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Genetic diversity and relationships among *Glaucium* (Papaveraceae) species by ISSR Markers: A high value medicinal plant

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Abstract. *Glaucium* Mill. (horned poppy), belonging to the family Papaveraceae, is represented by a total of 25 species worldwide, and especially distributed throughout Western, Northern and Eastern Asia, Europe, Northern Africa, and Australia. As a country, Iran harbors relatively more species of the genus *Glaucium* (11-13 species) and hence, this country is considered as the hot spot of the genus. As a result, we conducted a molecular analysis of the data for this genus due to the relevance of these species of plants. We employed 75 plants from seven species and seven provinces that were randomly picked for this investigation. Five primers were used to amplify genomic DNA, yielding 78 bands, 73 of which were polymorphic (97.78%). ISSR primers have a great capability to recognise polymorphic loci among *Glaucium* species, as evidenced by the high average PIC and MI values obtained. The genetic similarity of seven samples was calculated to be between 0.77 and 0.92. *Glaucium corniculatum* var. *corniculatum* and *Glaucium elegans* var. *elegans* showed the lowest similarity, while *Glaucium oxylobum* and *Glaucium grandiflorum* had the highest similarity, according to Inter-Simple sequence repeats (ISSR) markers analysis. The following are the study's goals: 1) Is it possible to identify *Glaucium* species using ISSR markers? 2) In Iran, how are these taxa genetically structured? 3) what is the inter-species relationship? According to this study, ISSR markers can be utilized to distinguish species.

Keywords: Iran, species identification, population structure, *Glaucium* species, ISSR markers.

INTRODUCTION

Having a better understanding of any biological investigations requires determining the exact boundaries of a species. As a result, in the context of biology, species delimitation is a topic that receives a lot of attention (Collard & Mackill 2009, Wu *et al.* 2013; Esfandani-Bozchaloyi *et al.* 2018a, 2018b, 2018c, 2018d; Pandey *et al.* 2008). Additionally, the research of intra-specific

levels of genetic diversity and the examination of genetic sequence of wild populations are essential for the development of effective conservation methods (Fujita *et al*., 2012; Hendrixson *et al*., 2013; Mckay *et al*., 2013). Chelidoniodeae Ernest, Eschscholzioideae Ernest, Papaveroideae Ernest, and Platystemonoideae Ernest were the four subfamilies of the Papaveraceae s. str (Ernest 1962-Kadereit 1993).

Later on, Kadereit *et al.* (1994) included the subfamily of Platystemonoideae in Papaveroideae as well. *Glaucium* is a genus belonging to Papaveraceae subfam. Chelidonoideae Ernest that contains about 23 species (Kadereit 1993). Fedde (1909) listed 20 species, ten varieties, and one subvariety, but Boissier (1867) only approved 12 species. Mory (1979) divided the genus into two segments based on fruit dehiscence, morphological and structural characteristics of leaves, stems, seeds, and pollen grains: *G.* sect. *Acropetal* Mory, with four species having acropetal dehiscence, and *G.* sect. *Glaucium*, with 18 species having basipetal dehiscence. The genus can be discovered in both dry and wet environments throughout Europe's Atlantic coasts and the Canary Islands, as well as Mongolia's Altai (Mory 1979). (Kadereit 1993).

In Iran, it was represented by 11 (Cullen 1966) to 13 (Mobayen 1985; Gran and Sharifnia 2008) species, of these, five are endemics: *G. calycinum* Boiss., *G. contortuplicatum* Boiss., *G. elegantissimum* Mobayen, *G. mathiolifolium* Mobayen and *G. golestanicum* Gran & Sharifnia.

Several taxonomic investigations have demonstrated that seed and trichome micromorphology can be used to classify and delimitate taxa at all taxonomic levels and even across plant families (Barthlott 1981, Krak and Mraz 2008, Salmaki *et al.* 2009, Satil *et al.* 2011, Salimi Moghadam *et al.* 2015, Tavakkoli and Assadi 2016, Arabi *et al.* 2017). Gran and Sharifnia also researched the seed ornaments of 14 *Glaucium* taxa in Iran (2008).

Light microscopy (LM) and scanning electron microscopy (SEM) were used to examine the seeds and trichomes of 15 *Glaucium* taxa found in Iran (Tavakkoli and Assadi, 2019). The seeds are semicircular to reniform in shape, however reniform and extended reniform seeds have been seen in *G. oxylobum* and *G. elegans*, respectively. The sculpturing of the testa surface are verrucate–rugulate (most frequent type), verrucate–granulate, verrucate–perforate, verrucate–lineolate, rugulate–granulate, rugulate and ocellate. Their findings reveal that seed and ovary trichome micromorphological traits give helpful and critical information for separating species and taxa within species, as well as a diagnostic key for the taxa.

Glaucium taxa were researched in terms of their morphological, palynological, and phylogenetical characteristics, according to Fatma Mungan Kiliç et al (2019). Several of these characteristics differ between taxa, particularly in micromorphology and the establishment of clades in phylogenetic trees based on matK and ITS3-6 DNA sequence data, according to their findings.

The genus *Glaucium* of Turkey was separated into subsections Glabrousae and Pubescentae based on the results of DNA investigations and morphological data (stem trichomes). For researching genetic diversity, molecular markers are a useful tool. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers are among the most commonly utilized advanced genetic markers for diversification assessments (Pharmawati *et al.* 2004). The RAPD method is quick and easy to use, and it doesn't need any clear insight of sequences. Using a single primer of any nucleotide sequence, the approach detects nucleotide sequence polymorphism (Moreno *et al.*, 1998). A single 16-18 bp. long primer consists of a repeating sequence attached at the 3' or 5' end of 2-4 arbitrary nucleotides is used to amplify DNA for ISSR markers. The method is faster, easier, less expensive, and more repeatable than RAPD (Esfandani-Bozchaloyi *et al.* 2017a, 2017b, 2017c, 2017d; Collard & Mackill 2009, Wu *et al.* 2013). The current study used new gene-targeted molecular markers, namely ISSR markers, to assess the genetic diversity and relationships among different *Glaucium* species. Because this is the first research of ISSR markers in the *Glaucium* genus, we conducted a molecular analysis on 75 collected specimens from seven *Glaucium* species. We attempt to respond to the following questions: 1) Does the researched species have infraspecific and interspecific genetic diversity? 2) Is there a link between genetic distance and geographical distance among these species? 3) How do populations and taxa differ genetically? 4) Does the *Glaucium* genus exchange genes with other *Glaucium* species in Iran?

MATERIALS AND METHODS

Plant materials

During the months of July to August 2016, 75 individuals representing seven geographical populations of *Glaucium* species were sampled in the Iranian provinces of Lorestan, Guilan, Mazandaran, Esfahan, Golestan, Hamadan, and Kohgiluyeh, as well as Boyer-Ahmad (Table 1).

75 plant accessions (eight to thirteen samples from each population) were collected from seven distinct pop-

Table 1. Voucher details of *Glaucium* species in this study from Iran.

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*G. corniculatum* var. *corniculatum* (L.) Curtis	Kohgiluyeh and Boyer-Ahmad	38°52'37"	47°23'92"	1144
Sp2	*G. elegans* var. *elegans* Fisch. & C.A.Mey.	Mazandaran, Haraz road, Emam Zad-e-Hashem	32°50'03"	51°24'28"	1990
Sp3	*G. oxylobum* var. *oxylobum* Boiss. & Buhse	Guilan, Sangar, Road sid	29°20'07"	51°52'08"	1610
Sp4	*G. flavum* var. *serpieri* (Heldr.) Halácsy	Esfahan:, Ghameshlou, Sanjab	38°52'373	47°23'92"	1144
Sp5	*G. fimbrilligerum* Boiss.	Lorestan, Oshtorankuh, above Tihun village	33°57'12"	47°57'32"	2500
Sp6	*G. contortuplicatum* var. *cantortuplicatum* Boiss.	Golestan, gorgan	34°52'373	48°23'92"	2200
Sp7	*G. grandiflorum* Boiss. & A.Huet	Hamedan, Nahavand	38°52'373	47°23'92"	1144

Figure. 1. Map of Iran shows the collection sites and provinces where *Glaucium* species were obtained for this study.

ulations of different eco-geographic features and stored in -20 until used for ISSR analysis. Table 1 and Fig. 1 provide more information on the geographical distribution of accessions.

Morphological studies

Morphometry was performed on eight to thirteen samples from each species. A total of 36 morphological features (13 qualitative, 23 quantitative) were investigated. The data was normalized (Mean=0, variance=1) and used to calculate Euclidean distance for clustering and ordination analysis (Podani 2000). Corolla form, bract shape, calyx shape, calyx length, calyx width, calyx apex, calyx margins, bract length, corolla length, corolla width, corolla apex, leaf length and width, leaf apex, leaf margins, leaf shape, leaf gland, and bract margins are among the morphological features analyzed.

DNA extraction and ISSR assay

Young leaves were utilized at random from one to twelve plants in each of the populations studied. Silica gel powder was used to dry them. To extract genomic DNA, the CTAB activated charcoal procedure was applied (Esfandani-Bozchaloyi *et al.* 2019). The purity of the extracted DNA was tested using an 8% agarose gel. 22 primers from the UBC (University of British Columbia) series were evaluated for DNA amplification for the ISSR study. Based on band reproducibility, ten primers were chosen for ISSR study of genetic diversity (Table 2).

PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany).

The reactions and amplifications were carried out in a Techne thermocycler (Germany) using the following program: Initial denaturation at 94°C for 5 minutes, then 40 cycles of 1 minute at 94°C, 1 minute at 52-57°C, and 2 minutes at 72°C. A last extension step of 7-10 minutes at 72°C brought the reaction to a close. Running the amplification results through a 1% agarose gel and staining with ethidium bromide revealed the amplification products. Using a 100-bp molecular size ladder, the fragment size was determined (Fermentas, Germany).

Data analyses

Morphological investigations

First, morphological characters were normalized (Mean = 0, Variance = 1) and utilized to calculate Euclidean distance between taxonomic pairs (Podani 2000).

The UPGMA (Unweighted paired group using average) ordination methods were utilized to group the plant specimens (Podani 2000). ANOVA (analysis of variance) was used to show morphological differences between

groups, while a biplot of PCA (principal components analysis) was employed to determine the most variable morphological features among the populations investigated (Podani 2000). For multivariate statistical analysis of morphological data, Hammer *et al.* (2012) employed PAST version 2.17 (Hammer *et al.* 2012).

Molecular analyses

The ISSR bands were coded as binary characters (presence = 1, absence = 0) and utilized to analyze genetic diversity. To quantify the capacity of each primer to distinguish polymorphic loci among the genotypes, two measures, polymorphism information content (PIC) and marker index (MI), were utilized to assess its discriminatory ability (Powell *et al.* 1996). MI = PIC× EMR is the formula for calculating MI for each primer, where EMR is the product of the number of polymorphic loci per primer (n) and the fraction of polymorphic fragments (β) (Heikrujam *et al.* 2015). For each primer, the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were measured. The number of effective alleles, Nei's gene diversity (H), Shannon information index (I), and percentage of polymorphism (P percent = number of polymorphic loci/number of total loci) were all calculated (Weising *et al*, 2005, Freeland *et al.* 2011).

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

Neighbor Joining (NJ) clustering and Neighbor-Net networking were based on Nei's genetic distance between populations (Freeland *et al.* 2011, Huson & Bryant 2006). The Mantel test was used to see if there was a link between the analyzed populations' geographical and genetic distances (Podani 2000). PAST ver. 2.17 (Hammer *et al.* 2012) and DARwin ver. 5 (2012) software were used to conduct these searches.

For demonstrating genetic differences between the populations, the AMOVA (Analysis of molecular variance) test (with 1000 permutations) was utilized, which was performed in GenAlex 6.4 (Peakall & Smouse 2006). The genetic organization of populations was investigated using the Bayesian-based model STRUCTURE analysis (Pritchard *et al.* 2000) and GenoDive ver. 2's maximum likelihood-based K-Means clustering approach (2013). Data were evaluated as dominating markers for STRUCTURE analysis (Falush *et al.* 2007). By using ΔK value, the Evanno test was run on the STRUCTURE output to identify the right number of K. (Evanno *et al.*, 2005). Two summary statistics, pseudo-F and Bayesian Information Criterion (BIC), give the best fit for k in K-Means clustering (Meirmans, 2012). Gene flow was calculated by (i) using PopGene ver. 1.32 (1997) to calculate Nm, an estimate of gene flow from Gst, as follows: Nm = 0.5(1 - Gst)/Gst. This method takes into account the same amount of gene flow in all populations (Yeh *et al.* 1999).

RESULTS

Species identification and inter-relationship. Morphometry

In quantitative morphological features, ANOVA revealed significant differences (P<0.01) among the samples analyzed. PCA analysis was used to discover the most changeable characteristics among the taxa investigated. The first three factors accounted for more than 75% of the overall variation. Characters like corolla form, calyx shape, calyx length, bract length, and leaf shape had the largest correlation (>0.7) in the first PCA axis, with 33 percent of total variation, whereas leaf apex, corolla length, leaf length, and leaf width influenced PCA axis 2 and 3 accordingly. Because the findings of several clustering and ordination approaches were similar, a PCA plot of morphological features is shown here (Fig. 2). Plant samples from different species were put together and generated various groups in general. This finding indicates that the examined species were divided into various groups based on both quantitative and qualitative morphological characteristics. We found no transitional forms in the specimens that we looked at.

Species identification and genetic diversity

To examine genetic links among *Glaucium* species, five ISSR primers were tested; all of the primers yielded replicable polymorphic bands in all seven *Glaucium* species. Figure 3 depicts the ISSR amplification produced by the ISSR-2, ISSR-4 primer. Seven *Glaucium* species yielded a total of 73 amplified polymorphism bands. The amplified fragments had different size from 100 to 3000 bp. ISSR-3 had the most polymorphic bands (22), whereas ISSR-2 had the fewest (only 7), with an average of 14 polymorphic bands per primer. The average PIC of the 5 ISSR primers was 0.22, ranging from 0.14 (ISSR-3) to 0.29 (ISSR-5). The primers' MI ranged from 2.85 (ISSR-2) to 5.47 (ISSR-5), with an average of 3.7 per primer.

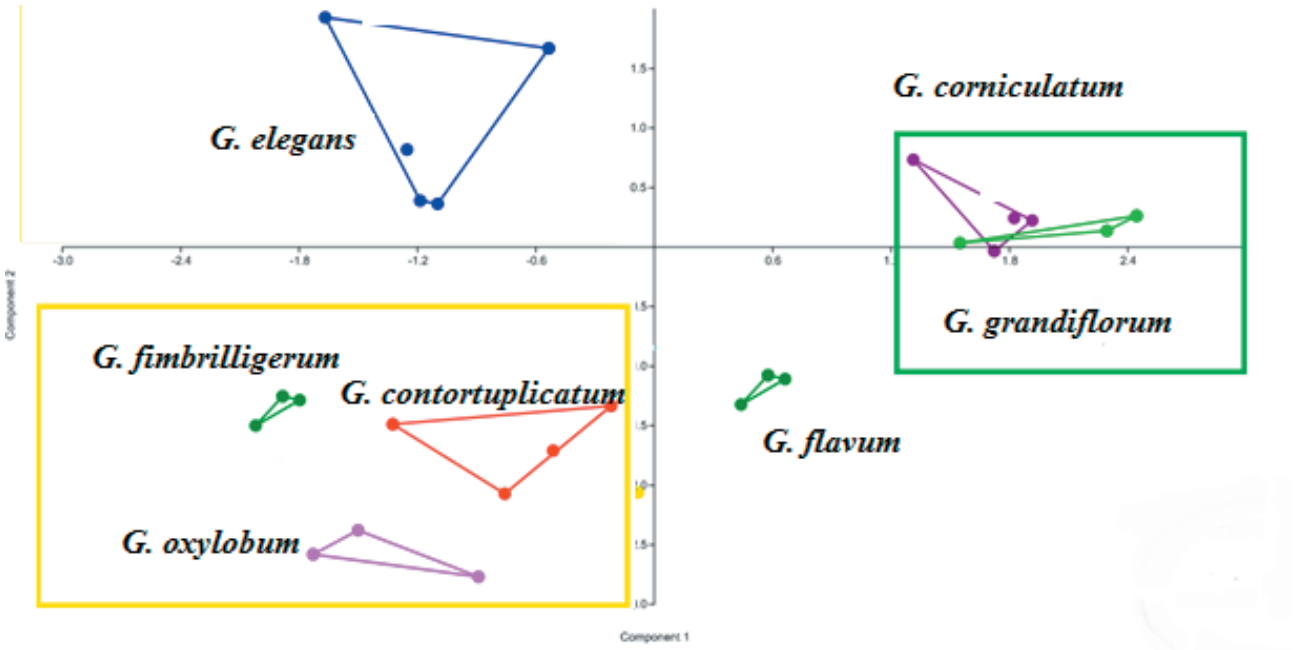

Figure 2. PCA plots of morphological characters revealing species delimitation in the *Glaucium* species.

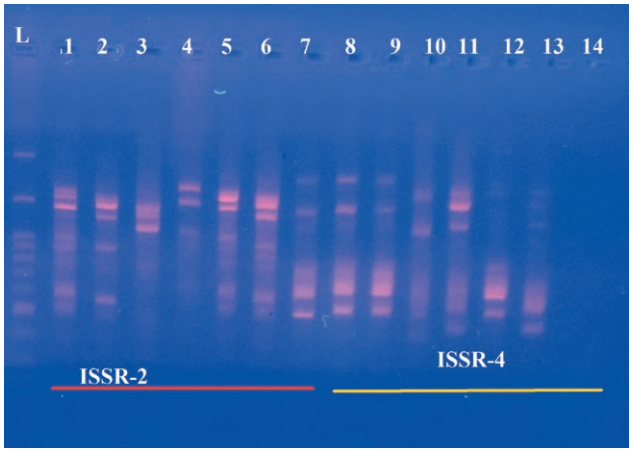

Figure 3. Electrophoresis gel of studied ecotypes from DNA fragments produced by ISSR-2, ISSR-4; 1, 8= *G. corniculatum* var. *corniculatum*; 2, 9= *G. elegans* var. *elegans*; 3, 10= *G. oxylobum*; 4, 11= *G. flavum* var. *serpieri*; 5, 12=; *G. fimbrilligerum;* 6, 13= *G. contortuplicatum*; 7, 14= *G. grandiflorum*

ISSR primers had an EMR ranging from 2.56 (ISSR-4) to 6.23 (ISSR-5), with an average of 4.6 per primer (Table 2). The primers with the highest EMR values were thought to be more useful in separating the genotypes. For all 7 *Glaucium* species amplified with ISSR primers, the genetic parameters were computed (Table 3). Unbiased predicted heterozygosity (H) ranged from 0.10 to 0.30 (*Glaucium corniculatum* var. *corniculatum*), with a mean of 0.21. With a mean of 0.26, Shannon's information index (I) showed a similar pattern, with the maximum value of 0.38 in *Glaucium corniculatum* var. *corniculatum* and the lowest value of 0.15 in *Glaucium contortuplicatum* var. *cantortuplicatum*. *Glaucium oxylobum* var. *oxylobum* has a number of alleles (Na) ranging from 0.261 to 0.667.

The effective number of alleles (*Ne*) ranged from 1.011 (*Glaucium contortuplicatum* var. *cantortuplicatum*) to 1.495 (*Glaucium elegans* var. *elegans*). The AMOVA test revealed a substantial genetic difference (P = 0.001) between the species investigated. It was discovered that 55 percent of overall variance occurred between species and 45 percent occurred within species (Table 4). Furthermore, significant Nei's GST (0.88, P = 0.001) and D est (0.389, P = 0.001) values revealed genetic difference between these species. In comparison to within-species genetic diversity, these findings demonstrated a larger distribution of genetic variety among *Glaucium* species. Because the findings of other clustering and ordination approaches were similar, NJ clustering is reported here (Figure 4). In general, two main clusters appeared in the NJ tree (Figure 4). Populations of *Glaucium fimbrilligerum*, *G. contortuplicatum*, and *G. oxylobum* were put in the first major cluster, separated from the other species by a great distance. Two sub-clusters made up the second major cluster. The first sub-cluster consisted of *Glaucium corniculatum* var. *corniculatum* and *G. grandiflorum* plants, whereas

Table 2. ISSR primers used for this study and the extent of polymorphism.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
ISSR-1	DBDACACACACACACACA	14	14	100.00%	0.22	2.66	3.55	4.45
ISSR-2	GGATGGATGGATGGAT	8	7	84.99%	0.25	4.91	4.43	2.85
ISSR-3	GACAGACAGACAGACA	22	22	100.00%	0.14	5.34	5.55	5.44
ISSR-4	AGAGAGAGAGAGAGAGYT	13	13	100.00%	0.27	2.88	2.56	3.85
ISSR-5	ACACACACACACACACC	12	12	100.00%	0.29	1.23	6.23	5.47
Mean		16	14	97.78%	0.22	3.5	4.6	3.7
Total		78	73					

Note: TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CAAT box- derived polymorphism (CBDP) primers.

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

SP	N	Na	Ne	I	He	UHe	%P
G. corniculatum var. *corniculatum* (L.) Curtis	13.000	0.358	1.380	0.384	0.30	0.31	66.50%
G. elegans var *elegans* Fisch. & C.A.Mey.	8.000	0.299	1.495	0.231	0.18	0.23	44.38%
G. oxylobum var. *oxylobum* Boiss. & Buhse	13.000	0.667	1.062	0.24	0.224	0.213	44.73%
G. flavum var. *serpieri* (Heldr.) Halácsy	8.000	0.499	1.067	0.19	0.181	0.14	49.26%
G. fimbrilligerum Boiss.	9.000	0.261	1.034	0.172	0.13	0.13	33.15%
G. contortuplicatum var. *cantortuplicatum* Boiss.	11.000	0.545	1.011	0.15	0.10	0.10	23.53%
G. grandiflorum Boiss. & A.Huet	13.000	0.352	1.083	0.23	0.22	0.14	45.05%

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

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	48	1201.364	22.789	17.154	55%	55%
Within Pops	50	104.443	1.805	1.888	45%	
Total	98	1355.807		19.060	100%	

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

the second sub-cluster consisted of *G. flavum* var. *serpieri* and *G. elegans* var. *elegans* plants. In general, ISSR data aligns well with morphological data in terms of species relationships. This is in line with the AMOVA and genetic diversity factors discussed previously. The species are genetically distinct. These findings show that ISSR molecular markers can be utilized to classify *Glaucium* species. The Nm analysis by Popgene software also produced mean Nm= 0.768, that is considered very low value of gene flow among the studied species. Isolation by distance (IBD) occurred among the *Glaucium* species tested, as the Mantel test with 5000 permutations revealed a substantial correlation ($r = 0.87$, $p=0.0002$) between genetic distance and geographical distance. The genetic identity of Nei and the genetic distance between the species examined (Table not included). *Glaucium corniculatum* var. *corniculatum* and *G. elegans* var. *elegans* had the highest degree of genetic similarity (0.92), according to the findings. Between *G. oxylobum* and *G. grandiflorum*, there was the least genetic resemblance (0.77). The low Nm value (0.768) indicates minimal gene flow or ancestrally shared alleles between the species investigated, as well as considerable genetic divergence between and within *Glaucium* species. The $\Delta K = 6$ was obtained by STRUCTURE analysis and the Evanno test. The Organization plot (Figure 5) revealed further details regarding the genetic structure of the species investigated, as well as common ancestral alleles and/or gene flow between *Glaucium* species. Due to shared common alleles, this plot demonstrated genetic affinity between *G. corniculatum* var. *corniculatum* and *G. grandiflorum* (similarly colored, No. 1, 7) and *G. fimbrilligerum* and *G. contort-*

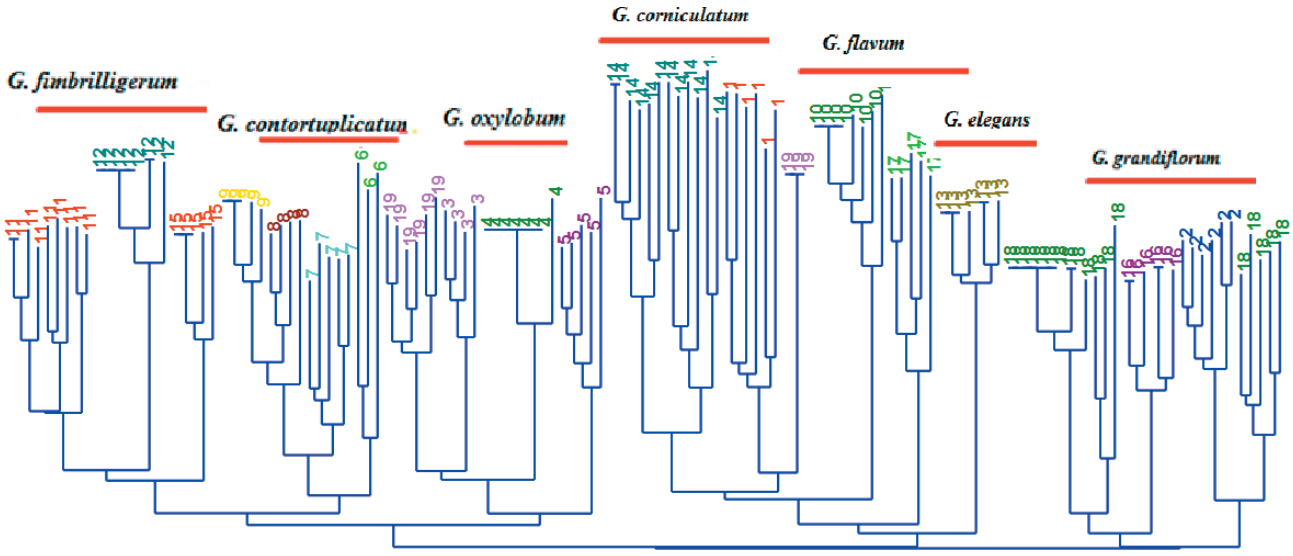

Figure 4. UPGMA tree of ISSR data revealing species delimitation in the *Glaucium* species.

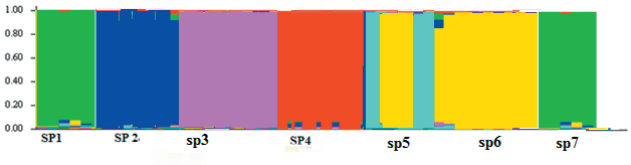

Figure 5. STRUCTURE plot of *Glaucium* species based on ISSR data. 1= *G. corniculatum* var. *corniculatum*; 2= *G. elegans* var. *elegans*; 3= *G. oxylobum*; 4= *G. flavum* var. *serpieri*; 5=; *G. fimbrilligerum;* 6= *G. contortuplicatum*; 7= *G. grandiflorum.*

uplicatum (sp No. 5,6). This aligns with the NJ dendrogram that was previously displayed. The allele compositions of the other species are different.

DISCUSSION

In the biology of long-term evolution of a taxon or population, genetic diversity plays a significant role. The foundation for a taxon's existence, expansion, and evolution. To recognize the taxonomy, origin, and evolution of a taxon, it is necessary to investigate its genetic diversity. Furthermore, such research will provide a theoretical foundation for the conservation, development, use, and breeding of germplasm resources (Lubbers *et al.*, 1991; Ma, *et al.*, 2021; Peng, et al 2021; Jia, *et al.*, 2020; Karasakal, *et al.*, 2020a; 2020b). The recent study discovered fascinating information about genetic divergence, genetic differentiation, and physical differences in Iran's north and west. The degree of genetic diversity inside a species is intimately linked to its breeding technique; the higher the percentage of open pollination/cross breeding, the higher the level of genetic divergence in the clade under investigation (Meusel *et al.*, 1965). A primer's PIC and MI features aid in establishing its efficacy in genetic diversity analysis. According to Sivaprakash *et al.* (2004), the level of polymorphism may be more directly related to an indicator technique's ability to address level of genetic diversity. PIC values of zero to 0.25 indicate very low genetic variation among genotypes, 0.25 to 0.50 indicate a mid-level of genetic diversity, and 0.50 indicate a high level of genetic diversity (Tams *et al.*, 2005; Si *et al.*, 2020; Sun *et al.*, 2021). The PIC values of the ISSR primers in this study ranged from 0.14 to 0.29, with a mean value of 0.22, indicating that ISSR primers had a mid-level ability to determine genetic diversity among *Glaucium* species. In the *Glaucium* taxon, all five primer pairs demonstrated good polymorphism. For the species under investigation, a total of 78 alleles were discovered. The total number of polymorphic bands per primer ranged from 8 to 22, and the average allele number in loci was 16.

In most studies, population size is limited to several vegetative accession (Meusel *et al.,* 1965; Uotila, 1996).

This population may have experienced genetic drift, as evidenced by the high degree of FIS and minimal genetic diversity.

The isolation of the population and absence the gene flow led to fragmentation of the *Glaucium* populations. Between genetic diversity parameters and population size were showing positive correlations that confirmed various studies (Leimu *et al.* 2006). The positive association across genetic diversity and size of population

can be explained in two ways (Leimu *et al*., 2006). 1- A positive connection may indicate the existence of an extinction vortex, in which a decrease in population size reduces genetic variety, resulting in inbreeding depression. Plant fitness separates populations depending on habitat quality changes, which is the second cause (Vergeer *et al*., 2003).

Low genetic variety, according to Booy *et al.* (2000), can impair plant fitness and limit a population's capabilities to react to changes in environmental conditions by selection and adaptation. Within populations, 45 percent of genetic variety was achieved, while 55 percent of genetic variance was gained among the assessed groups. The reproductive system in plant species is important member of the primary elements controlling the distribution of genetic variation (Duminil, 2007). Couvet (Booy *et al*., 2000) found that one migrant per generation is insufficient to maintain the long-term existence of small populations, but also that the numbers of immigrants is governed by phenotypic traits and population genetics (Vergeer *et al*., 2003).

Despite the fact that the genetic variations across the three groups were identical, they were statistically meaningful. For the lack of distinctions across isolated groups, there are two explanations. The initial hypothesis proposed that genetic variety within and between populations demonstrates gene flow patterns, resulting in population fragmentation (Dostálek *et al*., 2010). According to the second hypothesis, populations that are geographically close are more clearly related through gene transfer than species that are divided by a great distance.

The morphological, palynological, and phylogenetic parameters of ten *Glaucium* taxa were investigated (Fatma Mungan Kiliç *et al*., 2019). Although several of the morphological attributes of the taxa surveyed were matched with those listed in Cullen's Flora of Turkey (Cullen, 1965), certain properties were revealed to be different. In particular, the results of Mory's (1979) study were compared to those acquired by our methods. In this assessment, the morphological and palynological characteristics were determined to be the most equivalent. Gran and Sharifnia (2008) identified *G. haussknechtii* as homologous with *G. grandiflorum* depending on 28 qualitative and 37 quantitative features in a micromacromorphological examination of 18 *Glaucium* taxa.

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 results of phylogenetic analyses showed that the *Glaucium* taxa were grouped into two main clades in the ML trees based on the *matK* and ITS3-6 DNA sequences, which is in compatible with the hairness of their stems, petal color and testa outline of the seeds. The taxa included in these two sub-clades were also compatible with ovary tubercle.

Finally, the findings of this study revealed that primers obtained from ISSR were more successful than other molecular markers in determining the genetic diversity of the *Glaucium* genus. In addition, the dendrogram and PCA clearly distinguished *Glaucium* species, demonstrating that the ISSR approach is more effective in identifying *Glaucium* species.

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