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Research Article

Genetic Diversity and Morphometric Analysis in *Hesperis* L. (Brassicaceae) Using SRAP

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Abstract

There are 46 species of *Hesperis* plant around the world, 6 of these species grow in Iran. Humans have used some of the *Hesperis* species for their beneficial aspects over centuries. These species are mainly found in damp areas, specifically in the phytogeographic region of Euro-Siberian. As stated by Busch, Central Asia as well as the Mediterranean region are considered the origin location of the *Hesperis*. Their genetic diversity is evaluated by the Sequence-related amplified polymorphism (SRAP). Their genetic diversity and characteristics are also examined by considering their morphology and acquired molecular data. 65 specimens of 4 *Hesperis* species from 5 provinces have been gathered. The PCR (Polymerase Chain Reaction) technique employing 5 selective primers has been performed on the four mentioned *Hesperis* species, resulting in 85 DNA bands (total loci number), with each species producing between 10 to 25 amplified fragments. The results of SRAP[1] markers indicated that the least similarity appeared between two *H. straussii* and *H. odorata* species. A remarkable sign of isolation has been observed among these species, according to the results of the Mantel test. Furthermore, the genetic affinity of *Hesperis* taxa can be determined and deciphered by SRAP as well. Moreover, the conservation and biodiversity programs can benefit from these findings, and they also can be utilized in Iran to select an appropriate ecotype for pastures and forages.

Keywords: *Hesperis*; Gene Flow; Genetic Diversity; Morphometric Analysis; Sequence-related amplified polymorphism

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1. Introduction

Hesperis L. genus grows in the temperate northern hemisphere, which has a moderate climate, and its range is extended over Southwest Asia, Caucasia, South, and Central Europe, as well as West China, and mountainous regions of Mongolia. As reported by Tzvelev, (1959); Dvorak (1980), about 50 taxa of the *Hesperis* genus can be found all around the world. 27 species have been determined in Turkey (Cullen, 1965; Davis et al., 1988; Duran 2005, Duran & Ocak 2005), 14 species in Europe (Ball, 1964a), 5 species in Iraq (Dvorak, 1980), and 3 species in Italy (Pignatti, 1982). In Iran, according to Dvořák (1968), this genus is classified into 11 species, whereas, Assadi & al. (2017) report that there are only 6 species of them. The mentioned species belong to different sections including, *Hesperis* (Dvořák), *Diaplictos* (Dvořák), and *Pachycarpos* (Fourn). The first attempts at classification of the *Hesperis* species in sectional form were conducted by Andrzejowski (1821),

[1] Sequence-related Amplified polymorphism

whereas, Dvořák (1973) classified them in a subgeneric form, in which certain characteristics of the species, including, palynological, cytological, and morphological were employed. Taxonomists still continue to provide novel infrageneric classifications (Duran & al. 2003; Duran, 2009). Some specific *Hesperis* species have benefited people for centuries (Duran et al., 2003; 2009). Therefore, these species have been the earlier ones being studied and classified in the botanical history. Taxonomists took their hair features as the key morphological characteristic to study. It is worth mentioning that hair features are considered the only difference among certain species. Nevertheless, the environmental conditions affect this characteristic leading to variations. As a result, such characteristics shouldn't be taken into account as the main basis of identification and classification, yet they can be considered as extra information in cytogenetic, palynologic, and molecular studies.

Different areas in Turkey have been considered for collecting six species of the *Hesperis* genus, which were then analyzed for their phylogenetic relationships among supraspecific, specific, and infraspecific classifications using RAPD analysis (Aras et al., 2009). Both morphological features and the results obtained from the RAPD analysis indicate that the classification of certain species, including *H. pendula* (Sect. *Pachycarpos*), *H. schischkinii* (Sect. *Mediterranea*), *H. cappadocica* (Sect. *Contorta*), *H. bicuspidata* (Sect. *Hesperis*), and *H. breviscapa kotschyi* (*Cvelevia* Section), should be distinct. The evolutionary relationships across the Cruciferae (Brassicaceae) family have been understood through pollen morphology studies (Brochmann, 1992).

For the purpose of studying the pollen features as well as the micro- and macromorphological characteristics of Iranian taxa's seeds, both Light Microscope (LM) and Scanning Electron Microscope (SEM) have been used for the first time; the studied taxa included three groups, sects. *Diaplictos*, *Hesperis*, and *Pachycarpos* (Eslami Farouji et al., 2018). *Hesperis* has not yet been subject to global molecular phylogenetic investigations. Nowadays, as a result of parallelism and convergence in morphology, older classification models are not consistent with modern biosystematics studies in certain members within the Brassicaceae family.

Therefore, the determination of every species' actual taxonomic placement has become more challenging (Franzke & al. 2011). Iran has the second-highest diversity of *Hesperis* plants after Turkey (Assadi & al. 2017); although these plants have been investigated both at the infraspecific and infrageneric levels, challenges in their taxonomic classifications still remain (Eslami Farouji et al., 2018, unpublished).

This study has focused on four *Hesperis* species' molecular variations, which are found in Iran. The aims of this study are as follows: 1) assessing the genetic diversity of the under-study species, and 2) employing the NJ algorithm for examining relationships among the populations. The aforementioned analyses' results can considerably improve both breeding and conservation strategies.

2. Materials and Methods

2.1. Plants collection:

A total of 65 plant specimens were collected for this study. Table 1 presents the voucher of four *Hesperis* specimens collected from various provinces of Iran including Kohgilouye-Boirahmad, East Azerbaijan, Guilan, Kermanshah, and Lorestan between May and August over the years 2014 to 2020. 65 plant samples have been analyzed employing SRAP and morphometric analyses. For each population of four distinct species, 5 to 12 specimens were chosen considering the eco-geographic factors. These specimens were kept at -20 °C until their future usage. Table 1 also provides further details on these samples' geographical distribution and locations.

2.2. Morphological studies

12 specimens of each species have been analyzed by morphometric analysis. Accordingly, morphological features in both qualitative (10) and quantitative (11) ways have been investigated. Before calculation, the transformation was performed on the data. These species have been also examined for their various morphological characteristics, including their seeds, flowers, and leaves. Both ordination analyses along with Euclidean distance have been employed (Podani, 2000).

2.3. Sequence-related amplified polymorphism method

In this study, researchers randomly selected one to twelve plants and collected fresh leaves from them. Afterward, for the purpose of drying these leaves, they employed silica gel. The aforementioned protocol has been employed in order to extract the genomic DNA (Esfandani-Bozchaloyi et al. 2019). Subsequently, the SRAP assay was executed in accordance with the previous statement (Li and Quiros 2001). Table 2 presents five distinct primers used for the SRAP technique. The PCR technique was conducted with a total volume of 25µl reaction mixture, which contains 50 mM KCL, 0.2 µM of single primer, 3 U of Taq DNA polymerase, 10 mM of Tris-HCl buffer at pH 8, 20 ng of genomic DNA (Bioron, Germany), 0.2 mM of each dNTP (Bioron, Germany), and 1.5 mM of MgCl2. The Techne thermocycler (Germany) was employed to perform the mentioned PCR reaction.

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

Sp.	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
H. straussii Bornm.	Kermanshah, Kuh-e Bimar near Hukani village, Kerend,	34 ˚ 52'393"	46 ˚ 25' 92"	1133	HIAU 201677
	Kermanshah, Islamabad	34 ˚ 52'353	46 ˚ 27 92"	1143	HIAU 201678
H. hyrcana Bornm. & Gauba	Kohgilouye-Boirahmad, Fahlian,	30 ˚ 52'353	51 ˚ 27' 92"	1750	HIAU 201680
	East Azerbaijan, Kaleybar, Road side	38 ˚ 52'373	47 ˚ 23' 92"	1144	HIAU 201683
	Guilan, Gole rodbar, Road sid	37 ˚ 52'353"	49 ˚ 27' 92"	1143	HIAU 201684
	East Azerbaijan, Kaleybar, Shojabad	38 ˚ 52'393"	47 ˚ 25' 92"	1137	HIAU 201685
H. luristanica F. Dvořák	Lorestan, after Nojian, Wark waterfall	33 ˚ 52'353	48 ˚ 27' 92"	1330	HIAU 201686
	Lorestan, Khoramabad	33 ˚ 09' 55"	48 ˚ 55' 49 "	1450	HIAU 201687
	Lorestan, Azna	33 ˚ 09' 45"	48 ˚ 55' 39 "	1300	HIAU 201688
H. odorata F. Dvořák	Kermanshah, Parrou Mountain	34 ˚ 09' 55"	47 ˚ 55' 49 "	1600	HIAU 201689

Table 2. Information about SRAP and their results

Primer name	NTL[a]	NPL[b]	P[c]	PIC[d]	RP[e]
Em1-Me1	20	14	84.36%	0.55	44.77
Em2-Me2	10	10	100.00%	0.36	39.11
Em1-Me4	14	10	76.4%	0.45	47.26
Em2-Me4	25	25	100.00%	0.22	33.70
Em2-Me5	19	19	100.00%	0.30	40.91
Mean	16	15	92.00%	0.39	42.11
Total	85	78			212.90

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)

Firstly, in the initial step of the PCR process, denaturation, DNA samples were heated at 94°C for 5 minutes. Afterward, 40 cycles were executed, during which the samples went through 1 minute at 94°C, 1 minute at 52-57°C, and 2 minutes at 72°C. Lastly, at 72°C for 7-10 minutes, the last step of the PCR process, extension, was performed. Ethidium bromide was used to stain the samples. The sizes of DNA bands were estimated by comparing them to a 100 bp DNA ladder (Fermentas, Germany).

3. Data Analysis

The morphological traits were studied using the UPGMA[2] ordination approach. Accordingly, the morphological variations across the under-study species were evaluated by the ANOVA[3] test.

The differing morphological aspects among these species were recognized through the PCA[4]. Numerous multivariate statistical analyses, such as PC analysis have been performed employing the PAST software version 2.17 (Hammer et al., 2001).

3.1. Molecular analysis

The bands generated by the SRAP have been observed and recorded. If the bands are present, they get a score of 1, and if absent, they get a score of 0. The calculation was performed to determine every primer's NPL (number of polymorphism loci) as well as their NTL (total loci). Additionally, NPL/NTL was used to calculate the polymorphic ratio.

The concept of PIC has been introduced by Roldan-Ruiz et al. (2000). Rp (resolving power) of each marker is computed by Rp = ΣIb, where the Ib[5] is acquired using this formula: Ib= 1 - [2 x (0.5-p)]. P refers to the presence of bands in this formula (Prevost and Wilkinson, 1999). In order to determine the genetic affinity among these species, the pairwise genetic similarity between them has been assessed (Jaccard, 1908).

Peakall and Smouse, (2006) used GenAlEx 6.4 software was used to assess both Shannon's information index and unbiased expected heterozygosity. The gene flow between the under-study species has been evaluated using POPGENE software, ver.1.32 (Yeh et al., 1999).

[2] Unweighted paired group using average
[3] Analysis of variance
[4] Principal component analysis
[5] band informativeness

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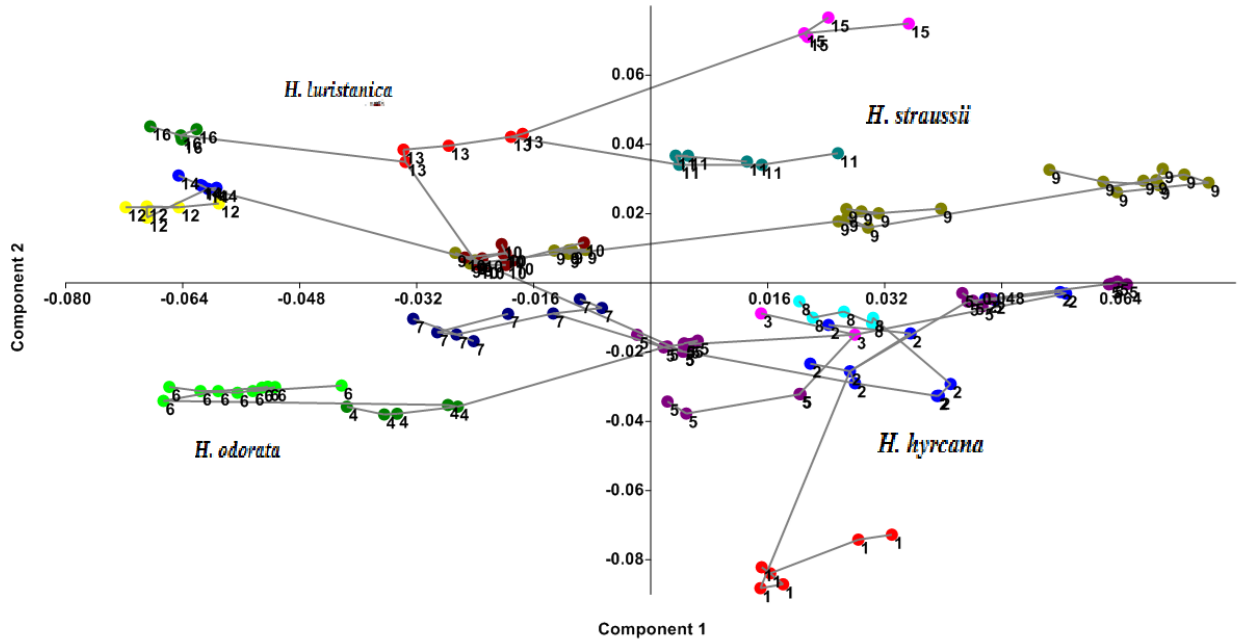

Figure 1. Analyzing the morphological characteristics of *Hesperis* species with a PCoA plot

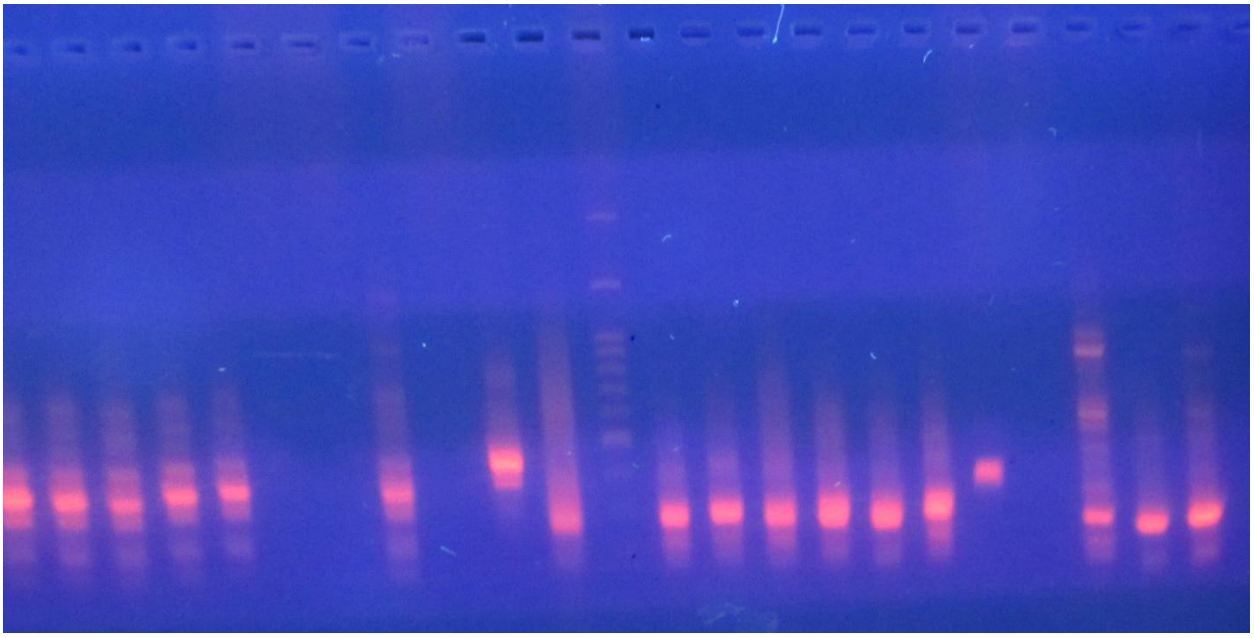

Figure 2. Electrophoresis gel of studied ecotypes from DNA fragments produced by SRAP profile; 1,5,9: *H. straussii*; 2, 6,10: *H. hyrcana*; 3,7,11: *H. luristanica;* 4, 8,12: *H. odorata*

The AMOVA test has been executed employing GenAlEx software (Peakall and Smouse 2006). PAST software ver. 2.17 was used to perform the Mantel test, with 5000 permutations (Hammer et al. 2001).

4. Findings

4.1. Morphometry

The quantitative morphology among the species was considerably different ($p<0.01$), as indicated by the ANOVA results. The cumulative variation explained by the PCA results was 62%. Accordingly, more than half of the total variations (57%) were captured by the first axis (PC1). It was observed that certain morphological traits, like calyx width, calyx length, corolla color, and corolla length, demonstrated a high correlation (>0.7). Fig. 1 provides a PCoA plot that has analyzed the *Hesperis* species' morphological traits. The differences in the morphological traits of *Hesperis* species were taken into account for classification purposes, and these differences were revealed through morphometric analysis, which led to their distinct classification.

3.2. Species identification and genetic diversity

For this study, 15 PCs were tested and 5 of them were found to be appropriate.

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Table 3. Genetic diversity parameters of the under-study *Hesperis* species

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

SP	N	Na	Ne	I	He	UHe	%P
H. straussii	20.000	0.113	1.099	0.222	0.27	0.32	38.23%
H. hyrcana	17.000	1.222	1.190	0.211	0.284	0.292	25.91%
H. luristanica	12.000	0.228	1.180	0.414	0.42	0.39	46.50%
H. odorata	15.000	0.288	1.011	0.131	0.12	0.15	14.78%

Table 4. Molecular variance analysis results

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	18	1456.364	80.711	20.144	70%	-
Within Pops	111	100.443	12.82	15.118	30%	70%
Total	130	1556.807	-	35.065	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, ($P < 0.001$)

The SRAP technique employed three primers, namely, Em2-Me4, Em3-Me1, and Em5-Me1, to generate the banding patterns of species, as shown in Fig. 2. This analysis led to the production of 78 amplified polymorphic bands that ranged from 150bp to 3000bp. The Em2-Me2 primer produced 10 polymorphic bands, which was the lowest number, whereas the Em2-Me4 produced 25, being the highest number. The average number of polymorphic bands generated by each primer was 15. Additionally, the average PIC amount for each of the 5 SRAP primers was 0.39, where the Em2-Me4 had the lowest value (0.22) and the Em1-Me1 had the highest (0.55). Moreover, as indicated by Fig.2 and Table 2, the average resolving power (RP) for each of these primers was 42.11, where the least amount belonged to EM2-Me4 with a value of 33.70 and the highest amount for Em1-Me4 with a value of 47.26. The genetic parameters calculated for the *Hesperis* species have been presented in Table 3. The average unbiased heterozygosity (H) was 0.30, where *H. odorata*, with a value of 0.15, presented the lowest, and *H. luristanica,* with a value of 0.39, demonstrated the highest amounts. The Shannon's information index demonstrated a value of 0.414 for *H. luristanica,* being the highest value, while a value of 0.131, the lowest value, was observed for *H. odorata.* The highest number of observed alleles (Na), with a value of 0.133, was found in *H. straussii*, while the lowest amount (1.222) was observed in *H. hyrcana*. Additionally, the highest number of effective alleles (Ne), with a value of 1.190, was found in *H. hyrcana*, while the lowest amount (1.011) was observed in *H. odorata*.

The *Hesperis* species appeared to exhibit considerable genetic variations ($p = 0.01$) among them, as stated by the results of the AMOVA test. Table 4 provides the results of the AMOVA test, which show a lower amount of genetic variations within these species, while 70% of the variation was observed among them. The *Hesperis* species appeared to be genetically differentiated, which was further supported by the genetic statistics, i.e., D-est values (0.287, $p = 0.01$) and Nei's G_{ST} (0.356, $p = 0.01$). Since the results obtained from different clustering and ordination approaches were similar, Fig. 3 demonstrates the NJ tree clustering results. Specimens from distinct *Hesperis* species were grouped into similar clusters, resulting in the formation of different groups. Therefore, it can be concluded that the under-study *Hesperis* species can be divided into two main distinct clusters or groups based on their molecular traits. The plant specimens we examined did not contain any intermediate forms. Additionally, Fig. 3 also provides the NJ tree grouping the *Hesperis* species into two major clusters. The *H.straussii* appeared clearly distinct from other groups being placed within the first major group. The second major group comprised two sub-clusters: the first included *H. odorata*, while the second one included two species, *H. luristanica,* and *H. hyrcana*.

There was a significant correlation between geographical distances and the genetics of the species (p=0.0002, r = 0.33), indicating isolation by distance. Additionally, it was stated the score of gene flow across them was $N_m = 0.621$. A supplementary Table details Nei's genetic identity and diversity among these species. According to the results, it was found that the most genetic similarity (0.94) was observed between *H. odorata* and *H. luristanica.* However, the least amount (0.72) appeared between *H. straussii* and *H. odorata.*

The Evanno test, following the STRUCTURE analysis, was performed for the purpose of identifying the potential genetic groups. The determination of shared ancestral alleles and/or interspecific gene flow between the under-study species was done employing the admixture model. Two statistical methods, pseudo-F, and BIC, were employed; the k-means clustering analysis suggested k=4 for pseudo-F and k=2 for BIC.

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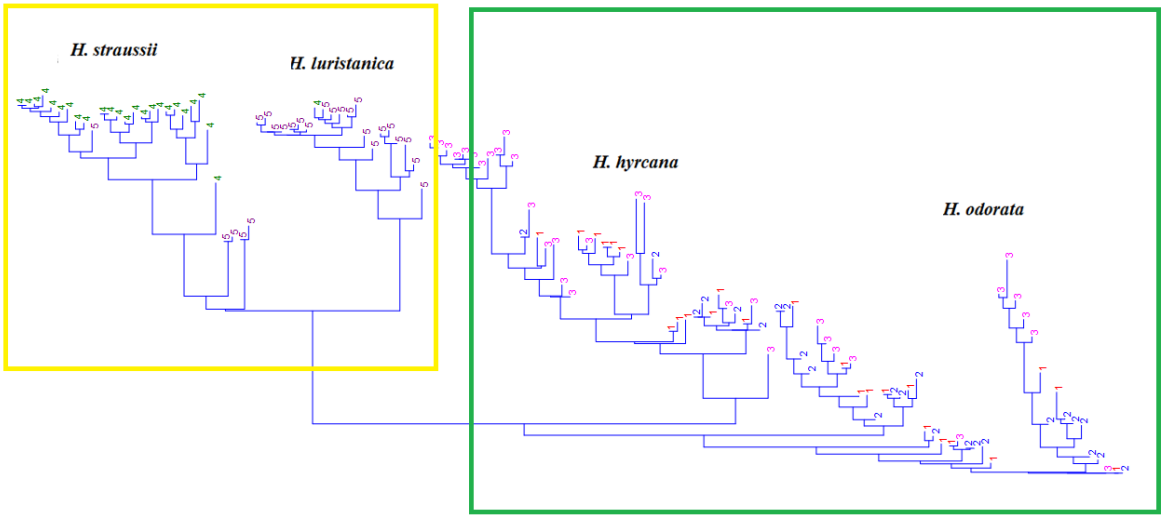

Figure 3. NJ tree of SRAP data revealing species delimitation in the *Hesperis* species

The presence of four genetic clusters is indicated by k=4. The results of both AMOVA and NJ clustering agree with k=4. The results of the Evano test on STRUCTURE analysis also aligned with the previously mentioned evaluations. The STRUCTURE analysis has been employed in order to provide further explanations of the under-study species' genetic structure (Figure not included), as well as their ancestrally shared alleles and/or gene flow. Furthermore, the results of the mentioned analysis demonstrated that species 1 and 2, as well as species 3 and 4, appeared to be genetically different from each other, with each species presented with a distinct color, which aligns with NJ dendrogram results. The remaining species also possess distinct allele combinations and have different genetics as well.

The gene flow among the under-study species appeared to be low as indicated by the low Nm value of 0.621; it also demonstrated that they still share some ancestor alleles. Additionally, the results of K-Means and STRUCTURE analyses aligned with the genetic stratification discussed above. No considerable signs of gene flow were observed in the population assignment test results, which were also in line with the Nm results.

5. Discussion

This study aims to employ the SRAP results (molecular data) of the *Hesperis* species, as well as their morphological features to assess the relations between them. The clear differentiation of the under-study species is confirmed by investigating their morphology, qualitative and quantitative aspects (AMOVA results). For delimitation and identification purposes of these species, their certain morphological aspects, including width of petal, stem hair, leaf hair, petiole hair, pedicel hair, and corolla color, can be used, according to the results of PCA analysis; therefore, it can be stated that these characteristics are of utmost importance in the context of taxonomy and plant systematics. Additionally, for studying genetic diversity, this study also explains that both molecular data and morphological traits are very important. The SRAP results, which present the genetic relations among the species, are generally in line with the results of morphometric analysis. Accordingly, this also aligns with the AMOVA and genetic diversity results. There appeared precise genetic differences between the studied species by the SRAP markers, explaining that these markers can be effectively used for their taxonomy and plant systematics as well.

Scientists must study the genetic diversity of the *Hesperis* species in Iran, considering the fact that they endure a lot of overexploitation and biodiversity threats. The studies on the species' genetic diversity are effective for the development of effective conservation strategies Various tools including proper primers as well as crucial indexes such as marker index[6] and Polymorphic information content[7] have been employed for performing genetic diversity research (Sivaprakash et al. 2004). The concept of varieties in each marker's ability of evaluating genetic diversity is widely accepted; additionally, more genetic diversity is associated with increased polymorphism (Sivaprakash et al. 2004). The PIC values have been assessed for SRAP primers in this study, averaging 0.39, where the lowest value being 0.22 and the highest at 0.55. As stated before the PIC values are used to assess genetic diversity, where low PIC values, 0 to 0.25, suggest that the genetic diversity is low, and values ranging from 0.25 to 0.50 indicate medium genetic diversity, and high PIC values, above 0.5, represent high genetic diversity among the species (Tams et al. 2005). Therefore, the SRAP markers have been proven to be useful tools for the evaluation of *Hesperis* species' genetic diversity. Accordingly, an average of 92% of

[6] MI

[7] PIC

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polymorphisms have been demonstrated employing these SRAP markers, in this research. The average PIC and RP values for SRAP markers were found to be 0.39 and 42.11, respectively. Our results based on the *Hesperis* species appeared to be higher than other experiments' results. Additionally, gene flow (N_m) across *Hesperis* species was observed to be low in this study. The geographical distance influenced the genetic variation of these species. According to the results of the Mantet test, the *Hesperis* species presented isolation by distance (IBD) among them. The differentiation of these species' populations was affected by local adaptation, genetic drift, and isolation, which are significant evolutionary processes (Frichot et al. 2013; De Kort et al. 2014). The *Hesperis* species' genetic diversity was further demonstrated by the high variation in specific genetic markers, i.e., *I*, *Ne*, *Na*, and *H*. Furthermore, the *Hesperis* species were clearly differentiated from one another, in accordance with the results of PCA and Dendrogram as well. The efficiency of the SRAP approach has been proven for *Hesperis* species analysis. Moreover, this study supports breeding and conservation strategies and may assist in finding proper ecotypes for pasture and forage.

The phylogenetic relations across six *Hesperis* taxa (supraspecific, specific, and infraspecific), collected from different parts of Turkey, have been examined using RAPD analysis (Aras et al., 2009). The results of the RAPD analysis support the classification of certain *Hesperis* species, including *H. pendula* (Sect. *Pachycarpos*), *H. schischkinii* (Sect. *Mediterranea), H. bicuspidata* (Sect. *Hesperis*), *H. kotschyi* (Sect. *Cvelevia*), *H. breviscapa,* and *H. cappadocica* (Sect. *Contorta*), into separate clusters; this idea is further consistent with their morphological traits. Nonetheless, the molecular analysis results didn't match their morphology, leading to the remodification of their evolutionary phylogenetic order based on two specimens, *H. kotschyi* and *H. breviscapa,* which were from the same section. Similarities in molecular and morphological traits were discovered for both the *H. breviscapa* and *H. kotschyi* species. Additionally, a specific species, *H. schischkinii,* has been re-evaluated in its infraspecific taxonomic status, employing RAPD analysis, considering the specimens with pubescent and/or glabrous fruits, displaying their allopatric and sympatric distribution.

The absence of distinctions across the characters of the isolated groups is described by two hypotheses, which are: 1) genetic diversity both among and within the members of these species is a result of gene flow, which can fragment the large population into smaller ones (Dostálek *et al.,* 2010); and 2) gene flow mostly occurs between the species that are closely located to each other leading to stronger interactions of them.

Authors Contribution
All the authors have participated sufficiently in the intellectual content, conception, and design of this work or the analysis and interpretation of the data (when applicable), as well as the writing of the manuscript.

Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable. request.

Conflict of interest
The author states that there is no conflict of interest.

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