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Morphometric analysis and genetic diversity in *Glaucium* (Papaveraceae) using sequence related amplified polymorphism

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Abstract. *Glaucium* belongs to the Papaveraceae family. *Glaucium* is a genus of annual, biennial, and perennial herbaceous plants that thrive on salty soils and near the sea. *Glaucium* is represented by a total of 10 taxa in Iran. Sequence-related amplified polymorphism was used to estimate genetic diversity. A combination of morphological and genomic data was used to identify genetic diversity and species features in Glaucium species. In eight provinces, 65 people connected to five *Glaucium* were gathered. Through polymerase chain reaction (PCR) amplification of five *Glaucium* species, a total of 144 (Number of total loci) (NTL) DNA bands were obtained. These bands were created by combining 10 different selective primers. The total number of amplified fragments varied from seven to twenty-six. The expected unbiased heterozygozity (H) ranged from 0.19 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) to 0.33 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) (*G. oxylobum* var. *oxylobum*). The genetic similarities between five species range from 0.63 to 0.88. The findings of clustering revealed two large groupings. The SRAP (Sequence-related amplified polymorphism) markers study revealed that *G. grandiflorum* and *G. oxylobum* var. *oxylobum* had the least similarity. This investigation also discovered a substantial indication of distance isolation (Mantel test results). The current findings indicate that sequence-related amplified polymorphism can discover and understand genetic affinity in *Glaucium* species. The current findings have consequences for biodiversity and conservation efforts. Aside from that, the current findings may pave the way for identifying acceptable ecotypes for grazing and pasture uses in Iran.

Keywords: population structure, gene flow, network, genetic admixture.

INTRODUCTION:

SRAP (sequence-related amplified polymorphism) is a PCR-based marker system (Gondal et al 2021; Dadzie et al 2021; Chimwamurombe et al 2020; Abeshu & Zewdu 2020).

It is one of the most efficient and straightforward marker systems for studying gene mapping and gene tagging in plant species (Si et al, 2020; Sun et al, 2021; Sun and Khayatnezhad 2021; Tao et al., 2021; Wang et al., 2021), and SRAP are potential markers for plant systematics and genetic diversity studies (Robarts and Wolfe 2014 Khayatnezhad and Gholamin 2021; Gholamin and Khayatnezhad 2020; 2021; Guo et al., 2021).

Poppy family (Papaveraceae) comprises of approximately 26 to 42 genera and 690 to 800 species in the world (Judd *et al*., 1999). The members of Papaveraceae are shrub, herbaceous perennials and annuals distributed in the temperate and the subtropical regions of the world. Among five genera of family Papaveraceae in Iran, *Glaucium*, *Hypecoum*, *Chelidonium* and *Roemeria* consist of 10, 1, 1 and 2 species, respectively (Rechinger and Cullen, 1966). *Glaucium* is found mostly in Atlantic Europe and Central Asia (Kaderiet 1993). The genus is divided into two sections, each containing four species, four subspecies, and two varieties: sect. *Acropetala* Mory has four species, four subspecies, two varieties and sects. *Glaucium,* which has 19 species, eight subspecies, and 16 variants (Mory 1979). It was represented by 11 (Cullen 1966) to 13 in Iran (Mobayen 1985; Gran and Sharifnia 2008).

Morover, Mobayen (1985) introduced two subspecies *G. fimbrilligerum* Boiss. subsp. *annuum* and *G. fimbrilligerum* subsp. *Ophyocarpum*. Azizian and Alishahi Norani (1997) studied anatomical characteristics of fruit and blade with emphasis on latex tubes in species of *Glaucium*. Furthermore, Carlquist and Hoekman (1985) studied anatomical structure of wood in Romneya and Dendromecon. Carlquist and Zona (1988) continued his studies in cooperation with Zona on structure of wood in Papaveraceae. Some anatomical features of midrib and fruit of *Glaucium* are of diagnostic value (Solereder, 1908; Metcalfe and Chalk, 1950). Several taxonomic investigations have demonstrated that seed and trichome micromorphology may be used for taxonomic categorization and delimitation at all taxonomic levels and across plant families (Ma et al., 2021a; 2021b; Peng et al., 2021; Ren et al., 2021). Arabi et al., 2017; Tavakkoli and Assadi, 2016).

Gran and Sharifnia also researched the seed ornamentations of 14 *Glaucium* species in Iran (2008). Light microscopy (LM) and scanning electron microscopy (SEM) was used to examine the seeds and trichomes of 15 species of the genus *Glaucium* found in Iran (Tavakkoli and Assadi 2019). The seeds are semicircular to reniform in shape. However reniform and elongated reniform seeds have been identified in *G. oxylobum* and *G. elegans*, respectively. The most common types of testa surface sculpturing include verrucate–rugulate, verrucate–granulate, verrucate–perforate, verrucate–lineolate, rugulate–granulate, rugulate, and ocellate. Their findings reveal that the micro-morphological properties of seed and ovary trichomes give important and substantial information for species and taxa within species separation, as well as a diagnostic key to the taxa. *Glaucium* taxa were studied in terms of morphological, palynological, and phylogenetic characteristics, according to Fatma Mungan Kiliç et al. (2019). Their findings reveal that several of these features change across species, particularly in micromorphology and the development of clades in phylogenetic trees based on matK and ITS3-6 DNA sequence data. The genus Glaucium of Turkey was separated into subsections Glabrousae and Pubescentae based on DNA investigations backed by morphological evidence (stem trichomes).

The present study investigated the molecular variation of five species in Iran. Objectives of the study were; a) to estimate genetic diversity; b) to evaluate population relationships using WARD approaches. There are consequences for breeding and conservation initiatives based on current findings.

MATERIALS AND METHODS:

Plants collection

Sixty-five (65) individuals were sampled. Five *Glaucium* species in west Azerbaijan, Mazandaran, Hamadan, Kurdistan, Esfahan, Semnan, Khorasan and Razavi Khorasan Provinces of Iran were selected and sampled during may-August 2014-2020 (Table 1). Morphometric and SRAP analyses on sixty five plant accessions were carried out. Based on additional eco-geographic criteria, five to twelve samples from each population belonging to five distinct species were chosen. Five samples were stored at - 20 °C till further use. Detailed information about locations of samples and geographical distribution of species are mentioned (Table 1 and Figure 1).

Morphological studies

Each species was subjected to morphometric analysis and twelve samples per species were processed. Qualitative (12) and quantitative (14) morphological characters

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

Taxa	Locality	Latitude	Longitude	Altitude(m)
G. fimbrilligerum Boiss.	Kurdestan, Sanandaj	35°19'18.75"	46°59'10.194"	1538
G. corniculatum var. *corniculatum* (L.) Curtis	West-Azarbaijan, Urumieh, Silvana	37.552673	45°4'33.7656"	1344
G. oxylobum var. *oxylobum* Boiss. & Buhse	Kurdestan, Sanandaj	38°22'18"	46°37'10"	1523
G. grandiflorum subsp. *grandiflorum* var. *grandiflorum* Boiss. & A.Huet	A.Huet Semnan, 20km NW of Shahrud	36°25'14"	54°15'32"	1345
G. contortuplicatum var. *cantortuplicatum* Boiss.	Mazandaran, 40 km Tonekabon to Janat abad	35°46'56"	51°23'29"	2383

Figure 1. Provinces and collection sites of *Glaucium* species.

were studied. Data were transformed before calculation. Different morphological characters of flowers, leaves, and seeds were studied. Ordination analyses were conducted while using Euclidean distance (Podani 2000).

Sequence-related amplified polymorphism method

One to twelve plants' worth of fresh leaves were utilized at random. Silica gel powder was used to dry them. Following the prior technique, the DNA was extracted (Esfandani-Bozchaloyi et al. 2019). According to the protocol, we ran the SRAP assays (Li and Quiros 2001). Ten SRAP were employed with various primer combinations (Table 2). Single primers, 20 ng of genomic DNA, and 3 U of Taq DNA polymerase (Bioron, Germany) were used in 25l of Tris-HCl buffer at pH 8; 50 mM of KCL; 1.5 mM of MgCl2; 10 mM of Tris-HCl buffer at pH 8 and 3 U Taq DNA polymerase (Bioron, Germany) were used in PCR reactions. The total volume of the reaction was 25 l. A Techne thermocycler was used for this PCR experiment (Germany).

Data Analyses

To evaluate morphological characteristics, the UPGMA (Unweighted paired group using average) ordination approach was used. To analyze morphological differences across species, an ANOVA (analysis of variance) was used. To find variable morphological features in *Glaucium* species, principal component analysis (PCA) was used. PAST software version 2.17 was used to conduct multivariate statistical studies, often known as PC analysis (Hammer et al. 2001).

Molecular analyses

Sequence-related amplified polymorphism (SRAP) bands were recorded. Presence and absence of bands were scored present (1) and absent (0), respectively. Total loci (NTL) and the number of polymorphism loci (NPL) for each primer were calculated. Mantet test was performed with 5000 permutations in PAST, version 2.17 (Hammer *et al*. 2001).

Comparing genetic divergence or genetic distances, as assessed by pairwise FST and related statistics, with geographical distances, as evaluated by the Mantel test, is one of the most used tools for examining spatial dynamics driving population structure. The Mantel test, as originally formulated in 1967, $Z_m = \sum_{i=1}^{n} \sum_{j=1}^{n} g_{ij} \times d_{ij}$ where *gij* and *dij* are, are the genetic and geographical distances between populations I and j, respectively. respectively, the genetic and geo-graphic distances between populations i and j, considering populations. Because Zm is is defined as the sum of product distances, its value is affected by the number of populations analyzed as well as the size of their distances. The Zm-value may be compared to a null distribution, and Mantel initially advocated using the standard normal deviation (SND), which is defined as SND =Zm/var(Zm)1/2 (Mantel 1967). PAST ver. 2.17 (Hammer et al. 2012) and DARwin ver. 5 (2012) software were used for these investigations. The AMOVA (Analysis of molecular variance) test (with 1000 permutations) created in GenAlex 6.4 4 (Peakall and Smouse 2006) was used to reveal genetic differences across the populations.

RESULTS

Morphometery

The ANOVA findings showed substantial differences ($p<0.01$) between the species in terms of quantitative morphological characteristics. Principal component analysis results explained 68% cumulative variation. The first PCA axis accounted for 59% of the overall variance.

The highest correlation (> 0.7) was shown by morphological characters such as calyx length, calyx width, corolla length, corolla color. The morphological characters of *Glaucium* species are shown in PCoA plot (Figure 2). Each species formed separate groups based on morphological characters. The morphometric analysis showed clear difference among *Glaucium* species and separated each groups.

Species identification and genetic diversity

Ten (10) suitable primer combinations (PCs), out of 25 PCs were screened in this research. Figure 3 illustrates the banding pattern of Em2-Me4, Em3-Me1 and Em5-Me1 primer by the SRAP marker profile. One hundered and thirty six (136) amplified polymorphic bands (number of polymorphic loci) were produced. These bands (fragments) had different range i.e. 150bp to 3000 bp. Maximum and minimum numbers of polymorphic bands were 22 for Em2-Me4 and 7 Em5-Me2, respectively. Each primer produced 13 polymorphic bands on average. The PIC ranged from 0.14 (Em4-Me1) to 0.63 (Em1-Me4) for the 10 SRAP primers, with an average of 0.42 for each primer The primers' RP varied from 12.24 (Em3-Me4) to 56.55 (Em3-Me1), with an average of 32.25. (Figure 3, Table 2).

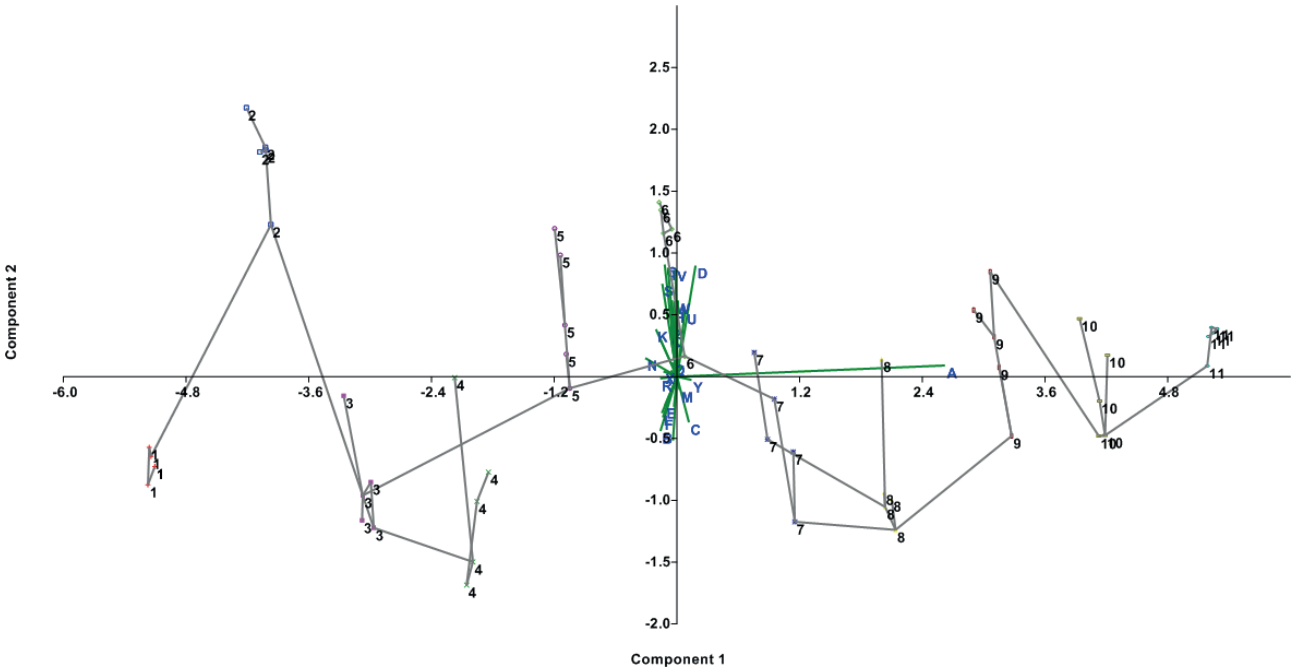

Figure 2. Morphological characters analysis of *Glaucium* species by PCA plot.

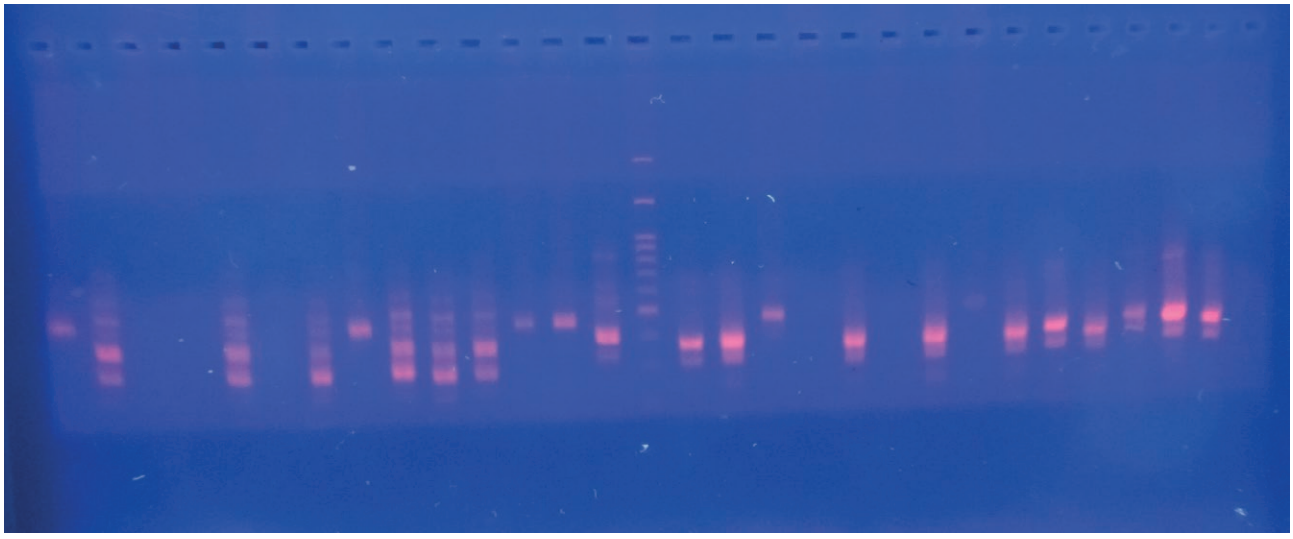

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile with primer Em2-Me4.

The calculated genetic parameters of *Glaucium* species are shown (Table 3). The unbiased heterozygosity (H) varied between 0.19 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) and 0.33 (*G. oxylobum* var. *oxylobum*) with a mean of 0.28. Shannon's information index (I) was maximum in *G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum* (0.444), where as we recorded minimum Shannon's information index in *G. oxylobum* var. *oxylobum* (0.231). The observed number of alleles (*Na*) ranged from 0.22 in *G. oxylobum* var. *oxylobum* to 1.445 in *G. corniculatum* var. *corniculatum*. The significant number of alleles (*Ne*) ranged from 1.029 (*G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum*) to 1.88 (*G. oxylobum* var. *oxylobum*).

Molecular Variance analysis reveals a substantial genetic difference ($p = 0.01$) between Glaucium species. The bulk of genetic diversity was found between species.

Table 2. SRAP primer information and results.

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em1-Me1	10	8	94.31%	0.33	23.77
Em2-Me2	17	17	100.00%	0.26	39.77
Em1-Me4	11	10	96.4%	0.63	20.46
Em2-Me4	22	22	100.00%	0.29	13.76
Em2-Me5	9	9	100.00%	0.34	40.99
Em3-Me4	13	13	100.00%	0.51	12.24
Em3-Me1	26	18	73.00%	0.20	56.55
Em4-Me1	11	11	100.00%	0.14	34.23
Em5-Me1	15	15	100.00%	0.57	48.55
Em5-Me2	7	7	100.00%	0.45	19.65
Mean	15	13	92.00%	0.42	32.25
Total	144	136			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 *Glaucium* species.

SP	N	Na	Ne	I	He	UHe	%P
G. fimbrilligerum	16.000	0.113	1.099	0.292	0.27	0.32	48.23%
G. corniculatum var. *corniculatum*	12.000	1.445	1.190	0.271	0.284	0.292	55.91%
G. oxylobum var. *oxylobum*	12.000	0.228	1.880	0.444	0.40	0.33	66.50%
G. grandiflorum subsp. *grandiflorum* var. *grandiflorum*	10.000	0.288	1.029	0.231	0.17	0.19	44.38%
G. contortuplicatum var. *cantortuplicatum*	15.000	0.772	1.095	0.288	0.35	0.27	62.05%

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

Analysis of Molecular Variance results

AMOVA findings revealed that 77% of the total variation was between species and comparatively less genetic variation was recorded at the species level (Table 4). Genetic difference between *Glaucium* species was highlighted by genetic statistics (Nei's G_{ST}), as evident by significant p values i.e. Nei's G_{ST} (0.699, p = 0.01) and D_est values (0.196, p = 0.01) Because several clustering and ordination approaches yielded comparable findings, NJ clustering is provided here (Figure 4). Plant samples from each species, which belong to a different part, were grouped together and created a single cluster. This finding indicates that the molecular characteristics analyzed may separate *Glaucium* species into two primary clusters or groupings. We found no transitional forms among the specimens analyzed. In general, two large clusters emerged in the NJ tree (Figure 4), populations *G. fimbrilligerum*; *G. contortuplicatum* and *G. oxylobum* were put in the first main cluster and were separated from the other species by a large distance.

The second major cluster included two sub-clusters. Plants of *G. corniculatum* var. *corniculatum* comprised the first sub-cluster, while plants of *G. grandiflorum* subsp. *grandiflorum* var. *grandiflorum* formed the second sub-cluster.

We detected strong correlation between geographical and genetic distances (r = 0.29, p=0.0002) and gene flow ($N_{m)}$ score of 0.388 was reported among species. Detailed information about genetic distances and genetic identity (Nei's) are described (Supplementary Table). The results indicated that *G. oxylobum* var. *oxylobum* and *G. fimbrilligerum* had the greatest degree of genetic similarity (0.88). On the contrary to this, *G. grandiflorum* and *G. oxylobum* var. *oxylobum* (0.63) had lowest genetic resemblance.

To determine the ideal number of genetic groups, we used STRUCTURE analysis followed by the Evanno test. In the species analyzed, we employed the admixture model to show interspecific gene flow or / and ancestrally shared alleles. According to pseudo-F, K-Means clustering yielded k = 5 and BIC yielded k = 3. K = 5 is con-

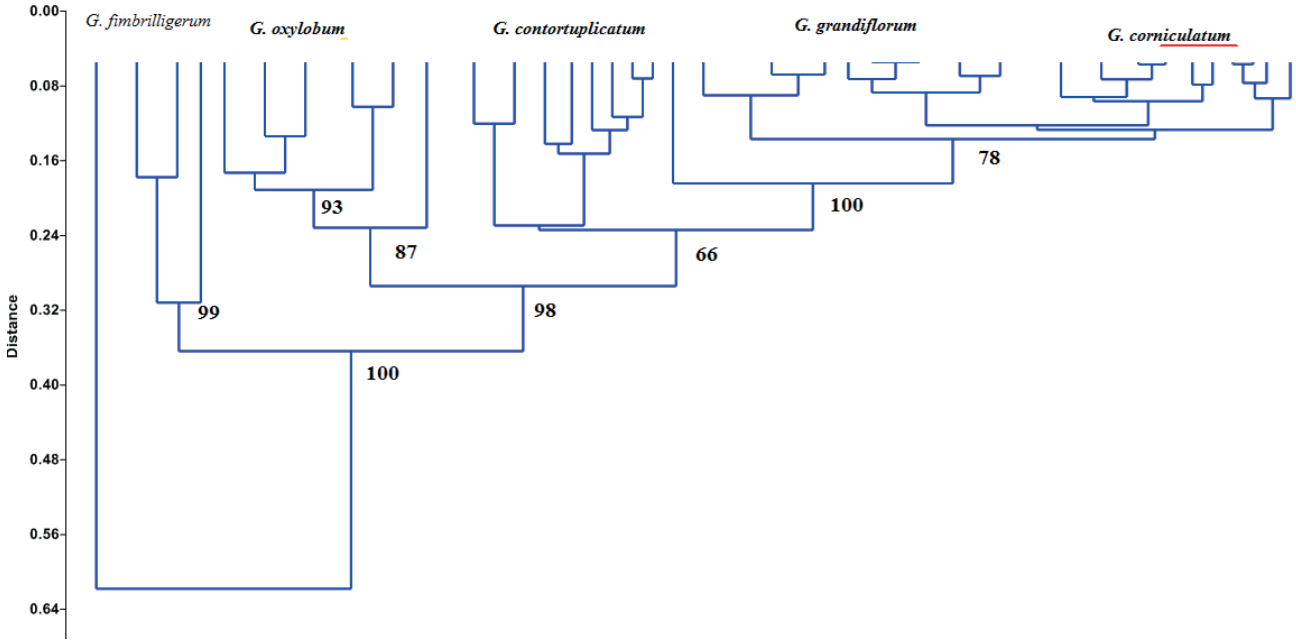

Figure 4. Dendrograms of *Glaucium* species.

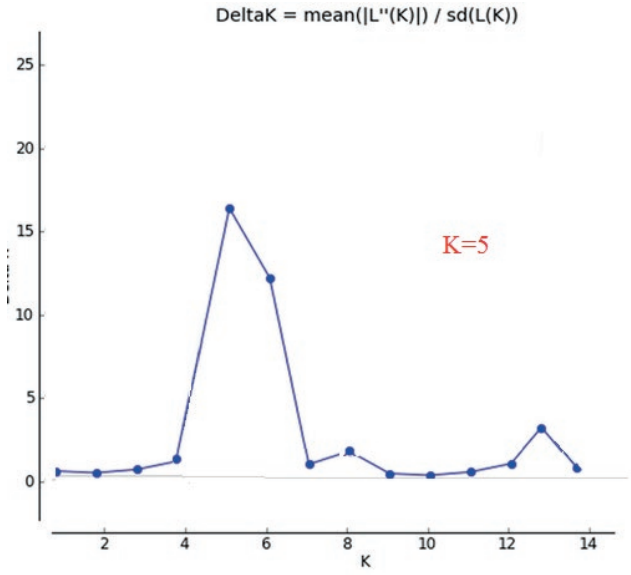

Figure 5. Evanno's test of SRAP data in *Glaucium* populations studied.

sistent with the NJ grouping and AMOVA. K = 5 indicates the existence of five genetic groups. The Evanno test on STRUCTURE analysis yielded a similar result, with a large peak at k = 5. The Organization plot (Fig. 5, 6) revealed further information about the genetic structure of the species analyzed, as well as common ancestral alleles and/or gene flow among *Glaucium* species.

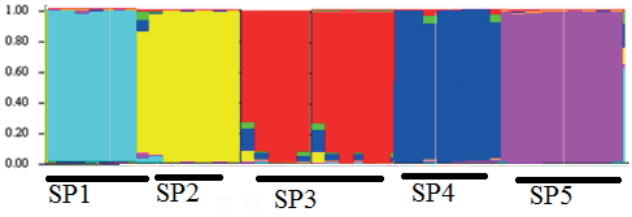

Figure 6. STRUCTURE plot of SRAP data in *Glaucium* populations studied.

This plot demonstrated the genetic difference between species 1 and 2 (which were colored differently), as well as 3 and 4, 5. This is consistent with the Neighbor joining dendrogram that was previously provided. The other species' allele compositions are diverse, and they vary genetically from one another. The low Nm value (0.388) indicates limited gene flow or ancestrally shared alleles between the species studied and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among members of the studied species.

DISCUSSION

We employed morphological and molecular (SRAP) data to determine species relationships in *Glaucium* spe-

Table 4. Molecular variance analysis

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	11	1221.364	88.789	12.164	77%	77%
Within Pops	170	114.443	6.88	5.238	23%	
Total	181	1385.807		17.060	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, ($P < 0.001$).

cies in this work. Morphological analyses of *Glaucium* species showed that quantitative indicators (ANOVA test results) and qualitative characteristics are well differentiated from each other. PCA analysis suggests that morphological characters such as corolla color, pedicel hair, stem hair, leaf hair, petiole hair, width of petal have the potentials to identify and delimitate *Glaucium* species.

Principal component analysis results suggests the utilization of morphological characters to identify and delimitate *Glaucium* species. Morphological characters including corolla color, the pedicel hair, the stem hair, the leaf hair, the petiole hair,width of petal play key role in plant systematics and taxonomy. Our work also highlighted the significance of morphological characters and molecular data to identify and study species genetic diversity. In general, genetic relationships obtained from SRAP data coincides with morphometric results. This is in accordance with the parameters of AMOVA and genetic diversity results. SRAP molecular markers detected clear genetic difference among species. These results indicate that SRAP have potentials to study plant systematics and taxonomy in *Glaucium* members.

Given the negative impact of biodiversity threats and overexploitation of *Glaucium* plant species in Iran, it is necessary to conduct genetic diversity studies on *Glaucium* species. Genetic diversity based studies pave our understanding to develop conservation strategies (Esfandani-Bozchaloyi *et al.* 2017). Genetic diversity studies are conducted through appropriate selection of primers and indexes including Polymorphic information content (PIC) and marker index (MI) are important indexes to fathom genetic variation in species (Hou *et al.,* 2021; Huang *et al*., 2021). Common logic suggests that different makers have different abilities to assess genetic diversity, and usually, genetic diversity is linked with polymorphism (Jia *et al.,* 2020; Karasakal *et al.,* 2020a; 2020b; Khayatnezhad and Gholamin 2020a; 2020b). In this research, we reported PIC values of SRAP primers from 0.14 to 0.63, with a mean value of 0.42. PIC values indeed show low and high genetic diversity among genotypes. Values between zero and 0.25 indicate minimal genetic diversity; values between 0.25 and 0.50 indicate moderate genetic diversity. Additionally, values greater than 0.5 are linked with a high level of genetic diversity (Tams et al. 2005; Wasana et al 2021; Hopla et al 2021; Fikirie et al 2020). Present results highlighted the efficiency of SRAP markers to estimate genetic diversity in *Glaucium* species. In our study, SRAP markers detected average percentage of polymorphism (92%). Additionally, the current study findings indicated the average PIC values of SRAP makers (0.42) and the average RP (resolving power) values of SRAP markers (32.25). Current research results also described average PIC values of SRAP *Glaucium* species have a lot more markers that show how well they're doing now than other species have had (Maria et al. 2007; Dana et al. 2007). These current reported values are higherIn the recent study, low gene flow (N_m) was detected among *Glaucium* species. The present study also depicted a significant correlation between genetic and geographical distances. Our findings revealed that isolation by distance (IBD) existed between *Glaucium* species (Mantet test results). Several mechanisms, such as isolation, local adaptation, and genetic drift, shape the species or population differentiation (Frichot *et al.* 2013; De Kort *et al.* 2014). The amount of variation in Na, Ne, H, and I indices showed that there was a lot of genetic variation in *Glaucium* species.

The magnitude of variability among Dendrogram and principal component analysis results showed clear difference among *Glaucium* species. This shows the high utilization of the SRAP technique to identify *Glaucium* species. Our results have implications for conservation and breeding programs. Furthermore, it may identify suitable ecotypes for forage and pasture. There are two possible explanations for why isolated populations don't have any differences from each other. The first hypothesis said that genetic diversity within and between populations shows how gene flow happens, which led to smaller populations (Dostálek et al., 2010). The second hypothesis is that people who live close to each other are better connected through gene flow than people who live far away.

The morphological, palynological, and phylogenetic features of ten *Glaucium* taxa were studied (Fatma Mungan Kiliç et al., 2019). A total of 10 Although some of the morphological characters of the taxa examined were following the information contained in Flora of Turkey (Cullen 1965), it was noticed that some of their properties were different. In addition, the data yielded from Mory's (1979) study and those yielded as a result of our measurements were compared. In this comparison, the major similarity was observed in terms of the morpho-

logical and palynological characters. In a micromacromorphological study performed by Gran and Sharifnia (2008) of 18 *Glaucium* taxa, the species *G. haussknechtii* has been recognized as synonymous with *G. grandiflorum* based on the analyses of 28 qualitative and 37 quantitative characters. According to Fatma Mungan Kiliç et al (2019) the *Glaucium* taxa were divided into two groups with respect to stem hairs. Taxa with pubescence stems were *G. corniculatum* subsp. *corniculatum* and *G. corniculatum* subsp. *refractum*, *G. grandiflorum* var. *grandiflorum*, *G. grandiflorum* var. *torquatum, G. grandiflorum* var. *haussknechtii* and *G. secmenii,* while the taxa with hairless stems were *G. flavum*, *G. leiocarpum, G. acutidentatum* and *G. cappadocicum*. The findings of phylogenetic analysis revealed that the *Glaucium* taxa were classified into two major clades using matK and ITS3-6 DNA sequences, which is consistent with the hairiness of their stems, petal color, and seed testa outline. The taxa included in these two sub-clades were also compatible with ovary tubercle.

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