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Original scientific article

GENE FLOW AND GENETIC STRUCTURE BETWEEN POPULATIONS OF *Hesperis* L. (BRASSICACEAE) SPECIES USING MOLECULAR MARKERS

Kuanhong MENG[1*],Jia YAO[2], CongYing HE[1], Hakimeh MORABBI HERAVI[3]

[1]Ningbo Institue of Oceanography Ningbo, Zhejiang, 315832, China
[2]School of Civil Engineering and Architecture, NingboTech University Ningbo, Zhejiang, 315100, China
[3]Department of Statistics, University of Bojnord, Iran

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Genetic variability and populations' structure were studied in seven geographical populations of *Hesperis* L. Genetic diversity parameters were determined in these populations. 5 of 10 random amplified polymorphic DNA primers produced 62 reproducible bands with average of 7.1 bands per primer and 55% of polymorphism. *Hesperis hyrcana* showed the highest number of effective allele (Ne), Shannon index (I) and genetic diversity (H). The highest values of genetic diversity were obtained in *Hesperis hyrcana*. NJ tree grouped the populations in two different clusters/groups, indicating their genetic difference which is discussed in details. The results of this study showed that the level of genetic variation in *Hesperis* is relatively high. NJ-based dendrogram showed a close relationship between members of *Hesperis straussii* and *Hesperis hyrcana*, while the *Hesperis luristanica* protected population differ the most from the other populations. Principal component analysis, however, showed some minor differences with NJ-based dendrograms.

Keyword: Genetic variability, Gene flow, *Hesperis*; Random Amplified Polymorphic DNA.

INTRODUCTION

One of the most important aspects of biological diversity for conservation strategies is the genetic diversity, particularly in rare, and narrow endemic species (MILLS and SCHWARTZ, 2005). Most authors agree that longstanding evolutionary potential of a species necessitates maintenance of genetic diversity (FALK and HOLSINGER, 1991). Similarly, most geneticists regard

Corresponding author: Kuanhong Meng, Ningbo Institue of Oceanography Ningbo, Zhejiang, 315832, China, E-mail: guxing_ge@163.com

population size as a significant factor in preserving genetic diversity (TURCHETTO *et al.*, 2016). In fragmented populations, this is critical as they are more susceptible due to allelic resources' loss and bigger population differentiation through genetic drift (reduces heterozygosity and subsequent allele fixation) and inbreeding depression (develop homozygosity within populations) (FRANKHAM, 2005; ZHANG *et al.*, 2020; ZHANG *et al.*, 2016; WANG *et al.*, 2020). Understanding of genetic variability and inter-, and intra-population diversity of rare and endemic species is therefore necessary for their conservation and management (e.g. CIRES *et al.*, 2012; 2013).

The genus *Hesperis* L. (Brassicaceae) comprises biennial and perennial herbs (DURAN *et al.*, 2003) and consists of 46 species worldwide (AL-SHEHBAZ *et al.*, 2006), mainly occurring in different parts of Europe, Caucasus, Transcaucasia, and to a lesser extent in northern and central Asia, and mostly in Turkey with 28 species (DURAN, 2008; ARAS *et al.*, 2009). The genus is represented by 11 (DVOŘÁK, 1968) or six species (ASSADI *et al.*, 2017) belonging to sections *Hesperis* Dvořák, *Diaplictos* Dvořák and *Pachycarpos* Fourn. in Iran. The first subgeneric (DVOŘÁK, 1973) and sectional (ANDRZEJOWSKI, 1821) studies of *Hesperis* were evaluated from morphological, cytological, and palynological characters, but still taxonomists try to introduce new infrageneric classifications (DURAN, 2016). *Hesperis* can be readily distinguished from the rest of the Brassicaceae by having stalked glands with uniseriate stalks terminated with a unicellular gland (AL-SHEHBAZ *et al.*, 2006). Among palynological, cytological and trichome characters, DURAN *et al.* (2003) used traditional morphological characters, e.g., life form, stem height, leaf shape, and diverse fruit characters for section classification. The genus was firstly revised by ANDRZEJOWSKI (1821) and he placed the genus in a single section (*Deilosma* Andrz.). Later, the taxonomy of the genus was treated by several researchers and the genus was divided into various sections on the basis of morphological characters; 2 sections (*Hesperidium* DC. and *Deilosma* Andrz.) by DE CANDOLLE (1824), 3 sections (*Hesperidium*, *Deilosma* and *Pachycarpos* Fourn. Emend. Tzvelev) by FOURNIER (1866) and TZVELEV (1959), and 2 sections (sect. *Purpureae* Boiss. and *Lividae* Boiss.) by BOISSIER (1867). DVORAK (1973) separated it into 5 subgenera (*Hesperis*, *Mediterranea* Borbás, *Cvelevia* Dvorak, *Contorta* Dvorak and *Diaplictos* Dvorak). CULLEN (1965) did not divide the species of the genus growing in Turkey into sections. Despite numerous studies on the infrageneric and infraspecific classification of the genus, problems have not been clarified yet (SAVULESCU, 1955). Previous study on species delimitation and species relationships performed in this genus.

According to ARAS *et al.* (2009) the phylogenetic relations among infraspecific, specific and suprespecific categories of 6 taxa of the genus *Hesperis* collected from different parts of Turkey were investigated by RAPD analysis. Their results of the RAPD analysis support the idea that *H. bicuspidata* (Sect. *Hesperis*), *H. schischkinii* (Sect. *Mediterranea*), *H. pendula* (Sect. *Pachycarpos*), *H. brevicapa*, *H. kotschyi* (Sect. *Cvelevia*) and *H. cappadocica* (Sect. *Contorta*) species need to be placed into different sections according to morphological characters.

After Turkey, Iran is the second country in terms of *Hesperis* diversity (ASSADI *et al.*, 2017) and despite several infrageneric and infraspecific studies of *Hesperis*, its taxonomic problems have not been resolved yet. But there are no attempt to study genetic diversity, ecological adaptation and intra- and inter-specific differentiation along with morphometric

studies on *Hesperis* of Iran. Therefore, we performed morphological and molecular study of 60 collected specimens of 3 species in *Hesperis.* Molecular markers are considered an important tool in order to provide data in terms of genetic polymorphisms between different individuals in a population. Molecular markers are commonly used in genetic analysis, fingerprinting, linkage mapping, germplasm characterization, and molecular breeding. RAPD analysis using PCR along with short arbitrary sequence primers has been reported sensitive to detecting variation at level of individuals. The benefits of this method are: a) a large number of samples are tested easily and efficiently using less quantity of material; b) the DNA amplicons are independent of ontogenetic expression; c) several genomic regions may be sampled with likely infinite numbers of markers (SONIYA *et al.*, 2001; ESFANDANI-BOZCHALOYI *et al.*, 2017 a, 2017b, 2017c, 2017d).

MATERIALS and METHODS

Plant sampling

A total of 60 individuals were collected from eco-geographically different populations representing three *Hesperis* species in East Azerbaijan, Lorestan, Kermanshah, Guilan, Provinces of Iran during July–August 2017–2019 (Table 1). All of the samples were used during RAPD and morphometric analysis and stored for further use in -20 °C.

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

Sp.	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
1. *H. straussii* Bornm.	Kermanshah, Kuh-e Bimar near Hukani village, Kerend,	34 ° 52'393"	46 ° 25' 92"	1133	HIAU 201677
	Kermanshah, Islamabad	34 ° 52[illegible]	46 ° 27 92"	1143	HIAU 201678
	East Azerbaijan, Kaleybar, Road	[illegible] 73	47 ° 23' 92"	1144	HIAU 201683
2. *H. hyrcana* Bornm. & Gauba	Guilan, Golrodbar, Road sid	37 ° 52'353"	49 ° 27' 92"	1143	HIAU 201684
	East Azerbaijan, Kaleybar, Shojabad	38 ° 52'393"	47 ° 25' 92"	1137	HIAU 201685
	Lorestan, after Nojian,	33 ° 52'353	48 ° 27' 92"	1330	HIAU 201686
3. *H. luristanica* F. Dvořák	Wark water fall Lorestan, Khoramabad	33 ° 09' 55"	48 ° 55' 49 "	1450	HIAU 201687

Morphological studies

One to twelve samples from each species were used for morphometric analysis. A total of 25 morphological (10 qualitative, 15 quantitative) characters were examined. The obtained data were standardized (Mean= 0, variance= 1) and used to assess Euclidean distance for clustering and ordination analyses (PODANI, 2000).

DNA Extraction and RAPD Assay

In each of the populations studied, fresh leaves from one to twelve plants were used randomly. Leaves were dried with silica gel prior to DNA extraction. In order to obtain genomic DNA, the CTAB-activated charcoal protocol was used (ESFANDANI-BOZCHALOYI *et al.*, 2019). By running on 0.8 percent agarose gel, the quality of extracted DNA was examined. A total of 25 Operon Technology Decamer RAPD Primers (Alameda, Canada) belonging to OPA, OPB, OPC, OPD sets were used. Among them, five primers were selected with simple, enlarged and rich bands of polymorphism (Table 2). PCR reactions were performed in a 25µl volume mixture containing the following component: Tris-HCl buffer (10 mM) at pH 8; KCl (50 mM); $MgCl_2$ (1.5 mM); dNTPs (0.2 mM); primer (0.2 µM); genomic DNA (20 ng) and of *Taq* DNA polymerase (3 U). In Techne thermocycler (Germany), the amplification reactions were carried out with the following PCR settings: 5 min initial denaturation at 94 °C; 40 cycles of 1 min at 94 °C; 1 min at 52-57 °C and 2 min at 72 °C. The reaction was completed by 7–10 min extension at 72 °C. The PCR amplified products were detected by running on 1% agarose gel, preceded by staining with ethidium bromide. The size of fragments was measured using a ladder with a molecular size of 100 bp (Fermentas, Germany).

Data Analyses

Morphological Studies

Morphological characters (Mean = 0, Variance = 1) were first standardized and used to determine the Euclidean distance between taxa pairs (PODANI, 2000). The ordination methods of UPGMA (Unweighted paired group using average) were used for clustering the samples (PODANI, 2000). In order to demonstrate morphological variation between populations, ANOVA (Analysis of variance) was performed, while PCA (Principal Components Analysis) bi-plot was employed to identify the most variable characters (PODANI, 2000). PAST software version 2.17 (HAMMER *et al.*, 2012) was used for multivariate statistical analyses of morphological data.

Molecular Analyses

The obtained RAPD bands were coded as binary characters (absence = 0, presence = 1) and used for the study of genetic diversity. Using two parameters, marker index (MI), and polymorphism information content (PIC), the discriminatory capacity of primers was evaluated to characterize the ability of each primer to identify polymorphic loci among the genotypes (Powell et al. 1996). For each primer, MI was calculated as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (n), and the polymorphic fragment fraction (β) (HEIKRUJAM *et al.*, 2015). For each primer, the number of polymorphic bands (NPB) was determined followed by the effective multiplex ratio (EMR). Other parameters such as Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% = number of polymorphic loci/number of total loci) were also determined (WEIRING *et al.*, 2005; FREELAND *et al.*, 2011). The formula for calculation of Shannon's index was: I = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, where "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. The percentage of polymorphic loci, UHe, H' and PCA were determined by GenAlEx 6.4 software (PEAKALL and SMOUSE, 2006). For generating Neighbor

Joining (NJ) clusters and Neighbor-Net networking, Nei's genetic distance between populations was employed (FREELAND *et al.*, 2011; HUSON and BRYANT, 2006). The Mantel test determined the correlation between the geographical and genetic distances of the populations (PODANI, 2000). These tests were performed in PAST ver. 2.17 (HAMMER *et al.*, 2012) and DARwin software ver. 5 (2012). As implemented in GenAlex 6.4 (PEAKALL and SMOUSE, 2006), the AMOVA (Analysis of Molecular Variance) test (with 1000 permutations) was used to evaluate population genetic differences. Gene flow was estimated by calculating Nm, an estimate of gene flow from Gst in PopGene ver. 1.32 (1997) as: Nm = 0.5 (1 - Gst)/Gst. This method considers the equal amount of gene flow among all populations.

RESULTS

Species identity and relationships

Morphometry

ANOVA test showed substantial differences (P <0.01) between the studied species in quantitative morphological characteristics. PCA analysis was conducted to determine the most variable characters among the taxa analysed. It showed that over 64 % of the overall variance was composed of the first three variables. Characters such as seed shape, calyx shape, calyx length, bract length and basal leaf shape have shown the highest association (>0.7) in the first PCA axis with 43 percent of total variance. Characters affecting PCA axis 2 and 3 respectively were seed colour, leaf surface, corolla length, filament length, basal leaf length. Different ordination and clustering methods generated similar results. Therefore, PCoA plot of morphological characters are presented here (Figs 1). Samples of each species were separately grouped. This finding indicates that the studied species were divided into different classes by both quantitative and qualitative morphological features. Among the studies sample we did not find any intermediate forms.

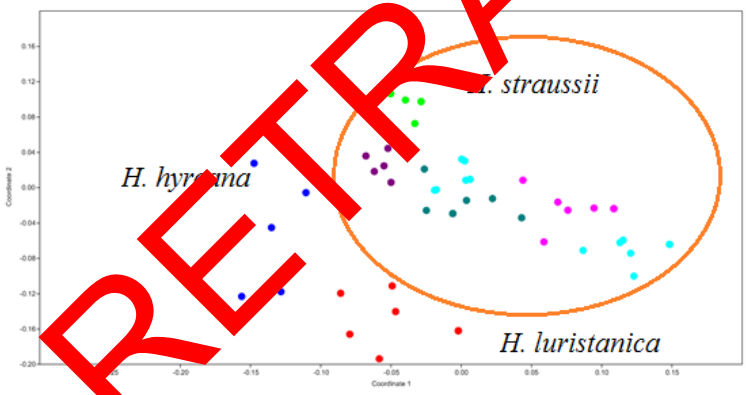

Fig 1. PCoA plots of morphological characters revealing species delimitation in the *Hesperis* species

Species Identification and Genetic Diversity

Five RAPD primers were screened in order to study genetic relationships within *Hesperis*. All primers generated reproducible polymorphic bands in three *Hesperis* species. In total, 62 amplified polymorphic bands were produced across three species. The size range of the amplified fragments was 100 to 3000 bp. The highest and lowest number of polymorphic bands was 18 for OPD-02 and 8 for OPC-04 with an average of 6.2 polymorphic bands per primer. The PIC of the five RAPD primers ranged from 0.43 (OPC-04) to 0.59 (OPB-01) with an average of 0.55 per primer. MI of the primers ranged from 3.25 (OPB-01) to 3.88 (OPC-04) with an average of 3.5 per primer. EMR of the RAPD primers ranged from 4.22 (OPB-01) to 8.56 (OPD-02) with an average of 7.87 per primer (Table 2). The primers with the high EMR values were considered to be more informative in distinguishing the genotypes.

Genetic parameters were determined for all the 3 *Hesperis* species amplified with RAPD primers (Table 3). The range of Unbiased expected heterozygosity (*H*) was 0.23 (*Hesperis straussii*) to 0.31 (*Hesperis hyrcana*) (mean: 0.28). A similar trend was depicted by Shannon's information index (*I*), with the highest value of 0.39 found in (*Hesperis hyrcana*) and the lowest value of 0.14 found in (*Hesperis straussii*) (mean: 0.30). The observed number of alleles (*Na*) varied between 0.261 in *Hesperis straussii* and 0.555 in *Hesperis hyrcana*. The range of effective number of alleles (*Ne*) was 1.014 (*Hesperis straussii*) to 1.042 (*Hesperis luristanica*).

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

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPB-01	5′-GTTTCGCTCC-3′	17	15	93.31%	0.59	5.55	4.22	3.25
OPB-02	5′-TGATCCCTGG-3′	11	11	100.00%	0.47	3.32	8.55	3.45
OPC-04	5'-CCGCATCTAC-3'	11	8	83.79%	0.43	5.11	6.34	3.88
OPD-02	5′-GGACCCAACC-3′	20	18	93.74%	0.57	5.66	8.56	3.67
OPD-03	5′-GTCGCCGTCA-3′	13	12	92.31%	0.54	4.21	6.60	3.55
Mean		7.1	6.2	93.68%	0.55	4.97	7.86	3.5
Total		71	62					

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 RAPD primer.

AMOVA test revealed substantial genetic variation (P = 0.01). It showed that 73% of total variation was interspecific and 27% was intra-specific (Table 4). In addition, genetic differentiation of was demonstrated by significant Nei's GST (0.654, P = 0.001) and D_est values (0.459, P = 0.01). Compared to intra-species, these results revealed a greater distribution of interspecific genetic diversity.

Table 3. Genetic diversity parameters in the studied Hesperis species.

SP	N	Na	Ne	I	He	UHe	%P
H. straussii	9.000	0.261	1.014	0.142	0.20	0.23	43.99%
H. hyrcana	6.000	0.555	1.021	0.39	0.35	0.31	68.33%
H. luristanica	4.000	0.344	1.042	0.20	0.23	0.29	57.53%

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	11	1261.364	55.789	19.764	73%	
Within Pops	110	334.443	4.777	4.898	27%	73%
Total	121	1643.777		24.040	100%	

Different clustering and ordination methods produced similar results therefore, only MDS plot and NJ dendrograms of RAPD data are presented here (Fig. 2 and 3). In general, plant samples of each species were grouped together and formed a separate group. This result shows that RAPD data can delimit *Hesperis* species. In the studied specimens we did not encounter intermediate forms (Fig. 2).

Two main clusters were produced in the NJ tree (Fig. 3). The first main cluster comprised of: *Hesperis straussii* and *Hesperis hyrcana* were separated from the rest of the species and join the others with a great distance and comprised the first cluster. The second cluster comprised of*; Hesperis luristanica*. Relationships obtained from RAPD data usually agree well with the relationship of species obtained from morphological data. This is in accordance with the parameters of AMOVA and genetic diversity previously reported. *Hesperis* species are genetically well distinguished from each other. These findings show that RAPD molecular markers can be used in the taxonomy of *Hesperis* species. The Nm analysis by Popgene software also produced a mean Nm= 0.643, that is deemed a low value of gene flow. A strong association (r= 0.543, p=0.0002) between genetic- and geographical distance was demonstrated by Mantel test with 5000 permutations. It indicates that isolation by distance (IBD) has occurred among these species.

The results of Nei's genetic identity and the genetic distance (Table not included) show the highest genetic similarity (0.74) between *Hesperis straussii* and *Hesperis hyrcana.* Lowest of genetic similarity was shown between *Hesperis straussii* and *Hesperis luristanica* (0.50). Lower Nm value (0.643) is an indicator of limited gene flow or ancestrally shared alleles between different species and indicating high genetic differentiation among and within *Hesperis* species.

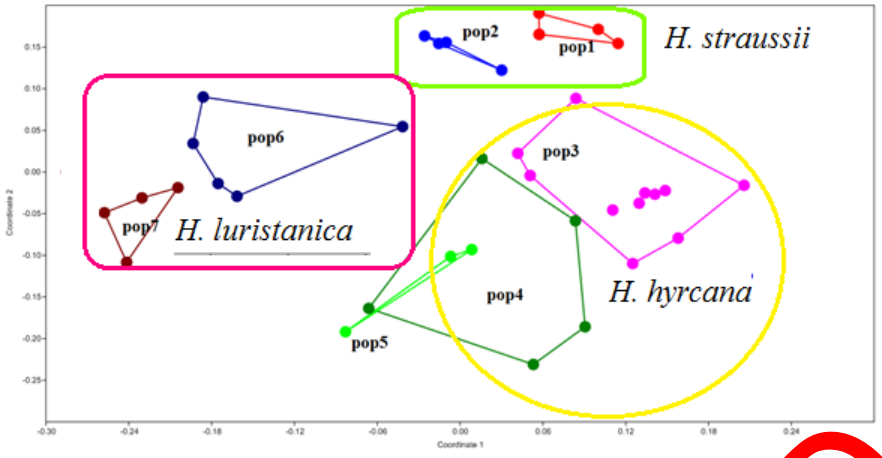

Fig. 2. MDS plot of RAPD data in *Hesperis* populations studied.

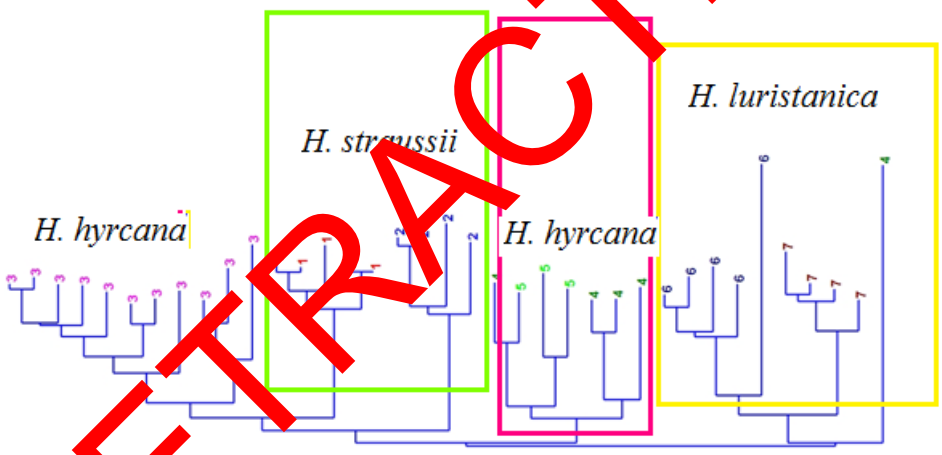

Fig 3. NJ tree of RAPD data revealing species delimitation in the *Hesperis* species

STRUCTURE analysis followed by Evanno test also produced delta K = 3. Therefore, we do have at least 3 genetic groups in the studied species: 1) – specimens with green and blue segments. These are accessions of *Hesperis straussii* and *Hesperis hyrcana* and 2) – specimens having red colored segments as dominant colour. These are accessions of *Hesperis luristanica.*

Admixture ancestry model used in STRUCTURE revealed genetic affinity between *Hesperis straussii* and *Hesperis hyrcana* due to shared common alleles (Fig. 4). This is in agreement with NJ tree presented before.

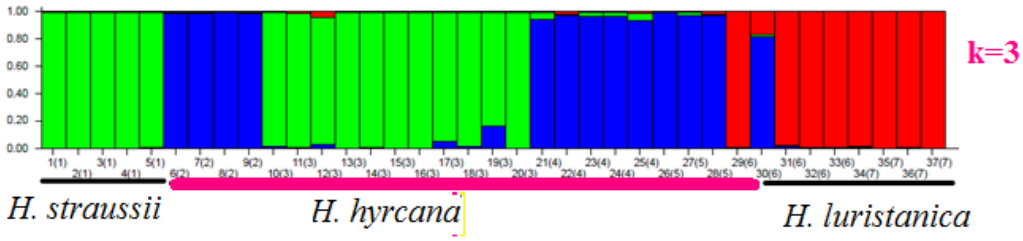

Figure 4. STRUCTURE plot of *Hesperis* species based on RAPD data.

DISCUSSION

Genetic diversity is a fundamental element of biodiversity and its conservation is indispensable for long-term survival of any species in unstable environments (MILLS and SCHWARTZ, 2005). Genetic diversity is non-randomly distributed among different populations and is influenced by numerous factors such as geography, dispersal mechanisms, breeding systems, life span etc. Changes in environment often lead to variation in genetic diversity levels among populations, and under adverse circumstances, populations with little variability are generally deemed less adapted (FALK and HOLSINGER, 1991; CONGFEN, *et al.*, 2021; ZHAO, *et al.*, 2021; XU, *et al.*, 2021). Most authors recognize that genetic diversity is fundamental to preserving the long-term evolutionary potential of a species (FALK and HOLSINGER, 1991). Experimental and field research has shown that habitat fragmentation and population decline have reduced the effective population size in the last decade. Similarly, majority of geneticists regard population size as a significant factor in preserving genetic variation (TURCHETTO *et al.*, 2016). In fragmented populations, this is very important because it is more vulnerable because of allelic richness loss and increased population differentiation via genetic drift and inbreeding depression (FRANKHAM, 2005; YANG, *et al.*, 2021; YIN, *et al.*, 2021; MIAO, *et al.*, 2021). Information of inter- and intra-population genetic diversity is therefore important for their conservation and management (e.g. ESFANDANI-BOZCHALOYI *et al.*, 2018a; 2018b; 2018c; 2018d).

We used morphological and molecular RAPD molecular data to test species relationships in *Hesperis* species in the current analysis. Morphological studies showed that both quantitative (the ANOVA test result) and qualitative characters are well distinguished from each other (the PCoA plot result). Furthermore, PCoA analysis suggests that morphological characters such as bract length, stipule length, bract shape, calyx shape, petal shape, stem-leaf length and width, petal length and width may be used in the delimitation of

species groups. This morphological differentiation is attributed to quantitative and qualitative characters.

Genetic Structure and Gene Flow

A primer's PIC and MI characteristics assist in assessing its usefulness in the study of genetic diversity. SIVAPRAKASH *et al.* (2004) asserted that the ability to overcome genetic diversity by a marker technique could be more explicitly linked to the degree of polymorphism. In general, the PIC value 0 to 0.25 suggests a very low genetic diversity among genotypes, a mid-level of genetic diversity between 0.25 and 0.50, and a value of 0.50 suggests a high level of genetic diversity, between 0.25 to 0.50 shows a mid-level of genetic diversity and value ≥0.50 indicates a high level of genetic diversity (TAMS *et al.* 2005). In this study, the RAPD primers' PIC values ranged from 0.43 to 0.59, with a mean value of 0.55, indicating a moderate level ability of RAPD primers in determining genetic diversity.

According to ARAS *et al.,* (2009) the phylogenetic relations among infraspecific, specific and supraspecific categories of 6 taxa of the genus *Hesperis* collected from different parts of Turkey were investigated by RAPD analysis. Their results showed that the RAPD analysis support the idea that *H. bicuspidata* (Sect. *Hesperis*), *H. schischkinii* (Sect. *Mediterranea*), *H. pendula* (Sect. *Pachycarpos*), *H. breviscapa*, *H. kotschyi* (Sect. *Evelevia*) and *H. cappadocica* (Sect. *Contorta*) species need to be placed into different sections according to morphological characters. On the other hand, the phylogenetic orders of the sections according to morphological characters and according to molecular data displayed some differences and evolutionary phylogenetic orders of the sections were redesigned. The phylogenetic relations among species were based on the samples *H. breviscapa* and *H. kotschyi* which take place in the same section. The accordance of morphological and molecular similarities was noticed for *H. breviscapa* and *H. kotschyi* species. Besides this, infraspecific taxonomic situations of *H. schischkinii* samples having hairy and glabrous (non-hairy) fruits which show allopatric and sympatric spread were reassessed by RAPD analysis. In conclusion, the findings of this study showed that the primers derived from RAPD were more efficient than the other molecular markers in assessing the genetic diversity of *Hesperis* in Iran. In addition, the *Hesperis* species in the dendrogram and PCoA were clearly distinguished from each other, suggesting the greater efficiency of the RAPD technique in the identification of the genus.

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PROTOK GENA I GENETIČKA STRUKTURA IZMEĐU POPULACIJA *Hesperis* L. (BRASSICACEAE) VRSTA KORIŠĆENJEM MOLEKULARNIH MARKERA

Kuanhong MENG[1*],Jia YAO[2], CongYing HE[1], Hakimeh MORABBI HERAVI[3]

[1]Ningbo Institut za okeanografiju, Zhejiang, Kina
[2]Škola za civilno inžinjerstvo i arhitekturu, NingboTech Univerzitet Ningbo, Zhejiang, Kina
[3]Department of Statistics, Univerzitet Bojnord, Iran

Izvod

Genetička varijabilnost i struktura populacija proučavani su u sedam geografskih populacija *Hesperis* L. U ovim populacijama utvrđeni su parametri genetske raznolikosti. 5 od 10 nasumično amplifikovanih polimorfnih DNK prajmera proizvelo je 62 ponovljive trake sa prosekom od 7,1 trake po prajmeru i 55% polimorfizma. *Hesperis hyrcana* je pokazao najveći broj efikasnih alela (Ne), Shannon indeksa (I) i genetske raznolikosti (H). Najviše vrednosti genetske raznolikosti dobijene su u *Hesperis hircana*. NJ grupisalo je populacije u dva različita klastera/grupe, ukazujući na njihovu genetsku razliku o kojoj se detaljno govori. Rezultati ove studije pokazali su da je nivo genetskih varijacija u *Hesperis* relativno visok. Dendrogram zasnovan na NJ pokazao je blisku vezu između članova *Hesperis aussii* i *Hesperis hyrcana* hircana, dok se zaštićena populacija *Hesperis luristanica* najviše razlikuje od ostalih populacija. Analiza glavnih komponenata, međutim, pokazala je neke manje razlike sa dendrogramima zasnovanim na NJ.

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