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Original scientific paper

POPULATION GENETIC ANALYSIS AND EVIDENCE OF INTER-SPECIFIC INTROGRESSION IN *Helichrysum armenium* AND *H. rubicundum* (Asteraceae)

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Helichrysum armenium and *H. rubicundum* are two medicinally important plants of Iran that are distributed in various regions of the country. They are extensively used by locals as medicinal plants and a great negative selection pressure is applied on them. Therefore, due to importance of these plant species, we performed a population genetic study in both species. For this study, we used 66 randomly collected plants from 24 geographical populations of *Helichrysum armenium* and *H. rubicundu.* These species had areas of overlap and contact and we found some intermediate plants that were included in our study too. UPGMA and MDS analyses revealed morphological separation of these closely related species and placed intermediate plants in an intermediate position. ISSR analysis revealed inter-population genetic diversity and K-Means clustering and STRUCTURE analyses revealed populations' genetic stratification in both species. Genetic difference of the studied populations was not correlated to geographical distance. Triangle plot of Bayesian analysis and NeighborNet plot showed inter-specific gene flow. The studied populations showed plants with 2n = 2x = 14 and 2n = 4x = 28 chromosomes and differed significantly in their meiotic behavior. Therefore, a combination of genetic stratification, and genetic admixture as well as polyploidy and chromosomes structural changes, have played rule in *Helichrysum* diversification.

Key words: genetic admixture, *Helichrysum*, polyploidy,. Inter-specific gene flow

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INTRODUCTION

The genus *Helichrysum* Mill. (Gnaphalieae) is a large and taxonomically difficult genus, that is distributed throughout the African continent, Madagascar, the Mediterranean basin, Macaronesia, western and central Asia as well as India. It comprises about 500 to 600 species (GALBANY-CASALS *et al.* 2009). Most of the *Helichrysum* species have medicinal values and their secondary products like flavonoids, acetophenones, phloroglucinol, pyrones, triterpenoids and sesquiterpene have been used in folk medicine and are included in many pharmacopoeias. *Helichrysum armenium* D.C. and *H. rubicundum* (K. Koch) Bornm., are two medicinal species that grow in different regions of Iran and form several local populations. In some regions these closely related species grow in sympatry. Due to importance of these taxa as a source of folk medicine and extensive use by local people, we performed population genetic analysis with the aim to illustrate their population structure and possible inter-specific gene flow in the area of sympatry. Hybridization is considered an important evolutionary force in Gnaphalieae. Combination of morphological and genetic data have been used to identify hybrids in the genus *Helichrysum* as these plants exhibit intermediate features of the parental species (GALBANY-CASALS *et al.* 2012). Multilocus molecular markers including simple sequence repeat markers (SSRs) and inter simple sequence repeat markers (ISSR) are good genetic markers to identify hybrid plants (GASKIN & KAZMER, 2009; NOORMOHAMMADI *et al.* 2012). These molecular markers are known to reveal genetic diversity in *Helichrysum* species and (AZIZI *et al.* 2014).

MATERIALS AND METHODS

Plant materials

Sixty-six plant specimens were randomly collected from 24 geographical populations of *Helichrysum armenium* and *H. rubicundum* located in 5 provinces of Iran (Table 1 and Fig. 1). These two species grow in sympatry in some regions (for example in Tabriz, East Azarbayejan). In these regions, we encountered several plants with morphological characters of both studied species and considered as potential hybrids and added to our study.

Fig. 1. Distribtion map of *H. armenium and H. rubicundum* . Red dots=*H. armenium and* Green dots = *H. rubicundum*

Table 1. Helichrysum species and populations studied, their localities and voucher Number.

Sp.	Locality	Voucher No.	Latitude	Longitude	Elevation
H. armenium	Chenan, Kurdistan	HSBU2012277	46°56'53.00"E	35°26'17.13"N	1710
H. armenium	Abidar, Kurdistan	HSBU2012213	46°58'45.58"E	35°18'43.19"N	1536
H. armenium	6km Marivan-Sanandaj, Kurdistan	HSBU2012261	46°10'35.00"E	35°31'37.00"N	2500
H. armenium	Ariz passage, Kurdistan	HSBU2012211	45° 45.48"E	38°33'1.00"N	2200
H. armenium	Alaboolagh, Hamedan	HSBU2012215	48°26'8.00"E	35°48'37.00"N	2300
H. armenium	Mamakan, West Azarbayejan	HSBU2012203	44°46'17.96"E	37°59'26.64"N	1500
H. armenium	Garan passage, Kurdistan	HSBU2012210	46°23'59.75"E	35°29'57.84"N	2400
H. armenium	Ghasemloo, Urmia, West Azarbayejan	HSBU2012257	45° 9'1.48"E	37°20'46.22"N	1340
H. armenium	25km Khoy-Chaldoran, West Azarbayejan	HSBU2012207	44°57'8.00"E	38°33'1.00"N	1500
H. armenium	20km toOshnaveiyeh, West Azarbayejan	HSBU2012206	45° 06'.28"E	37° 02'.81"N	1500
H. rubicundum	Damavand, Alborz	HSBU2012250	52°11'52.01"E	35°56'55.31"N	2750
H. rubicundum	Poldasht-Jolfa, West Azarbayejan	HSBU2012249	45° 4'15.62"E	39°20'51.95"N	810
H. rubicundum	Jolfa- Marand, East Azarbayejan	HSBU2012278	45°30'49.71"E	38°52'7.19N	1621
H. rubicundum	Seroo, West Azarbayejan	HSBU2012279	44°38'34.00"E	37°43'39.00"N	1595
H. rubicundum	24 km Givi, Ardebil	HSBU2012256	48°28'23.71"E	37°44'59.40"N	1650
H. rubicundum	2 km Tabriz-Ahar, East Azarbayejan	HSBU2012248	46°51'8.03"E	38°24'26.11"N	1674
H. rubicundum	43 km to Givi, Ardebil	HSBU2012255	48°17'30.55"E	37°59'37.14N	1650
H. rubicundum	Lahrood, Zanjan	HSBU2012252	49°47'56.00"E	30°37'39.00"N	1300
H. rubicundum	13 kmNaghadeh, West Azarbayejan	HSBU2012266	45° 21'.04"E	36° 59'.80"N	1550
H. rubicundum	Rend village, Kaleibar, East Azarbayejan	HSBU2012253	44°37'45.07"E	39°19'1.93"N	1076
H. rubicundum	15 Km to Tabriz, East Azarbayejan	HSBU2012243	47° 1'8.69"E	38°21'6.98"N	1979
H. rubicundum	Ghasemloo valley, West Azarbayejan	HSBU2012280	45° 9'1.73"E	37°37'45.26"N	1200
H. rubicundum	22 Km to Tabriz, East Azarbayejan	HSBU2012244	46°40'50.34"E	38°18'5.85"N	1740
H. rubicundum	25 Km to Tabriz, East Azarbayejan	HSBU2012245	46°34'58.52"E	38°14'37.14"N	1764

DNA extraction and ISSR assay

Fresh leaves were collected randomly in each of the studied populations and dried in silica gel powder. Genomic DNA was extracted using CTAB with activated charcoal protocol (Sheidai et al. 2013). The quality of extracted DNA was examined by running on 0.8% agarose gel.

Ten ISSR primers; (AGC)5GT, (CA)7GT, (AGC)5GG, UBC810, (CA)7AT, (GA)9C, UBC807, UBC811, (GA)9A and (GT)7CA commercialized by UBC (the University of British Columbia) were used.PCR reactions were performed in a 25µl volume containing 10 mM Tris-

HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 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 amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, 30 S at 94°C; 1 min at 50°C and 1min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. The amplification products were visualized by running on 2% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Morphological study

Seventeen morphological characters (quantitative and qualitative) were studied such as the gemma length and width, the basal leaf width, the stem leaf width, the length and width of the upper leaves, the length and width of synflorescence, the length and width of receptacle, the width of involucre, phyllary number, the corolla length, pappus number, number of flowers, form of the involucre, gemma status and indumentum of the basal leaves.

The range of quantitative morphological characters (5 readings in each case) was determined. These characters and along with qualitative characters were coded as binary and multistate characters. For multivariate statistical analyses, Gower distance was determined for clustering.

UPGMA (Unweighted paired groups using average method) and Ward (minimum spherical cluster method) were used for grouping of the accessions after 100 times bootstrapping (PODANI 2000). Different ordination methods were applied on standardized data like PCA (Principal components analysis), PCoA (Principal coordinate analysis) and MDS (Multidimentional scaling) (PODANI 2000). Moreover, minimum spanning tree (MST) was performed to illustrate morphological affinity of the presumed hybrid plants. Data analyses were performed by using PAST ver. 2.17 (HAMER *et al.* 2012). Different clustering and ordination methods were used to check consistency of results and when the results are similar, only one of them is presented here.

Genetic analyses

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). The following genetic diversity parameters were determined in each population: percentage of allelic polymorphism, allele diversity (WEISING 2005), Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (FREELAND *et al.* 2011).

Nei's genetic distance was determined among the studied populations followed by Neighbor Joining (NJ) clustering and NeighborNet method of networking with 100 times bootstrapping (HUSON & BRYANT, 2006). The Mantel test was performed to study correlation between geographical distance and the genetic distance of the studied populations (PODANI, 2000). GenAlex 6.4 (PEAKALL & SMOUSE, 2006), PAST ver. 2.17 (HAMER *et al.* 2012), DARwin ver. 5 (2012), and SplitsTree4 V4.13.1 (2013) programs were used for these analyses.

Genetic difference among the studied populations and provinces were determined by: AMOVA (Analysis of molecular variance) test (with 1000 permutations), and 2- Nei's Gst analysis of GenoDive ver.2 (2013) (MEIRMANS & VAN TIENDEREN, 2004). Moreover, to avoid possible problem caused by dominant nature of ISSR markers, Hickory test (Hickory program ver. 1.0) that is a Bayesian approach was used to reveal populations' genetic differentiation (HOLSINGER *et al.* 2003, TERO *et al.* 2003).

New differentiation parameters such as G'ST est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (JOST, 2008), were also determined.

The genetic structure of populations was studied by STRUCTURE analysis (PRITCHARD *et al.* 2000) of dominant molecular markers (FALUSH *et al.* 2007) and K-Means clustering as done in GenoDive ver. 2. (2013) MEIRMANS, 2012). We used two summary statistics to present K-Means clustering, 1- pseudo-F and 2- Bayesian Information Criterion (BIC).

Non-metric multidimentional scaling (MDS) (PODANI, 2000) was performed to study genetic grouping of the populations and latent factor mixed models (LFMM) (FRICHOT *et al.* 2013) was used to test correlation between the studied molecular markers and geographical parameters (latitude and longitude) by LFMM program Version: 1.2 (2013).

The hybrid index as implemented in Genodive ver. 2 (2012) was used to study inter-specific gene flow. It is a quantitative estimate of the genetic contribution of two parental species or populations to an individual of unknown provenance. GenoDive uses the method of BUERKLE (2005) to calculate a maximum likelihood estimate of such a hybrid index.

Cytology

Twelve populations were analyzed for meiotic chromosome number and cytogenetic variability with regard to chiasma frequency and distribution and chromosome pairing (SHEIDAI *et al.* 2013). Squash technique was used for cytological studies performed on young flower buds randomly collected from at least 20 randomly collected plants in each population. Flowers' bud fixation and details of cytological preparation were according to our previous report (SHEIDAI *et al.* 2013).

ANOVA test (Analysis of variance) was performed to reveal cytogenetic difference of the studied populations, while cytogenetic similarity of the populations was studied by clustering method. Since some populations had plants with two different chromosome numbers, the relative chiasma values were obtained by dividing the actual numbers by the number of chromosomes and used for clustering (SHEIDAI *et al.* 2013).

RESULTS AND DISCUSSION

Morphometry

UPGMA clustering of morphological characters (Fig.2) separated *H. armenium* (plant numbers 1-25) and *H. rubicundum* (plant numbers 26-60) plants from each other in two different major clusters, and placed intermediate putative hybrid plants (plant numbers 61-66) in an intermediate position between the two species.

The MDS plot of morphological characters (Figure not given) not only supported UPGMA result, but also revealed morphological variability of the studied species. A higher degree of morphological variability was observed in *H. rubicundum* compared to *H. armenium*.

Inter-population morphological variability has been reported in several *Helichrysum* species (see for example, GALBANY-CASALS *et al.* 2006; AZIZI *et al.* 2014). Such high intra-specific morphological variability may be due to cross-pollination nature of *Helichrysum* species and to some extent due to local adaptations.

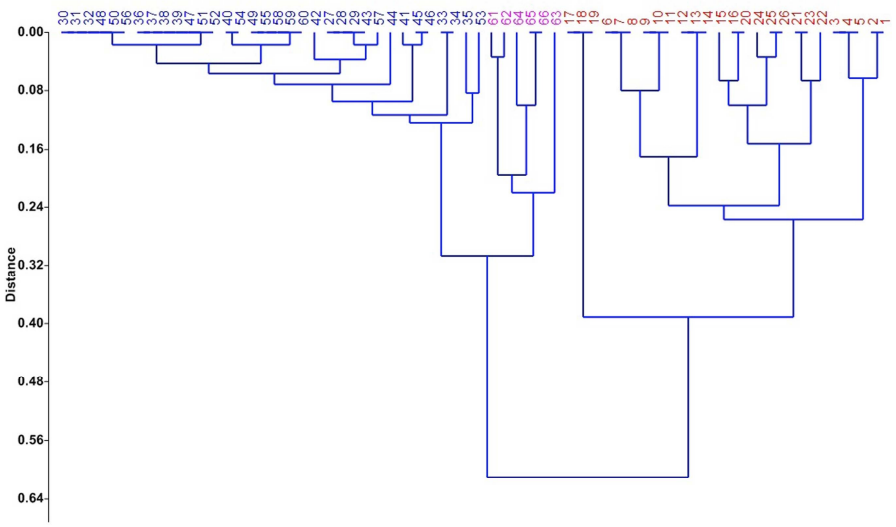

Fig. 2. UPGMA clustering of morphological characters revealing morphological separation of *H. armenium* (plant numbers 1-25) and *H. rubicundum* (plant numbers 26-60) and showing intermediate position of presumed hybrids (plant numbers 61-66).

Genetic diversity and population structure

Helichrisum armenium

Genetic diversity parameters determined in the studied populations are provided in Table 2. The highest value for genetic polymorphism (31.82%) was observed in Chatan population (Sanadaj, Kurdistan), followed by Mishodagh population (Marand, East Azarbayejan) (28.79%). These populations also had highest values for Shannon Information Index (0.192 and 0.159, respectively), and gene diversity (0.132, 0.159, respectively).

AMOVA test showed significant genetic difference among *H. armenium* populations (φPT value = 0.55, P = 0.01), indicating their genetic difference. This was supported by significant Gst (0.15, P = 0.01) and D_st (0.11, P = 0.01) values obtained as well as by Hickory (Theta = 0.20) test. AMOVA test revealed that 61% of genetic variability occurred due to within population difference, and 39% occurred due to among populations' difference.

NJ tree and PCoA grouping of the specimens based on ISSR data produced similar results. Therefore, NJ tree is presented and discussed here (Fig. 3). The NJ tree produced three major clusters. Chatan and Oshnavieh populations (Kurdistan and West Azarbayejan provinces, respectively) showed close genetic affinity and formed the first cluster. Similarly, Goran (Kurdistan) and Mamakan (West Azarbayejan) populations were placed in a single cluster, to which, Asadabad population (Hamedan) was joined with some distance. Finally, Marivan population (Kurdistan) was placed in a single cluster far from the other studied populations.

K-Means clustering also produced best clustering according to pseudo-F: k = 3, and best clustering according to Bayesian Information Criterion: k = 6, which are in agreement with NJ tree and AMOVA test. These results indicated the populations' genetic stratification.

Table 2. Genetic diversity parameters in Helichrysum species.

Pop	Ne	I	He	UHe	%P
H. armenium					
Pop1	1.225	0.192	0.132	0.176	31.82
Pop2	1.000	0.000	0.000	0.000	0.00
Pop3	1.153	0.130	0.088	0.106	22.73
Pop4	1.000	0.000	0.000	0.000	0.00
Pop5	1.150	0.128	0.088	0.117	21.21
Pop6	1.204	0.174	0.119	0.159	28.79
H. rubicundum					
Pop7	1.304	0.264	0.178	0.213	46.97
Pop8	1.000	0.000	0.000	0.000	0.00
Pop9	1.193	0.165	0.113	0.151	27.27
Pop10	1.193	0.165	0.113	0.151	27.27
Pop11	1.257	0.246	0.161	0.193	46.97
Pop12	1.246	0.211	0.144	0.192	34.85
Pop13	1.000	0.000	0.000	0.000	0.00
Pop14	1.150	0.128	0.088	0.117	21.21
Pop15	1.303	0.268	0.180	0.215	48.48
Pop16	1.000	0.000	0.000	0.000	0.00
Pop17	1.129	0.110	0.075	0.100	18.18
Pop18	1.000	0.000	0.000	0.000	0.00
Pop19	1.257	0.220	0.151	0.201	36.36
Pop20	1.290	0.254	0.170	0.205	45.45

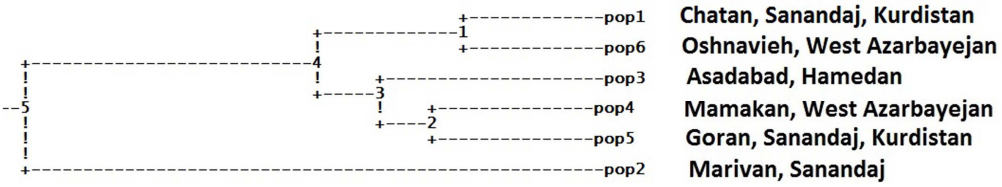

Fig. 3. NJ tree of molecular data in *H. armenium*.

H. rubicundum

In *H. rubicundum*, the highest value for genetic polymorphism (48.48%) was observed in Sero population (West Azarbayejan), followed by Damavand (Alborz, Karaj) and Givi (Ardebil) populations (46.97). These populations also had the highest value for Shannon Information Index and gene diversity.

AMOVA test produced significant difference among the studied populations as well as among *H. rubicundum* populations (φPT value = 0.36, P = 0.01), and revealed that 72% of total genetic variability occurred due to within populations' genetic variability and 28% due to among populations' genetic difference.

The NJ tree (Fig. 4) showed closer genetic affinity between Kaleibar (West Azarbayejan) and Givi (Ardebil) populations. Damavand (Karaj), population joined these two populations with some distance. Naghadeh and Boylarpush populations of West Azarbayejan were placed close to each other and along with the first 3 populations formed the first cluster.

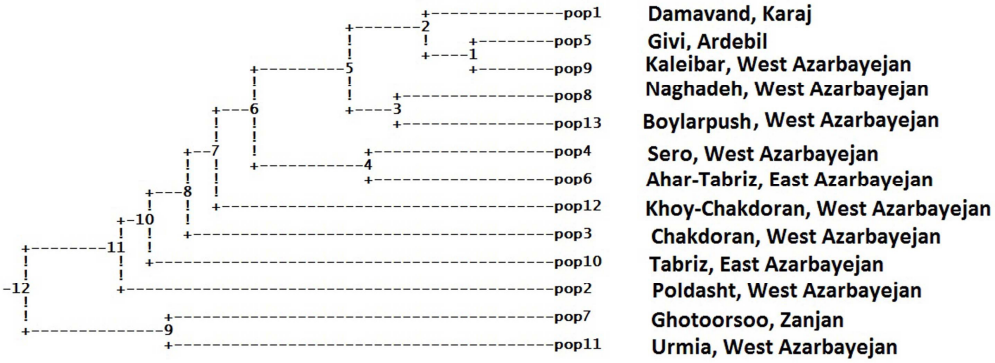

Fig. 4. NJ tree of molecular data in *H. rubicundum*.

Sero (West Azarbayejan) and Ahar-Tabriz (East Azarbayejan) populations formed the second cluster. The other studied populations were scattered in different clusters due to their genetic difference.

K-Means clustering also produced best clustering according to pseudo-F: k = 3, and best clustering according to Bayesian Information Criterion: k = 1, which is in agreement with NJ tree and AMOVA test. These results indicated the populations' genetic stratification.

The Mantel test performed between genetic distance and geographical distance of the studied populations did not produce significant correlation in both species.

Combined analyses

We could get good results for 50 combined analyses and therefore a data matrix of 50 X 66 was formed for genetic analyses. Genetic diversity parameters determined in two studied species are presented in Table 2.

STRUCTURE plot of the studied populations is presented in Fig. 5. The allele combinations are presented by different colors. Difference in the proportion of colored segments indicated the populations' difference in their allele frequency. For example, plants 4-7 in *H. armenium* contained light brown colored segments as the main allelic forms and were different from other plants in this species. Similarly, populations 14, 18 and 20 of *H. rubicundum*, had violet colored segment as the major allelic forms that differed from the other studied plant populations in this species. Therefore, STRUCTURE plot showed some degree of genetic divergence among plant populations in both studied species. Our results are in agreement with AZIZI *et al.* (2014) report. However, SMISSEN *et al.* (2006) reported a weak geographic structure in the endemic complex species of *H. lanceolatum* in New Zealand.

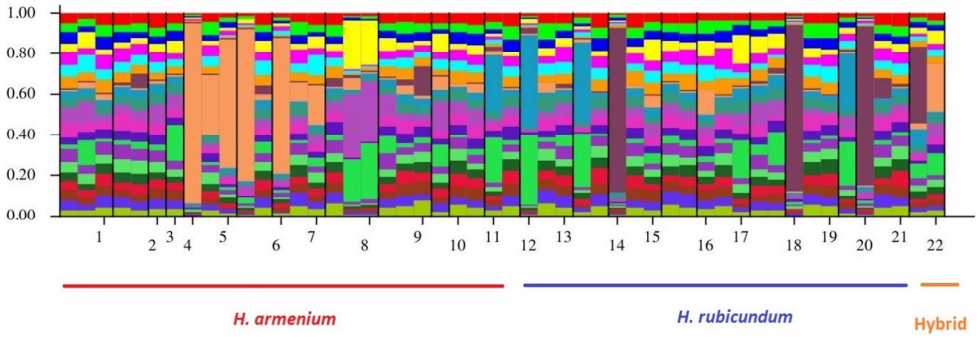

Fig. 5. STRUCTURE plot of molecular data in *H. armenium* and *H. rubicundum*.

Inter-specific gene flow

Estimated mean Nm value obtained as an indicator of gene flow among the studied species produced Nm value of >1.00, that is considered to be high value.

Reticulogram and NeighboeNet diagram produced similar results therefore, only NeighborNet diagram is presented here (Fig. 6). Both analyses revealed that some of the populations from both species were intermixed and were placed in a single cluster due to genetic similarity. This figure revealed occurrence of gene flow between members of different populations in each species (for example populations 10 and 11 of *H. armenium*, and populations 13 and 17 of *H. rubicundum*) and also between members of the two studied species (For example, populations 11 and 12). This result is in agreement with the Mantel test result in both *H. armenium* and *H. rubicundum*.

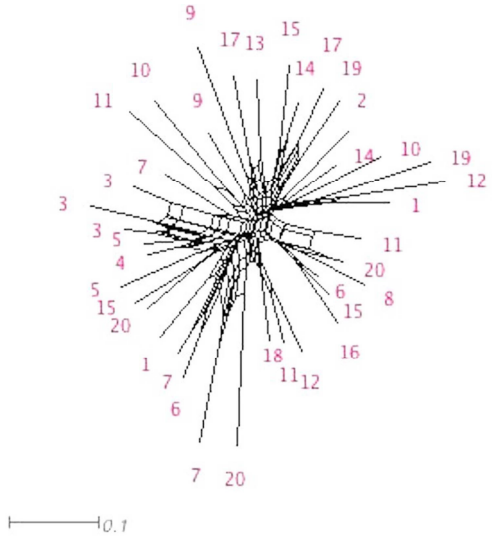

Fig. 6. Neighbor-Net plot of molecular data in *H. armenium* and *H. rubicundum*.

Finally, triangle plot of Bayesian analysis (Fig. 7) produced another support for inter-specific gene flow. It showed genetic admixture of the studied species as the members of these species were placed in different corners of triangle in intermix.

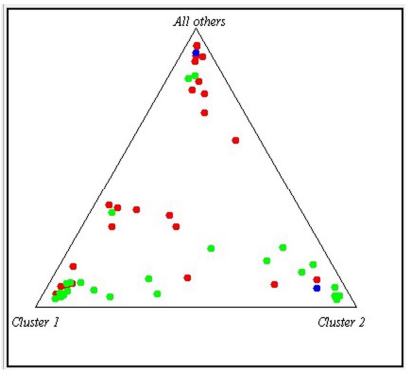

Fig. 7. Triangle plot of molecular data in *H. armenium* and *H. rubicundum*.
Red dots = *H. armenium*, Green dots = *H. rubicundum*.

AMOVA test produced significant difference (P= 0.01) between the two species and K-Means clustering produced the Best k = 2. Therefore, in spite of inter-specific gene flow between *H. armenium* and *H. rubiduncum*, they remained genetically as two separate entities (species).

LFMM test result showed that none of the ISSR loci studied was correlated to the geographical parameters in both studied species. All ISSR loci had $-\log_{10}(p)$ value of <2, which is

not significant. Therefore these loci are not adaptive, and some other reasons like genetic drift might have played rule in genetic divergence of the studied populations.

Potential hybrids

During this study we encountered with plants with intermediate morphological characters from both studied species. These plants formed a distinct group in UPGMA tree and MDS plot of morphological characters as presented before (Figs. 2 and 3).

We performed MST analysis to reveal morphological affinity of the presumed hybrids to the potential parental species (Fig. 8). The MST plot revealed that some plants were morphologically closer to *H. armenium* (plants 4 and 5 in Fig. 8), while some others were closer to *H. rubicundum* (plants 1 and 2 in Fig. 8). This morphological result is in agreement with genetic result obtained by triangle plot presented before (Fig. 7). The latter plot also showed that some plants with intermediate morphological characters were genetically closer to *H. armenium*, while some others were closer to *H. rubicundum*.

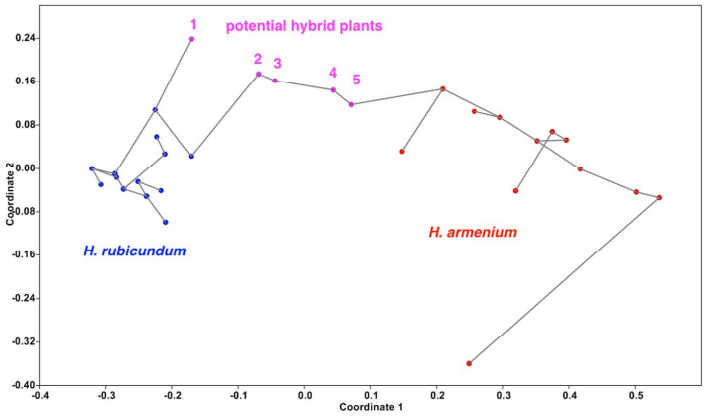

Fig. 8. Minimum spanning tree (MST) of morphological characters.

We performed population assignment and Hybrid index tests to illustrate genetic affinity between putative hybrid plants and their potential parental species i.e. *H. armenium and H. rubicundum*. Three-nine randomly studied parental plants and putative hybrids were used in this analysis.

Population assignment test result is presented in Table 3. The result revealed that some plants of *H. armenium* are genetically close to *H. rubicundum* (for example, plants number 3, 10 and 11). Similarly, and vise versa (plants number 12 and 28 of *H. rubicundum* were close to *H. armenium*). Moreover, plants number 37 and 39 (putative hybrid plants) were close to *H. rubicundum* while, plant number 38 was close to *H. armenium*.

We obtained 100% hybrid index value (h = 1) and genetic resemblance for plant number 37 with *H. armenium* as the reference population, and h = 0.54 for plant No. 38. Similarly, a higher h value (0.76) was obtained for plant No. 38, when *H. armenium* was the reference population. Therefore, both population assignment and Hybrid index methods revealed that

different degrees of gene flow between *H. armenium* and *H. armenium* had resulted to the formation of hybrid plants. A similar phenomenon reported in the other *Helichrysum* species by GALBANY-CASALS *et al.* (2006, 2011, 2012) and SMISSEN *et al.* (2007).

Table 3. Population assignment test based on maximum likelihood.

Individual	Current	Inferred	Lik_max	Lik_home	Lik_ratio	Pop001	Pop002
1	Pop001	Pop002	-30.567	-36.248	11.362	-36.248	-30.567
2	Pop001	Pop001	-28.103	-28.103	0	-28.103	-33.628
3	Pop001	Pop002	-34.706	-38.712	8.012	-38.712	-34.706
4	Pop001	Pop001	-20.296	-20.296	0	-20.296	-32.157
5	Pop001	Pop001	-26.133	-26.133	0	-26.133	-31.521
6	Pop001	Pop001	-31.46	-31.46	0	-31.46	-39.722
7	Pop001	Pop001	-19.156	-19.156	0	-19.156	-27.495
8	Pop001	Pop001	-19.379	-19.379	0	-19.379	-26.882
9	Pop001	Pop001	-36.073	-36.073	0	-36.073	-36.542
10	Pop001	Pop002	-34.75	-49.345	29.19	-49.345	-34.75
11	Pop001	Pop002	-20.258	-23.988	7.459	-23.988	-20.258
12	Pop002	Pop001	-29.617	-33.868	8.503	-29.617	-33.868
13	Pop002	Pop002	-26.416	-26.416	0	-28.752	-26.416
14	Pop002	Pop002	-50.175	-50.175	0	-64.449	-50.175
15	Pop002	Pop002	-32.6	-32.6	0	-33.462	-32.6
16	Pop002	Pop002	-41.474	-41.474	0	-50.264	-41.474
17	Pop002	Pop002	-26.488	-26.488	0	-31.857	-26.488
18	Pop002	Pop002	-35.81	-35.81	0	-55.066	-35.81
19	Pop002	Pop002	-32.337	-32.337	0	-44.783	-32.337
20	Pop002	Pop002	-29.111	-29.111	0	-36.709	-29.111
21	Pop002	Pop002	-40.919	-40.919	0	-52.423	-40.919
22	Pop002	Pop002	-25.555	-25.555	0	-35.403	-25.555
23	Pop002	Pop002	-31.913	-31.913	0	-44.287	-31.913
24	Pop002	Pop002	-39.326	-39.326	0	-61.161	-39.326
25	Pop002	Pop002	-33.3	-33.3	0	-38.65	-33.3
26	Pop002	Pop002	-21.69	-21.69	0	-28.397	-21.69
27	Pop002	Pop002	-27.196	-27.196	0	-34.135	-27.196
28	Pop002	Pop001	-26.957	-36.535	19.156	-26.957	-36.535
29	Pop002	Pop002	-39.638	-39.638	0	-42.756	-39.638

30	Pop002	Pop002	-23.073	-23.073	0	-30.13	-23.073
31	Pop002	Pop002	-28.638	-28.638	0	-40.295	-28.638
32	Pop002	Pop002	-34.721	-34.721	0	-45.099	-34.721
33	Pop002	Pop002	-32.322	-32.322	0	-45.043	-32.322
34	Pop002	Pop002	-28.728	-28.728	0	-35.582	-28.728
35	Pop002	Pop002	-41.754	-41.754	0	-56.245	-41.754
36	Pop002	Pop002	-29.331	-29.331	0	-49.55	-29.331
37	Pop003	Pop002	-45.85	---	---	-68.033	-45.85
38	Pop003	Pop001	-28.249	---	---	-28.249	-32.089
39	Pop003	Pop002	-23.369	---	---	-23.856	-23.369

Populations, number: 1 = *Helichrysumarmenium*, 2 = *H. rubicundum*, and 3 = potential hybrids.

Cytological variability

Meiotic studies were performed in populations of *H. armenium* and *H. rubicundum*. Details of data obtained for polyploidy level, chiasma frequency and chromosome pairing are provided in Table 4, Figs. 9 and 10.

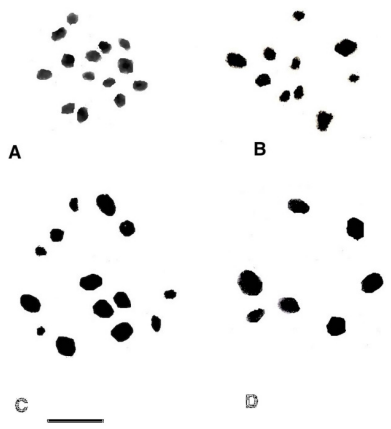

Fig. 9. Representative meiotic cells in *H. armenium* and *H. rubicundum*.
A = Meiocyte showing 2n = 28 in Khoy-Chaldoran population of *H. rubicundum*. B = Meiocyte showing 2n = 14 and few univalent in 2 Km Tabriz-Ahar population of *H. rubicundum*. C and D = Meiocytes showing 2n = 28 and 2n = 14 in Mishodagh and Boylarpush populations of *H. armenium*. Bar line = 10 µm

Cytological study showed the occurrence of 2n = 2x = 14 and 2n = 4x =28 in the studied populations. Previously 2n = 8x = 56 chromosome number was reported for *H. armenium* (Chariat-Panahi et al. 1982), while 2n = 4x = 28 was cited for *H. rubicundum* (GHAFFARI, 1989).

Table 4. Relative cytogenetic data in Helichrysum species and populations.

Speceis	Population	n	TOX	TX	IX	UNI	ROD	RB	IV
H. armenium	Mishodagh	14	1.21	0.31	0.90	0.18	0.32	0.45	0.00
	Garan passage	7, 14	1.21	0.40	0.81	0.19	0.40	0.40	0.00
	Boylarpush	7	1.54	0.34	1.20	0.06	0.34	0.60	0.00
	Ariz passage	14	1.48	0.49	0.99	0.07	0.39	0.52	0.01
	Oshnavieh	7, 14	1.39	0.58	0.81	0.04	0.54	0.41	0.01
	Chetan	7, 14	1.34	0.60	0.74	0.05	0.56	0.37	0.01
	Abidar	7, 14	1.49	0.41	1.08	0.07	0.36	0.54	0.01
H. rubicundum	23 Km Givi, Ardebil	7, 14	1.49	0.66	0.83	0.04	0.45	0.43	0.04
	43 Km Givi, Ardebil	7, 14	1.39	0.53	0.86	0.08	0.46	0.43	0.02
	Poldasht be Jolfa	7, 14	1.57	0.63	0.95	0.04	0.38	0.48	0.05
	SoroChaldoran	7, 14	1.24	0.57	0.67	0.10	0.55	0.35	0.00
	Ghasemloo valley	7, 14	1.44	0.56	0.89	0.01	0.53	0.44	0.01
	Rend village	7, 14	1.48	0.54	0.93	0.03	0.46	0.47	0.02
	Naghadeh	7, 14	1.49	0.53	0.95	0.01	0.50	0.48	0.01
	Khoy-Chaldoran	7, 14	1.46	0.65	0.81	0.03	0.48	0.41	0.04
	15 Km Tabriz-Ahar	7, 14	1.63	0.33	1.31	0.03	0.31	0.65	0.00
	2 Km Tabriz-Ahar	7, 14	1.26	0.60	0.66	0.07	0.60	0.33	0.00
	22 Km Tabriz-Ahar	7, 14	1.42	0.53	0.90	0.03	0.51	0.46	0.00
	Damavand 1, Alborz	7, 14	1.53	0.40	1.13	0.02	0.40	0.56	0.01
	Damavand 2, Alborz	14	1.46	0.45	1.01	0.06	0.42	0.51	0.01

Abbreviations: TOX = Total chiasmata per bivalent, TX = Terminal chiasmata per bivalent, IX = Intercalary chiasmata per bivalent, UNI = No. Univalents per cell, ROD = No. of rod bivalents per cell, RB = No. of ring bivalents per cell, IV = No. ofquadrivalents per cell.

The highest value for total chiasma/ bivalent occurred in 15 Km Tabriz-Ahar (1.63), while the highest value of terminal chiasmata/ bivalent occurred in 23 Km Givi, Ardebil (0.66). Similarly, the highest value for intercalary chiasmata/ bivalent was observed in Boylarpush (1.20). The populations studied formed mostly ring and rod bivalents, although few univalent and quadrivalents were formed in some of the studied populations (Table 4, Fig. 9, B).

Pearson' coefficient of correlation determined between cytogenetic features revealed significant positive correlation between total, terminal and intercalary chiasmata and ring bivalents ($P = 0.05$). It showed significant negative correlation between chiasma parameters and number of univalents and rod bivalents ($P = 0.05$) (Fig. 10). However, no correlation was found between meiotic features and longitude, latitude and elevation. This result indicated that cytological differences are not correlated with geographical parameters as was previously observed also for ISSR loci studied.

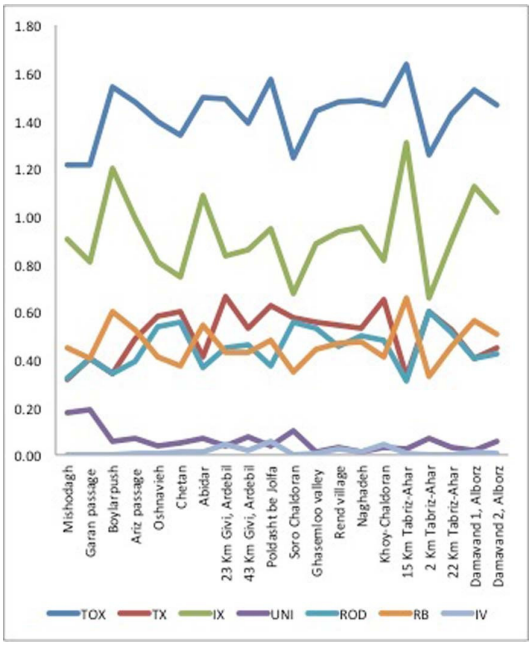

Fig. 10. Plot of cytological data showing patterns of variation in *H. armenium* and *H. rubicundum*. Abbreviations: TOX = Total chiasmata per bivalent, TX = Terminal chiasmata per bivalent, IX = Intercalary chiasmata per bivalent, UN = No. Univalents per cell, ROD = No. of rod bivalents per cell, RB = No. of ring bivalents per cell, IV = No. of quadrivalents per cell.

ANOVA (Analysis of variance) test performed among plants with 2n = 4x = 28 revealed significant difference (P = 0.01) for all meiotic features, while the same test performed among plants with 2n = 2x = 14 did not produce significant difference.

Chiasma frequency and distribution is under genetic control and significant change in their frequency is considered as a mean for genetic recombination and diversity that can be used by plants with similar chromosome number to adapt to their local environment (SHEIDAI *et al.* 2013). Our data showed that significant degree of change had occurred for genes that control chiasma formation and localization in plants with 2n = 4x = 28.

UPGMA clustering of *H. armenium* and *H. rubicundum* based on meiotic characteristics revealed their inter-population cytogenetic variability (Figs. 11 and 12). In *H. armenium*, the studied populations were grouped in three clusters. Oshnavieh (West Azarbayejan province) and Chetan (Kurdistan) populations showed meiotic similarity and were placed in one cluster, while Garan passage (Kurdistan) and Mishodagh populations formed the second cluster. Abidar, Ariz passage and Boylarpush differed cytogenetically from the other two clusters and formed the third cluster.

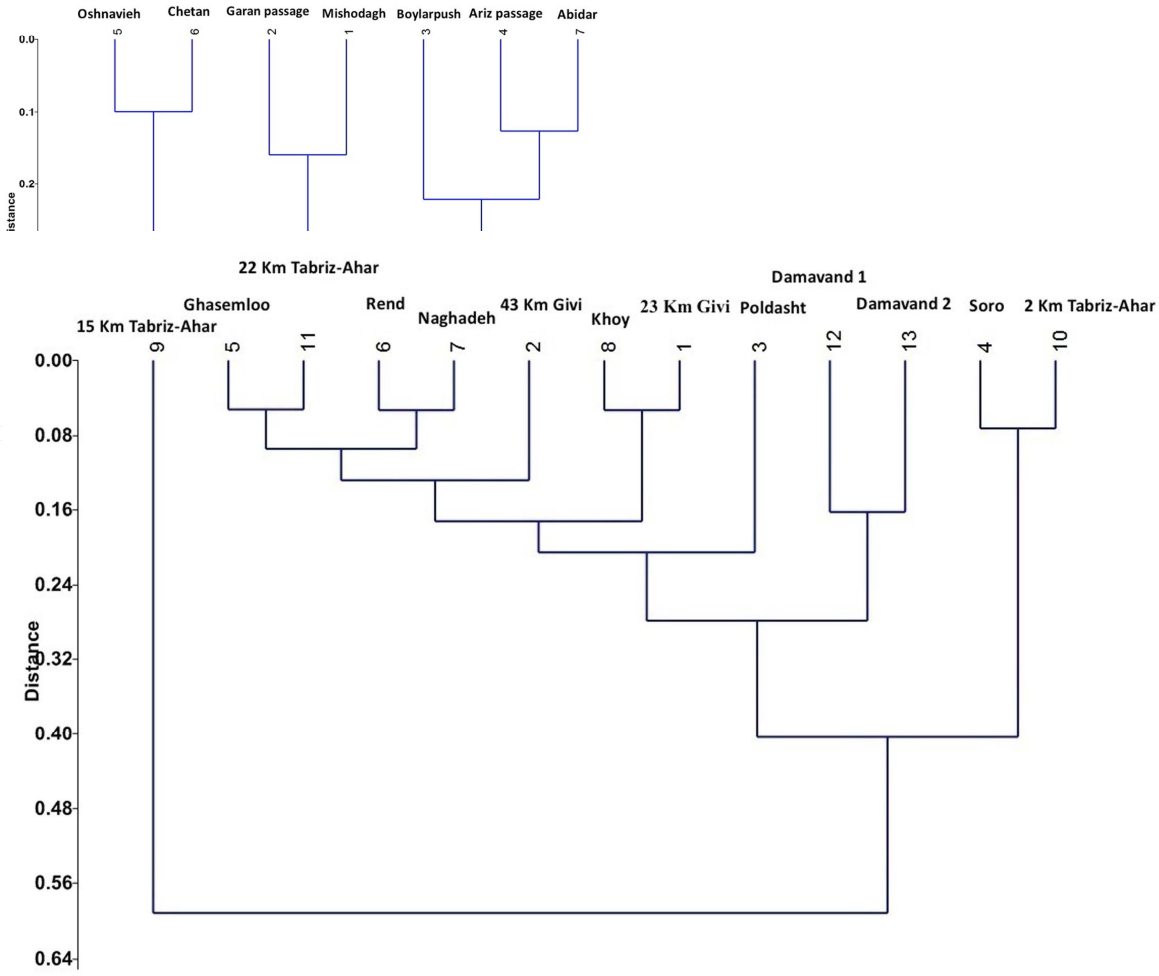

Fig. 12. UPGMA tree of cytological data *H. rubicundum*.

In *H. rubicundum*, 15 Km Tabriz-Ahar differed greatly in its cytogenetic features and formed a separate cluster (Fig. 12). Plants studied from two different locations of Damavand also formed a separate cluster, while Soro and 2 Km Tabriz-Ahar joined them with some distance. Polyploidy and chromosomal structural changes along with hybridization have been considered as important mechanism of speciation in *Helicrysum* (GALBANY-CASALS *et al.* 2012; AZIZI *et al.* 2014). Therefore, a combination of genetic variability, gene flow and genetic admixture as well as polyploidy and chromosomes structural changes, have played rule in *Helichrysum* diversification.

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REFERENCES

AZIZI, N., M. SHEIDAI, V. MOZAFARIAN and Z. NOORMOHAMMADI (2014): Genetic, cytogenetic and morphological diversity in *Helicrysum leucocephalum* (Asteraceae) populations. Biologia 69, 566-573.

BUERKLE, C.A. (2005): Maximum-likelihood estimation of a hybrid index based on molecular markers. Mol Ecol Notes. *5*, 684-687.

CHARIAT-PANAHI, M.S., H. LESSANI and D. CARTIER (1982): Etude caryologique de quelques espèces de la flore de l'Iran. Rev Cytol Biol Veg Bot. *5,* 189–197.

FALUSH, D., M. STEPHENS and J.K. PRITCHARD (2007): Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes. doi: 10.1111/j.1471-8286.2007.01758.x

FREELAND, J.R., S. KIRK and D. PETERSEN (2011):. Molecular Ecology, 2nd ed. WILLY-BLACKWELL, London. 2011; pp. 449.

FRICHOT, E., S.D. SCHOVILLE, G. BOUCHARD and O. FRANCOIS (2013): Testing for Associations between Loci and environmental Gradients Using Latent Factor Mixed Models. Mol Biol Evol. *30*, 1687-1699.

GALBANY-CASALS, M., J.M. BLANCO-MORENO, N. GARCIA-JAXAS, I. BREITWIESER and R.D. SMISSEN (2011): Genetic variation in Mediterranean Helichrysum italicum (Asteraceae; Gnaphalieae): do disjunct populations of subsp. microphyllum have a common origin? Plant Biol. *13*(4), 678–687. http://dx.doi.org/10.1111/j.1438-8677.2010.00411.x.

GALBANY-CASALS, M., P. CARNICERO-CAMPMANY, J.M. BLANCO-MORENO and R.D. SMISSEN (2012): Morphological and genetic evidence of contemporary intersectional hybridisation in Mediterranean Helichrysum (Asteraceae, Gnaphalieae). Plant Biol. *14*, 789-800.

GALBANY-CASALS, M., N., GARCIA-JACAS, C., LLORENC SAEZ and A. SUSANNA (2009): Phylogeny, biogeography, and character evolution in Mediterranean, Asiatic, and Macaronesian Helichrysum (Asteraceae, Gnaphalieae) inferred from nuclear phylogenetic analyses. Int J Plant Sci. *170*, 365-380. DOI: 10.1086/596332.

GALBANY-CASALS, M., L., SAEZ, and C. BENEDI (2006): A taxonomic revision of *Helichrysum* sect. *Stoechadina* (Asteraceae, Gnaphalieae). Can J Bot. *84*, 1203-1232.

GASKIN, J.F. and D.J. KAZMER (2009): Introgression between invasive saltcedars (*Tamarix chinensis* and *T. ramosissima*) in the USA. Biol Invasions. 11, 1121–1130. DOI 10.1007/s10530-008-9384-1.

GHAFFARI, S.M. (1989): Chromosome studies in Iranian Compositae. Iran J Bot *4*, 189–196.

HAMER, OM., D.A.T HARPER and P.D. RYAN (2012): PAST: Paleontological Statistics software package for education and data analysis. Palaeontol Elect. *4,* 9.

HOLSINGER, K.E. and P.O. LEWIS (2003): Hickory: a package for analysis of population genetic data V1.0. Available at http://www.eeb.uconn.edu; Accessed 2003.

HUSON, D.H. and D. BRYANT (2006): Application of Phylogenetic Networks in Evolutionary Studies, Mol Biol Evol. *23*(2), 254-267. Software available from www. splitstree.org.

JOST, L (2008): GST and its relatives do not measure differentiation. Mol Ecol. *17*, 4015-4026.

MEIRMANS, P.G. and P.H. VAN TIENDEREN (2004): GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. Mol Ecol Notes. *4*, 792-794.

MEIRMANS, P.G. and P.H. VAN TIENDEREN (2012): The effects of inheritance in tetraploids on genetic diversity and population divergence. Heredity *110*, 131–137.

NOORMOHAMMADI, Z., H. SAMADI-MOLAYOOSEFI and M. SHEIDAI (2012): Intra-specific genetic diversity in *O. cuspidata* subsp. *cuspidata* in Hormozgan province. Genet Mol Res. *11*, 707-716.

PEAKALL, R. and P.E. SMOUSE (2006): GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes. *6*, 288-295.

PODANI, J. (2000): Introduction to the Exploration of Multivariate Data [English translation], Leide, Netherlands: Backhuyes.

SHEIDAI, M., K. KOLAHIZADEH, Z. NOORMOHAMMADI, N. AZANI and M. NIKOO (2012): Correlation between geography and cytogenetic diversity in Pomegranate (*Punica granatum* L.) cultivars. Acta Bot Bracil. *26*(4), 948-957.

SMISSEN, R.D., I. BREITWIESER and J.M. WARD (2006): Genetic diversity in the New Zealand endemic species *Helichrysum lanceolatum* (Asteraceae : Gnaphalieae). New Zealand J Bot. *44*(3), 237-247.

SMISSEN, R.D., I BREITWIESER and J.M. WARD (2007): Genetic characterization of hybridization between the New Zealand everlastings *Helichrysum lanceolatum* and *Anaphalioides bellidioides* (Asteraceae: Gnaphalieae). Bot J Linnean Soc. *154*(1), 89-98.

TERO, N., J. ASPI, P. SIIKAMAKI, A. JAKALANIEMI and J. TUOMI (2003): Genetic structure and gene flow in a metapopulation of an endangered plant species, *Silene tatarica*. Mol Ecol. *1*2, 2073-2085.

WEISING K., H. NYBOM, K. WOLFF and G. KAHL (2005): DNA Fingerprinting in Plants. Principles, Methods, and Applications. (2nd ed.), Boca Rayton, Fl., USA: CRC Press, pp. 472.

POPULACIONO - GENETIČKA ANALIZA I EVIDENCIJA UNUTAR SPECIJSKE INTROGRESIJE KOD *Helichrysum armenium* and *H. rubicundum* (Asteraceae)

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Izvod

Helichrysum armenium i *H. rubicundum* su značajne lekovite biljke za Iran i proširene su u različitim regionima Irana. Vršena su istraživanja 66 slučajno sakupljene biljke iz 24 populacije *Helichrysum armenium* and *H. rubicundu.* UPGMA and MDS analizama su potvrđene morfološke separacije ovevrlo bliske biološke vrste a intermedijalne biljke su smeštene u intermedijalnu poziciju. ISSR analize su potvrdile inter-populacioni genetički diverzitet a analize K- sredine , grupisanja i strukture su pokazale genetičku u ispitivanim vrstama. Kombinacijom genetičke startifikacije i genetičkog mešanja kao i poliploidija i strukturalne promene hromozoma su igrale ulogu kod *Helichrysum* diverzifikaciji.

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