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Original scientific article

SPECIES IDENTIFICATION AND POPULATION STRUCTURE ANALYSIS IN *Hesperis* L. (Brassicaceae)

Zhiwei ZHANG[1,*], Hangcheng YU[2], Shuowen FENG[1], Amir Abbas MINAEIFAR[3]

[1]School of Architecture and Civil Engineering, Chongqing Metropolitan College of Science and Technology, Yongchuan,Chongqing, 402167, China
[2]College of Forestry ,Guizhou University,Guiyang,Guizhou,550000,China
[3]Department of Biology. Payame Noor University. P.O. Box19395-3697 Tehran, Iran

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In biology, biogeography, ecology, and conservation, species identification is critical. *Hesperis* L. is a Brassicaceae (Cruciferae) genus of 46 species found across the temperate northern hemisphere, from South and Central Europe to Southwest Asia, Caucasia, and the mountainous portions of West China and Mongolia. According to the latest therapies, *Hesperis* is expressed in Iran by six species. Despite the fact that several *Hesperis* species are widely distributed throughout Iran, nothing is known about their genetic diversity, divergence techniques, or dispersion patterns. As a result, we examined 122 accessions from six *Hesperis* species gathered from various locations in Iran using genetic (ISSR markers) and morphological techniques. A total of ten ISSR markers were employed in this study. The Nei's genetic distance was used to measure genetic distances, and descriptive statistics of folks were used to produce genetic parameters. There were a total of 118 polymorphic bands found. The study's aims are as follows: 1) Is it possible to identify *Hesperis* species using ISSR markers? 2) In Iran, what is the genetic make-up of these taxa? 3) How do interspecies relationships work? According to the conclusions of this research, morphological and ISSR data may be utilized to identify species. The *Hesperis* species are genetically distinct yet share significant genes, according to the AMOVA and STRUCTURE analyses.

Keywords: ISSR, morphology, species identification, STRUCTURE analyses

Corresponding author: Zhiwei Zhang, School of Architecture and Civil Engineering, Chongqing Metropolitan College of Science and Technology, Yongchuan,Chongqing , 402167, China. E-mail:nasar.nas1990@gmail.com; xiaowei988211@163.com

INTRODUCTION

In a variety of biological domains, such as ecology, biogeography, and plant conservation, species delimitation is used (ZHENG *et al.*, 2021; ZHU *et al.*, 2021; YIN *et al.*, 2021). Both tree-based and non-tree-based techniques differentiate species. In the first scenario, species are divided into clades (phylogenetic species concept), while non-tree-based methodologies define species based on gene flow analyses (biological species concept.

WIENS and PENKROT (2002) advocated for species delimitation based on DNA, morphological, and character data. The later authors employed coalescent simulations and data from several loci to examine the species constraints. They demonstrated how population genetics influences the establishment of species boundaries. By using amplified fragment length polymorphism (AFLP) molecular markers, used population genetics approaches to the species delimitation issue in Narcissus Linnaeus (1753: 289). (Amaryllidaceae J.St.-Hil. nom. cons). *Hesperis* L. (Brassicaceae) is a biennial and perennial plant with 46 species worldwide (AL-SHEHBAZ *et al.*, 2006). It is found mostly in Europe, the Caucasus, Transcaucasia, and to a lesser degree in northern and central Asia, with 28 species principally in Turkey (DURAN *et al.*, 2003, DURAN, 2008; ARAS *et al*., 2009). In Iran, the genera *Hesperis* Dvoák, *Diaplictos* Dvoák, and *Pachycarpos* Fourn. are represented by 11 (DVORAK, 1968) or six (ASSADI *et al.*, 2017) species belonging to the sections *Hesperis* Dvoák, *Diaplictos* Dvoák, and *Pachycarpos* Fourn. Although morphological, cytological, and palynological features were used to assess the first subgeneric (DVORAK, 1973) and sectional (ANDRZEJOWSKI, 1821) investigations of *Hesperis*, taxonomists continue to strive to present new infrageneric categories (DURAN *et al.*, 2003; DURAN, 2016). The stalked glands with uniseriate stalks terminating in a unicellular gland differentiate *Hesperis* from the rest of the Brassicaceae (AL-SHEHBAZ *et al*., 2006). Traditional morphological parameters, such as life form, stem height, leaf shape, and different fruit traits, were employed by DURAN *et al.* (2003) for sectional categorization between palynological, cytological, and trichome characters. ANDRZEJOWSKI (1821) was the first to update the genus, placing it in a single section (*Deilosma* Andrz.). Several researchers later treated the taxonomy of the genus. The genus was divided into various sections based on morphological characters: DE CANDOLLE (1824) divided the genus into two sections (*Hesperidium* DC. and *Deilosma* Andrz.), FOURNIER (1866) divided the genus into three sections (*Hesperidium*, *Deilosma*, and *Pachycarpos* Fourn. Emend. Tzvelev) divided (1867). It was divided into five subgenera by DVORAK (1973). (*Hesperis*, *Mediterranea* Borbas, *Cvelevia* Dvorak, *Contorta* Dvorak and *Diaplictos* Dvorak). The genus growing in Turkey were not divided into sections by CULLEN (1965). Despite multiple investigations on the genus' infragenetic and infraspecific classification, issues have yet to be resolved (FOURNIER, 1866; TZVELEV, 1959; DVORAK, 1968; 1973). There has been previous research on species delimitation and species relationships in this genus DURAN and OCAK (2005), DURAN (2009), PINAR *et al.* (2009), DURAN *et al.* (2011), DURAN (2016), PADURE *et al.* (2016), Natural selection and adaptation are reflected in the morphology of seed surfaces. As a consequence, it's important on both a broad and specialized level (BROCHMANN, 1992; BERNAND, 2000; KOUL *et al*., 2000). Phylogenetic connections between infraspecific, specific, and supraspecific categories of 6 species of the genus *Hesperis* collected from various locations of

Turkey were investigated using RAPD analysis, according to ARAS *et al*. (2009). *Hesperis* species include *Hesperis bicuspidata* (Sect. *Hesperis*), *Hesperis schischkinii* (Sect. *Mediterranea*), *Hesperis pendula* (Sect. *Pachycarpos*), *Hesperis breviscapa*, *Hesperis kotschyi* (Sect. *Cvelevia*), and *Hesperis cappadocica* (Sect. *Contor*). The Cruciferae taxonomic connections have been deciphered using pollen morphology as a framework (BROCHMANN, 1992).

The macro- and micromorphological properties of pollen and seeds from Iranian taxa belonging to three sections, comprising sects *Hesperis*, *Diaplictos*, and *Pachycarpos*, were evaluated for the first time using light (LM) and scanning electron microscopy (SEM) techniques (ESLAMI FAROUJI *et al*., 2018). *Hesperis* is yet to be investigated on a global scale in terms of molecular phylogenetics. Traditional categorization schemes mainly contradict recent biosystematics results between different Brassicaceae family members due to morphological feature parallelism and convergence. As a consequence, pinpointing each species' reallocation will be challenging (FRANZKE *et al*., 2011). Iran's taxonomic difficulties have yet to be addressed, despite multiple infrageneric and infraspecific *Hesperis* studies (ASSADI *et al*., 2017). (ESLAMI FAROUJI *et al*., 2018). However, little effort has been made to look into the genetic diversity, ecological adaptability, intra-, and inter-specific differentiation, or morphometric investigations of the Iranian *Hesperis*. As a result, we visually and genetically evaluated 122 *Hesperis* specimens from three regions. Molecular markers are commonly regarded as a useful tool for determining genetic differences between individual in a population. ISSR markers are PCR-based molecular markers that amplification of regions between two microsatellite sequences (ESFANDANI-BOZCHALOYI *et al*., 2018a; 2018b; 2018c; 2018d; BI *et al*., 2021; CHENG *et al*., 2021). These indicators may be used without any previous knowledge of the species' DNA. They may also be studied using basic procedures, are simple to handle, and only need a small quantity of DNA. We try to respond to the following questions: 1) Does the researched species have infraspecific and interspecific genetic diversity? 2) Is there a link between the genetic gap between these species and their geographical remoteness? 3) Can you tell me about the genetic differences across populations and taxa? 4) Is it feasible to cross-pollinate *Hesperis* species in Iran?

MATERIALS AND METHODS

Plant materials

One hundred twenty-two plant samples were collected from 18 geographical populations of six Hesperis species between 2016 and 2017, including *H. persica* subsp. *persica*, *H. persica* subsp. *Kurdica* (sect. *Pachycarpos*), *H. odorata*, *H. nivalis*, *H. luristanica* (sect. *Diaplictos*), *H. hyrcana*, and *H. straussii* (sect. *Hesperis*) (Table 1). Based on descriptions in Flora Iranica, Flora of Turkey, and other floras, specimens were given names (DVORAK, 1968; CULLEN, 1965; DURAN *et al*., 2003). The locations of the sample sites are described in detail (Table 1).

Table 1. Voucher details of Hesperis species and relative genera examined 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 ˚ 52'353"	46 ˚ 27 92"	1143	HIAU 201678
	Kohgilouye-Boirahmad, Fahlian,	30 ˚ 52'353	51 ˚ 27' 92"	1750	HIAU 201680
2. *H. hyrcana* Bornm. & Gauba	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
3. *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
4. *H. odorata* F. Dvořák	Kermanshah, Parrou Mountain	34 ˚ 09' 55"	47 ˚ 55' 49 "	1600	HIAU 201689
5. *H. nivalis* Boiss. & Hausskn.	Chaharmahal va Bakhtiari , Shahr-e Kord- Sabzkouh protected	320702.32	504432.6	2300	HIAU 201690
	Chaharmahal va Bakhtiari , Shahr-e kord Baba Heydar-Sefid daneh	321204.81	500311.98	2200	HIAU 201691
	Hamadan, Asadabad	354158.62	494730.34	1335	HIAU 201692
	Hamadan, Nahavand	351414.32	491807.09	1807	HIAU 201693
	Hamadan, Heidareh	35 080.23	49 8507.03	1320	HIAU 201694
6. *H. persica* Boiss. subsp. *persica*	East Azerbaijan, Kaleybar	38 ˚ 52'373	47 ˚ 23' 92"	1144	HIAU 201695
	Tehran, Darband	355003.36	512428.62	1700	HIAU 201696
7. *H. persica* subsp. *kurdica* (F. Dvořák & Hadac) F. Dvořák	East Azerbaijan, Kaleybar Cheshme Ali Akbar	38 ˚ 52'373	47 ˚ 23' 92"	1144	HIAU 201697

Morphological studies

Morphometry was performed on five models from each species. Thirty-seven morphological features (16 qualitative, 21 quantitative) were investigated. Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa (PODANI 2000). For grouping of the plant specimens, The UPGMA

(Unweighted paired group using average) ordination methods were used (PODANI 2000). ANOVA (Analysis of variance) were performed to show morphological difference among the populations while, PCA (Principal components analysis) biplot was used to identify the most variable morphological characters among the studied populations (PODANI 2000). PAST version 2.17 (Hammer *et al.* 2012) was used for multivariate statistical analyses of morphological data.

DNA extraction and ISSR assay

Fresh leaves were collected at random from 5-10 plants in each of the groups investigated. Silica gel powder was used to dry them. The CTAB activated charcoal procedure was applied (ESFANDANI-BOZCHALOYI *et al*., 2019). ISSR bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Discriminatory ability of the used primers was evaluated by means of two parameters, polymorphism information content (PIC) and marker index (MI) to characterize the capacity of each primer to detect polymorphic loci among the genotypes. MI is calculated for each primer as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (*n*) and the fraction of polymorphic fragments (β). The number of polymorphic bands (NPB) and the effective multiplex ratio (EMR) were calculated for each primer. Parameter like 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 determined (FREELAND *et al.* 2011). Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ 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. The percentage of polymorphic loci, the mean loci by accession and by population, UHe, H' and PCA were calculated by GenAlEx 6.4 software. Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (FREELAND *et al*. 2011). Mantel test checked the correlation between geographical and genetic distances of the studied populations (PODANI 2000). These analyses were done by PAST ver. 2.17 (HAMMER *et al.* 2012), DARwin ver. 5 (2012) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 were used to show genetic difference of the populations. Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers the equal amount of gene flow among all populations.

RESULTS

Species identification and inter-relationship

Morphometry

In quantitative morphological features, ANOVA revealed significant differences (P0.01) between the species investigated. PCA analysis was used to discover the most variable characteristics between the taxa investigated. It was discovered that the first three parameters accounted for nearly 78% of the overall variance. Characters including length, width, hairs and number of sepals, pedicle hair, and seed width showed the largest correlation (>0.7) in the first PCA axis. In contrast, leaf texture, stigma number, Fruit orientation, Fruit hair type, Petal length, and Stem hair type influenced PCA axis 2 and 3 accordingly (Fig. 1).

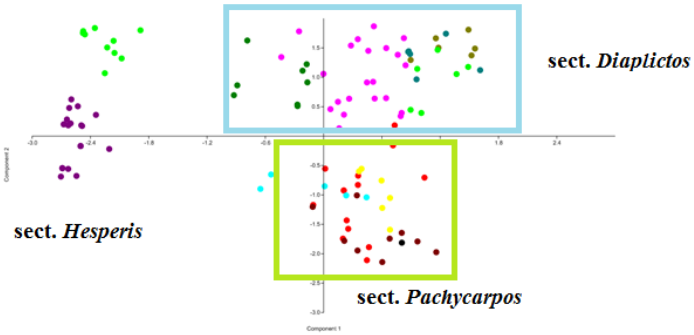

Figure 1. PCA plots of morphological characters revealing species delimitation in the *Hesperis* species

Because the findings of several clustering and ordination approaches were comparable, UPGMA clustering and a PCA plot of morphological features are shown here (Fig. 1). Plant samples from each species were divided into sections and created independent clusters. This finding demonstrates that the morphological characteristics evaluated may distinguish between *Hesperis* species. We didn't find any intermediate forms between the specimens we looked at.

In general, the UPGMA tree created two large clusters. *H. hyrcana* and *H. straussii* (sect. *Hesperis*) were separated in the first cluster by stem hair type, leaf shape, fruit hairs, petal color, and fruit orientation. Two sub-clusters made up the second main cluster. Due to morphological similarity, plants of *H. persica* subsp. *persica* and *H. persica* subsp. *Kurdica* from the *Pachycarpos* section formed the first sub-cluster. In contrast, plants of *H. odorata*, *H. nivalis*, and *H. luristanica* (sect. *Diaplictos*) formed the second sub-cluster. They differed by fruit length, leaf hair length, sepal length, sepal color, and fruit. The species were sorted into discrete groups by the PCA plot of morphological features (Fig. 1), with no intermixing.

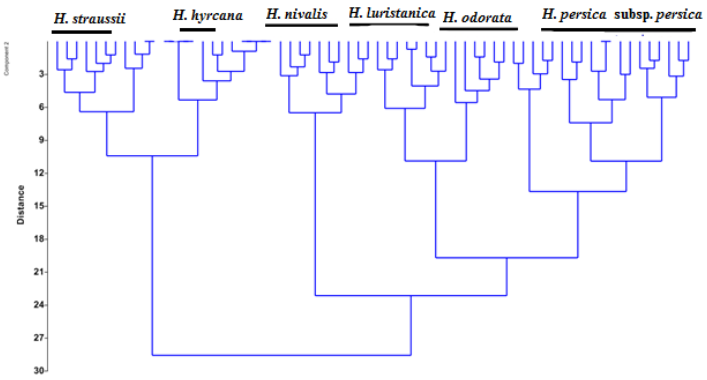

Figure 2. NJ tree of ISSR data in the studied *Hesperis* species

Species Identification and Genetic Diversity

All ISSR primers formed polymorphic bands. Genetic diversity characteristics assessed in the examined species (Table 2) indicated that *H. persica* subsp. *persica* (sp6) had the greatest genetic polymorphism (56.11 percent), while *H. luristanica* had the lowest level (3.23 percent) (sp3). The highest values for an effective number of alleles (Ne = 1.3) and Shannon information index (I =0.38) were found in *H. persica* subsp. *persica*.

Table 2. Genetic diversity parameters in the studied Hesperis species.

Pop	N	Na	Ne	I	He	UHe	%P
sp1	6.000	0.258	1.029	0.023	0.026	0.010	4.38%
sp2	8.000	0.429	1.097	0.084	0.056	0.060	16.13%
sp3	14.000	0.344	1.039	0.011	0.017	0.023	3.23%
sp4	12.000	0.925	1.279	0.233	0.155	0.162	32.09%
sp5	11.000	0.784	1.171	0.162	0.104	0.109	46.56%
sp6	12.000	1.347	1.304	0.381	0.174	0.182	56.11%
sp7	14.000	0.560	1.186	0.098	0.064	0.066	31.51%

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

Table 3. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	14	777.747	51.397	8.082	55%	
Within Pops	67	321.607	6.530	5.530	45%	55%
Total	81	1119.354		14.613	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).

H. hyrcana and *H. straussii* (sect. *Hesperis*) are isolated from the other examined species and link them with a considerable distance, according to a NJ tree based on Nei's genetic distance (Fig. 2). *H. odorata, H. nivalis*, and *H. luristanica* demonstrated close genetic similarity in this dendrogram (sect. *Diaplictos*). *H. persica* subsp. *persica* and *H. persica* subsp. *Kurdica* (sect. *Pachycarpos*) were also grouped. In general, species relationships derived from ISSR data are consistent with morphological data. This is consistent with the AMOVA and genetic diversity metrics previously reported. Genetically, the species are distinct from one another. The Popgene software's Nm study similarly generated a mean Nm= 0.37, which is considered a very low gene flow between the species investigated.

Isolation by distance (IBD) occurred between the *Hesperis* species tested, as the Mantel test with 5000 permutations revealed a strong correlation (r = 0.28, p=0.0001) between genetic distance and geographical distance.

The genetic identity of Nei and the genetic distance between the species examined (Table is not included). The findings revealed that *H. odorata* and *H. nivalis* had the greatest

degree of genetic similarity (0.85). Between *H. hyrcana* and *H. persica* subsp. *persica*, there was the least genetic resemblance (0.75).

DISCUSSION

Genetic diversity

One facet of biological variation that is highly essential for conservation efforts is genetic diversity. The size of a population is thought to be a significant factor in sustaining genetic diversity. Because of environmental stochasticity, genetic drift, and inbreeding, small populations are more prone to extinction than large ones. Within populations, genetic drift reduces heterozygozity and eventually leads to allele fixation, whereas inbreeding enhances homozygozity (MA *et al.*, 2021; PENG *et al.*, 2021; SI *et al*., 2021; JIA, *et a.l*, 2020). In general, a decrease in population size may lead to a loss of genetic variation due to inbreeding and genetic drift. Genetic variety loss may result in a loss of fitness and evolutionary potential to respond to environmental changes in the long run (LANDE 1993). For small population species conservation and management planning, measuring genetic variability and variation patterns within and within populations is critical.

Although members of the genus *Hesperis* have been included in several recent studies of Brassicaceae molecular phylogeny, the current work is the first to use molecular markers to investigate genetic connections in the genus *Hesperis*. The ISSR approach has been demonstrated to effectively distinguish between distinct *Hesperis* species.

Phylogenetic relationships between infraspecific, specific, and supraspecific categories of 6 species of the genus *Hesperis* collected from various locations of Turkey were studied using RAPD analysis, according to ARAS *et al.* (2009). According to their findings, the RAPD analysis supports the concept that *H. bicuspidata* (Sect. *Hesperis*), *H. schischkinii* (Sect. *Mediterranea*), *H. pendula* (Sect. *Pachycarpos*), *H. breviscapa*, *H. kotschyi* (Sect. *Cvelevia*), and *H. cappadocica* (Sect. *Contorta*) species. On the other side, there were some changes in the phylogenetic order of the sections based on morphological features and molecular data, and the evolutionary phylogenetic orders of the sections were revised. The evolutionary relationships between species were determined using samples of *H. breviscapa* and *H. kotschyi* from the same area.

The morphological and molecular similarities between *H. breviscapa* and *H. kotschyi* species were discovered. RAPD analysis was also used to review the infraspecific taxonomic circumstances of *H. schischkinii* samples with hairy and glabrous (non-hairy) fruits that indicate allopatric and sympatric dissemination.

ISSR markers were used to determine genetic variability within *Hesperis* species in this investigation. According to our findings, *H. luristanica* has a lower amount of genetic diversity (P: 3.23 percent, He: 0.017, I: 0.011). Biological features, reproductive mode, and breeding system have all been identified as significant influences on genetic diversity levels. The genetic diversity of outcrossing organisms is generally far greater than that of selfing species (HAMRICK and GODT 1989). Previous research revealed that *Hesperis'* mating strategy is mostly selfing (MIAO *et al.*, 2018; NIU *et al.*, 2021).

Several variables influence the genetic structure, including breeding systems, genetic drift, population size, and natural selection (HAMRICK and GODT, 1990). According to our genetic

analysis, the 122 individuals created a distinct separation between all groups; this finding suggested differentiation. The PCA also agrees with this finding (Fig. 1). The results of a molecular variance study of all populations revealed that 55 percent of genetic diversity was found across populations, whereas 45 percent was found within these populations (Table 3). To summarize, the current work demonstrated ISSR genetic markers in conjunction with morphological features to identify *Hesperis* species. There is some interspecific genetic mixing in *Hesperis* species, although the examined species are highly distinct throughout the speciation process and invasion of new environments.

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IDENTIFIKACIJA VRSTA I ANALIZA STRUKTURE POPULACIJE U *Hesperis* L. (*Brassicaceae*)

Zhiwei ZHANG[1,*], Hangcheng YU[2], Shuowen FENG[1], Amir Abbas MINAEIFAR[3]

[1]Škola za arhitekturu i civilno inžinjerstvo, Chongqing Metropolitan Koledž za nauku i tehnologiju, Yongchuan,Chongqing, 402167, Kina
[2]Koledž za šumarstvo, Guizhou Univerzitet, Guiyang,Guizhou, 550000, Kina
[3]Departman za biologiju, Payame Noor Univerzitet, P.O. Box19395-3697 Teheran, Iran

Izvod

Identifikacija vrsta je fundamentalno važna u oblastima biologije, biogeografije, ekologije i očuvanja. Rod *Hesperis* L. pripada porodici Brassicaceae (Cruciferae) i obuhvata oko 46 vrsta rasprostranjenih na širokom geografskom području u umerenoj klimi severne hemisfere koje se proteže od južne i centralne Evrope, jugozapadne Azije, Kavkaza, do planinskih regiona Zapadne Kine i Mongolije. Prema najnovijim podacima, Hesperis je u Iranu zastupljen sa šest vrsta. Uprkos velikoj rasprostranjenosti mnogih vrsta *Hesperisa* koje rastu u Iranu, nema dostupnih izveštaja o njihovoj genetskoj raznovrsnosti, načinu divergencije i obrascima širenja. Zbog toga smo uradili molekularne (ISSR markeri) i morfološke studije 122 uzoraka šest vrsta *Hesperisa* koje su prikupljene iz različitih staništa u Iranu. Korišćen je set od 10 ISSR markera. Genetske udaljenosti su procenjene na osnovu Jaccard koeficijenta sličnosti, a takođe je urađena deskriptivna statistika populacija za procenu genetskih parametara. Dobijeno je ukupno 118 polimorfnih traka. Ciljevi ovog rada su: 1) utvrditi da li ISSR markeri mogu da identifikuju vrste *Hesperisa*, 2) ispitati koja je genetika ovih taksona u Iranu, i 3) istražiti međuodnos vrsta? Ovo istraživanje je otkrilo da kombinacija morfoloških i ISSR podataka može identifikovati vrstu. Takođe, otkriveno je da kombinacija morfoloških i ISSR podataka može razgraničiti vrstu. Analiza AMOVA i STRUKTURA otkrila je da su vrste *Hesperisa* genetski diferencirane, ali imaju određeni stepen zajedničkih alela.

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