig. 1. Distribution map of the studied species of *Stellaria*.

Table 2. List of selected characters and their codes in morphological studies.

No.	**Characters**	**Numerical code**
1	Plant height	mm
2	Length of basal leaves	mm
3	Width of basal leaves	mm
4	Length of stem leaves	mm
5	Width of stem leaves	mm
6	Bract length	mm
7	Bract width	mm
8	Pedicel length	mm
9	Number of seeds per capsule	
10	Number of flowers per inflorescence	
11	Number of calyx	
12	Calyx length	mm
13	Calyx width	mm
14	Number of petal	
15	petal length	mm
16	Growth period	0-annual 1- perennial
17	Bract apex	0-acute 1- narrow 2- absence
18	State of stem	0-unbranched 1- branched
19	State of stem strength	0-thin 1- strong
20	Stem hairs	1-unilateral hair 2- multilateral hair
21	Cross-section of stem	1- rectangular 2- elliptical
22	Shape of basal leaves	1- linear- lanceolate 2- lanceolate 3- lanceolate – acuminate 4- ovate
23	Basal leaves apex	0-acute 1- narrow
24	Basal leaves petiole	0-absence 1- presence

Table 3. Genetic diversity parameters in the studied *Stellaria* species.

Pop	N	Na	Ne	I	He	UHe	P%
sp1	12.000	1.347	1.404	0.381	0.174	0.182	46.91%
sp2	8.000	0.429	1.097	0.084	0.056	0.060	16.13%
sp3	6.000	0.258	1.029	0.023	0.016	0.010	4.38%
sp4	12.000	0.925	1.279	0.233	0.155	0.162	22.09%
sp5	11.000	0.784	1.171	0.162	0.104	0.109	36.56%
sp6	14.000	0.344	1.039	0.014	0.021	0.023	3.98%
sp7	14.000	0.560	1.186	0.098	0.064	0.066	21.51%
sp8	10.000	0.441	1.036	0.033	0.022	0.023	6.53%

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.

DNA extraction and SCoT assay

Fresh leaves were randomly utilized from 5-10 plants in each group investigated. Silica gel powder was used to dry them. Genomic DNA was extracted using the CTAB activated charcoal technique (Doyle and Doyle, 1987). A 0.8 percent agarose gel was used to test the extracted DNA quality. Collard and Mackill (2009) created 25 SCoT primers; ten primers with distinct, expanded, as well as rich polymorphism bands were selected (Table 3). The PCR procedures were conducted in a 25µl volume including ten mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of every dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA, as well as 3 U of Taq DNA polymerase (Bioron, Germany). The preceding program was used to execute the amplifications and reactions in a Techne thermocycler (Germany): 5 minutes at 95°C for denaturation, accompanied by 37 cycles of 1 minute at 95°C, 1 minute at 50-56°C, and 1 minute at 72°C. A final extension phase of 5-10 minutes at 72°C concluded the reaction. The ethidium bromide staining was used to determine the amplification products on a 1% agarose gel. A 100 bp molecular size ladder was utilized to estimate the fragment size (Fermentas, Germany).

Table 4. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	14	727.747	51.327	8.082	58%	58%
Within Pops	67	391.607	5.530	5.530	42%	
Total	81	1119.354		13.612	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, ($P < 0.001$).

Data analyses

Morphological studies: According to Podani (2000), morphological traits were initially standardized (Mean = zero, Variance = 1) and employed for calculating Euclidean distance between taxa. The UPGMA (Unweighted paired group using average) method was utilized to group the plant specimens (Podani, 2000). ANOVA (Analysis of Variance) illustrated the morphological variation across populations. At the same time, the PCA (Principal Component Analysis) biplot was employed to discover the most variable morphological features among the analyzed populations (Podani, 2000). Multivariate statistical analyses of morphological data were performed using PAST version 2.17 (Hammer *et al.*, 2012).

Molecular analyses: The SCoT bands collected were encoded as binary characters (presence = 1, absence = 0). A variety of parameters were calculated, including Nei's gene diversity (H), the Shannon information index (I), the number of efficient alleles, as well as the polymorphism percentage (Weising *et al.*, 2005; Freeland *et al.*, 2011). Neighbor-Joining (NJ) clustering and Neighbor-Net networking depended on Nei's genetic distance between populations (Freeland et al., 2011; HUSON and BRYANT 2006). The Mantel test was used to determine the association between the analyzed populations' geographical as well as genetic distances (Podani, 2000). These evaluations were carried out using PAST ver. 2.17 (Hammer *et al.*, 2012), DARwin ver. 5 (2012), and SplitsTree4 V4.13.1 (2013) software. The AMOVA (Analysis of Molecular Variance) test (containing 1000 permutations) conducted in GenAlex 6.4 (Peakall and Smouse, 2006) and the Nei,s Gst analysis conducted in GenoDive ver.2 (2013) (Meirmans and Van Tienderen, 2004) were employed to show genetic differentiation across the populations. Furthermore, GST est = standardized measure of genetic differentiation (Hedrick, 2005) and D est = Jost measure of differentiation were used to study population genetic differentiation (JOST, 2008). Bayesian model STRUCTURE analysis and GenoDive ver. 2's maximum likelihood-based K-Means clustering approach (based on maximum likelihood) was used to examine the population's genetic structure (2013). Data were evaluated as dominating markers for STRUCTURE analysis. The admixture ancestry model was utilized with the correlated allele frequency model. The Evanno test was applied to the STRUCTURE result for calculating the appropriate number of K utilizing the delta K value (Evanno *et al.*, 2005). Two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), are used to identify the best fit for k in K-Means clustering (Meirmans, 2012).

Gene flow was calculated by utilizing PopGene ver. 1.32 (1997) to calculate Nm, and gene flow estimate from Gst, as Nm = 0.5(1 - Gst)/Gst. This technique assumes that all populations experience an equal amount of gene flow. (ii) Population assignment test using maximum likelihood, as conducted in GenoDive version 2. (2013), the existence of common alleles was assessed via using DARwin ver 5 to create a ventriculogram network employing the least square approach (2012).

Results and Discussion

Species identification and inter-relationship

Morphometry: ANOVA revealed significant variations in quantitative morphological features (P 0.01) across the species studied. PCA analysis was used to discover the most variable characteristics among the species investigated. It was discovered that the top three factors accounted for more than 73% of the overall variation. The parameters length, breadth, hairs, number of sepals, pedicle hair, the width of seed, and capsule form have the strongest correlation (>0.7) in the first PCA axis, accounting for 52 percent of total Variance. The texture of the leaf, the number of stigmas, the number and size of capsule sutures and length, the number of petals, petal existence, as well as stem branching were all factors impacting PCA axis 2 and 3 (figure excluded).

Numerous grouping and ordination techniques yielded the same findings. Figure 2 illustrates a UPGMA grouping and PCA plot of morphological characteristics. Plant samples from each species relating to a specific part were grouped and generated clusters separately in general. This study indicates that the morphological parameters examined could distinguish *Stellaria* species. We identified no transitional forms among the specimens we investigated.

Generally, the UPGMA tree created two large clusters (Fig. 2). The attributes seed shape, size, quantity per capsule, cauline leaves shape, and several stigmas, the *Mesostemma* taxa and *Myosoton aquaticum* created a distinct group in *Stellaria* in the first cluster. Two sub-clusters were

included in the second main cluster. Due to morphological similarity, *S. media* and *S. pallida* from the *Stellaria* section and *S. alsinoides* (*Pseudalsine* section) formed the first sub-cluster. In contrast, *S. persica, S. graminea,* and *S. holostea* (*Stellaria* section) formed the second sub-cluster. They were distinguished by hair absence, linear, sessile leaves, ten stamens, 3mer stigma, and deeply.

Species identification and genetic diversity

The entire SCoT primers obtained polymorphic bands. According to table two, the genetic variation characteristics evaluated in the examined species indicated that *S. media* (sp1) possessed the greatest amount of genetic polymorphism (46.91 percent), whereas *S. alsinoides* had the lowest level (3.98 percent) (sp6). *S. media* also exhibited the greatest effective number of alleles (Ne = 1.4) and Shannon information index (I = 0.38) values.

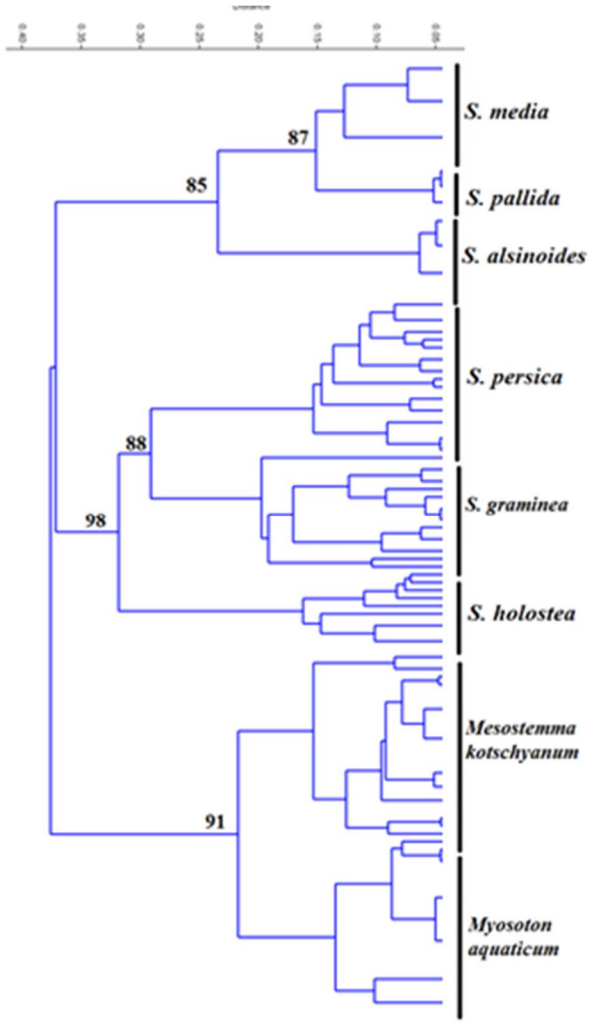

Fig. 2. Species delimitation in the *Stellaria* revealed by UPGMA clustering of morphological characters.

The AMOVA test revealed a substantial genetic difference between the examined species (P = 0.01). It demonstrated that 58% of overall Variance occurred across species, and 42% occurred within species (Table 4). Furthermore, substantial Nei's GST (0.41, P = 0.01) and D est values (0.169, P = 0.01) were found to indicate genetic divergence between these species.

The NJ tree created using Nei's genetic distance (not included) revealed that *Mesostemma* taxa and *Myosoton aquaticum* are genetically distant from the other examined species. This dendrogram revealed that *S. media* and *S. pallida* had a deep genetic affiliation. Likewise, *S. persica* and *S. graminea* (both in the *Stellaria* section) were positioned close together, with *S. holostea* joining them at a distance. In general, correlations between species derived from SCoT data correlate well with morphological data. This corresponds to AMOVA as well as genetic diversity parameters. The genetic differences between the species are substantial. Additionally, the Nm study performed by the Popgene program yielded a mean Nm=0.23, which is regarded as a meager amount of gene flow between the analyzed species. The Mantel test containing five hundred permutations revealed a significant association (r = 0.18, p=0.0001) between genetic and geographical distance, indicating that isolation by distance (IBD) happened among the *Stellaria* species tested.

The genetic identification of Nei and the genetic distance between the examined species were established (Table is excluded). These findings indicated that *S. media* and *S. pallida* had the greatest degree of genetic similarity (0.90). Between *Mesostemma* taxa and *Myosoton aquaticum,* the highest degree of genetic similarity was observed (0.64).

The species genetic structure

We conducted STRUCTURE analysis, followed by the Evanno test to determine the ideal number of genetic groupings. According to pseudo-F and BIC, K-Means clustering yielded k = 8 and k = 14, respectively. K = 14 is consistent with the NJ grouping and AMOVA. K = 8 indicates the existence of eight genetic collections. Ployed the admixture model to show interspecific gene flow or/and ancestrally shared alleles. The same result was achieved using the Evanno test on the STRUCTURE analysis, which revealed a significant peak at k = 8. (Fig. 3).

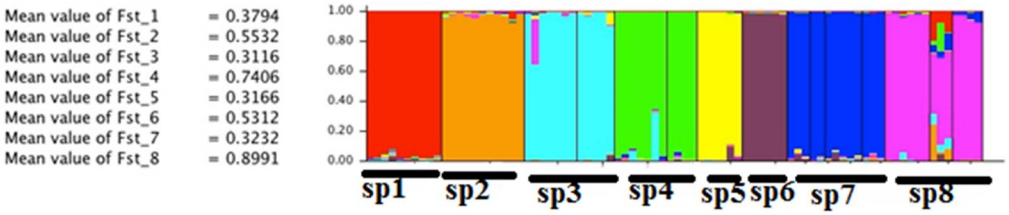

Fig. 3. STRUCTURE plot of *Stellaria* species based on SCoT data.

The STRUCTURE plot (Fig. 3) revealed further data about the species genetic structure investigated and common ancestral alleles and gene flow among Geranium species. The plot demonstrated the genetic distinction between species 1 and 2 (diversely colored) and between species 7 and 8. This is consistent with the Neighbor-joining dendrogram that was previously provided. The rest of the species vary in allele composition and are diverse genetically.

The low Nm value (0.23) supports genetic stratification as demonstrated by K-Means and STRUCTURE studies, indicating minimal gene flow or ancestrally shared alleles amongst the species investigated. The population assignment test concurred with the Nm finding that no evidence of considerable gene flow among the tested species.

Genetic diversity

Genetic diversity is a critical component of biological variation for conservation methods (Khayatnezhad, and Gholamin 2021; Gholamin, and Khayatnezhad 2020; 2021; Guo, *et al*., 2021). The size of the population is thought to be critical for preserving genetic variety. Because of environmental stochasticity, genetic drift, and inbreeding, small people are more prone to extinction than large ones. Genetic drift reduces heterozygosity and eventually allele fixation, but inbreeding enhances homozygosity within populations (Hou, *et al.*, 2021; Huang, *et al*., 2021; Jia, *et al.*, 2020; Karasakal, *et al.*, 2020a; 2020b; Khayatnezhad, and Gholamin 2020a; 2020b). Generally, population size declines may result in a loss of genetic diversity due to genetic drift and inbreeding. In the end, reduced genetic variation may decline fitness and the evolutionary potential to respond to environmental alterations (Lande, 1993; Ma, *et al.*, 2021a; 2021b; Peng, *et al* 2021; Ren, *et al*, 2021). The conservation and management of small population species rely heavily on characterizing patterns of genetic variability and variation within and across distinct populations.

The current research used SCoT markers to determine the genetic diversity within *Stellaria*. Our study shows that *S. alsinoiedes* exhibited a reduced amount of genetic diversity (P: 3.98 percent, He: 0.021, I: 0.014). Natural features, reproductive mode, and breeding system have been identified as significant factors influencing genetic diversity levels. Generally, outcrossing species have a far more significant genetic variation than selfing ones (Hamrick and Godt 1989; Nybom 2004). In the past, it was thought that S.'s mating system was mostly self-involved (Peterson, 1936).

Verkleij *et al.* performed isoenzyme studies on the hypotetraploid *S. media* and the diploid *S. pallida* (1980). Their findings revealed a variation in isoenzyme pattern between the species *S. medium* and *S. pallida* for five enzymes. In *S. media*, two of the five enzymes exhibited interpopulation variation. In *S. pallida*, there was essentially no fluctuation in the isoenzyme pattern that could be described by the species' persistent autogamous (cleistogamous) condition. It was not feasible to demonstrate the effects of polyploidy on the isoenzyme pattern and activity. *S. medium* and *S. pallida* are primarily self-fertile, and there is a crossing barrier between such species (Peterson, 1936), probably owing to *S. pallida's* diploidy (2n = 22) and S. media's hypotetraploidy (2n=40-44) (Scholte, 1978). Chinnappa and Morton (1984) used isozyme, RFLP, and RAPD analyses, as well as comparative morphological investigations, to study the genetic variation and phenotypic plasticity that contribute to population divergence within the *S. longipes* complex. Two factors contribute significantly to this species' success: (1) genetic variability resulting from polyploidy, facultative outbreeding, and interspecific gene flow; and (2) the development of phenotypic plasticity because of environmental-induced modifications in the genotypes' physiology and morphological expression. Although all genotypes were self–compatible, protandrous, gynodioecious, and partly gynodioecious, individuals were prevalent in the species (Philipp, 1975; Chinnappa, 1985). Chinnappa and Morton (1984) confirmed Philipp's (1972) and Chinnappa and Morton's (1984) findings that there is no association between chromosome number and morphology or reproductive biology (1974, 1976). Chinnappa and Morton (1991), relying on previous research (Chinnappa and Morton 1974, 1976, 1984; Macdonald *et al.*, 1987), advocated that the *Stellaria* taxa in issue be classified into an S. longipes complex with two subspecies: *S. longipes* Goldie subsp. *longipes* and *S. longipes* Goldie subsp. *arenicola* (Raup). The *S. longipes* subsp. *arenicola*'s evolution was thought to have begun with the colonization of a dune environment and a likely change in the breeding system to self-pollination. In its native habitat, *S. longipes* subsp. *arenicola* is interfertile with other *S. longipes* populations and intergrades, although field investigations show that *S. longipes* subsp. *arenicola* is predominantly self-pollinating (Macdonald *et al.*, 1987). Otherwise, *S. longipes* is a single polymorphic species with no well-defined infraspecific taxa (Chinnappa and Morton, 1991).

Conclusions

Numerous variables influence the genetic structure, breeding frameworks, hereditary float, populace estimate, and natural selection (Hamrick and Godt, 1990). Our genetic structure analysis revealed that the 112 individuals created a distinct divergence between all groups, a finding that is corroborated by the PCA (Fig. 2). The examination of molecular Variance in all populations uncovered that 58% of differing hereditary qualities happened over individuals, whereas 42% occurred inside these bunches (Table 4). The current research concludes that SCoT molecular markers, in conjunction with morphological features, are beneficial for identifying *Stellaria* species. While there are few interspecific genetic mixing in *Stellaria*, the examined species are highly distinct throughout the speciation procedure and invasion of new environments.

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