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Original scientific paper

CONTEMPORARY INTER-SPECIFIC HYBRIDIZATION BETWEEN *Cirsium aduncum* AND *C. Haussknechtii* (Asteraceae): EVIDENCE FROM MOLECULAR AND MORPHOLOGICAL DATA

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Cirsium aduncum Fisch. & C.A.Mey. Ex DC. and *C. haussknechtii* Boiss., (Asteraceae) are important medicinal plant species that grow in different geographical regions of Iran. We had no knowledge about population genetic structure, intra-specific and inter-specific gene flow and the presence of hybrid zone for this two species in Iran. Therefore, in order to provide data for conservation of these two medicinally important species, the population genetic analysis and morphometric studies were performed in 18 geographical populations of these species. ANOVA and MDS analyses revealed significant morphological difference among the studied populations in either species, while MDS plot showed morphological overlap in plants of these two species. AMOVA test revealed significant genetic difference among the studied populations. Mantel test showed positive significant correlation between genetic and geographical distances and the occurrence of isolation by distance. Population assignment test and STRUCTURE plot of genetic data revealed inter-specific introgression between these species.

Keywords: AMOVA, *Circium*, gene flow, population assignment, STRUCTURE analysis.

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INTRODUCTION

The genus *Cirsium* Mill. (Asteracea) contains about 250 perennial, biennial or rarely annual spiny species. These species grow in the Northern hemisphere, Europe, North Africa, Siberia, Central Asia, West and East Africa, as well as Central America (ZOMLEFER, 1994; BURES *et al*., 2004; SEGARRA-MORAGUES *et al*., 2007).

Cirsium species show wide ranges of ecological adaptations in places they grow and occupy different localities with regard to elevation, temperature and edaphic factors due to their genetic adaptability and plasticity. They also are well known for interspecific hybrid formation and wide gene flow, which helps their adaptation to various environmental conditions (BUREŠ *et al.*, 2004).

Most of *Cirsium* species are of medicinal values and have been used to reduce fever, blood cholesterol and triglyceride level and also used against jaundice. Some of the *Cirsium* species have been used as food plants (ORHAN *et al*., 2013; ABBET *et al*., 2014).

Population genetics study particularly analysis of genetic diversity is important in plant conservation and molecular ecology studies (FREELAND *et al*., 2011). It provides data on the genetic variability, gene flow versus population genetic isolation, population genetic fragmentation, the role of genetic drift, the bottleneck and any other evolutionary forces acting on populations' divergence (SHEIDAI *et al*., 2012; 2013; 2014). Population genetic studies can also trough light on hybridization and inter-specific gene flow between the species that grow in overlapping geographical regions (ALBALADEJO and APARICIO, 2007, IJBARI *et al*., 2014).

Cirsium aduncum Fisch. & C.A.Mey. Ex DC. and *C. haussknechtii* Boiss., have medicinal and food values and are widely used by locals. We have no information available about genetic diversity, population genetic structure and gene flow among these medicinal species in Iran. Therefore, the present investigation was carried out to provide data about population genetic structure and morphological variability of 18 geographical populations that may be of help in conservation of these species.

Multilocus molecular markers such as simple sequence repeat (SSR) markers and inter-simple sequence repeat (ISSR) markers are useful in studying introgressive hybridization (GASKIN and KAZMER, 2009). Therefore, for population genetic study and to illustrate of inter-species gene flow, we used ISSR markers that are reproducible, simple to work and not expensive and were shown to be informative for genetic diversity and population structure studies in the genus *Cirsium* (SEIF *et al*., 2012; NOUROOZI *et al*., 2013; SHEIDAI *et al*., 2013).

The genus *Cirsium* is well known for production of inter-specific hybrids and the occurrence of morphological variability and intergradation of diagnostic characters (DABEYDEEN, 1980). Therefore, we studied morphological variability in the studied species and populations too. The intergradation of diagnostic morphological characters along with genetic admixture have been considered as strong evidence for inter-specific genetic introgression (DABEYDEEN, 1980; GALBANY-CASALS *et al*., 2012; IJBARI *et al*., 2014).

MATERIALS AND METHODS

Plant materials

Sixty-one plant accessions were collected from 18 geographical populations of *Cirsium aduncum* Fisch. & C.A.Mey. Ex DC. and *C. haussknechtii* Boiss. Details of localities are provided in Table 1. Voucher specimens are deposited in Herbarium of Shahid Beheshti

University (HSBU). Fresh leaves were collected and used for DNA extraction and molecular study.

Table 1. Circium species and populations studied, their locality and voucher No.

Population	Species	Latitude	Longitude	Localities and voucher No.
1	*C. aduncum*	37°43'31.95"	46°58'0.19"	East Azarbaijan:Mianeh-Bostanabad, 1844 m, Nouroozi, 2010500-HSBU.
2	*C. aduncum*	38°21'39.77"	46°51'42.79"	East Azarbaijan: Tabriz-Ahar,1794m, Naji2010506HSBU.
3	*C. aduncum*	37°42'33.90"	46°58'50.34"	East Azarbaijan : Mianeh-Bostanabad , 1830 m , Naji , 2010507-HSBU.
4	*C. aduncum*	38°25'41.09"	47°15'5.17"	East Azarbaijan : Ahar-Meshkinshahr , 1257 m , Naji , 2010508-HSBU.
5	*C. aduncum*	38°20'40.23"	45°50'1.95"	East Azarbaijan : Marana-Tabriz , 1736 m , Naji , 2010509-HSBU.
6	*C. aduncum*	37°51'13.29"	46°47'9.22"	East Azarbaijan : Bostanabad-Mianeh , 1778 m , Nouroozi , 2010501-HSBU.
7	*C. aduncum*	38°22'47.45"	47°29'28.83"	Ardebil: Meshkinshahr, Mazraejahan, 1169 m, Nouroozi & Fathollahi, 8600170-HSBU.
8	*C. aduncum*	38°29'17.29"	48° 0'0.59"	Ardabil : Meshkinshahr-Ardebil , 1400 m , Nouroozi , 8600172-HSBU.
9	*C. aduncum*	38°26'50.12"	47°13'26.53"	East Azarbaijan : Ahar-Meshkinshahr , 1300 m , Nouroozi , 2010502-HSBU.
10	*C. aduncum*	38°27'1.91"	44°40'25.73"	West Azarbaijan : Qatur-khoy , 1500 m , Nourooz , 8600171-HSBU.
11	*C. aduncum*	37°38'33.40"	47° 4'46.38"	East Azarbaijan : Bostanabad-Mianeh , 1675 m , Nouroozi , 2010503-HSBU.
12	*C. aduncum*	38°23'1.50"	46°49'45.26"	East Azarbaijan : Ahar - tabriz , Goijabel , 1850 m , Nouroozi , 2010504-HSBU.
13	*C. haussknechtii*	37°54'28.96"	46° 9'47.88"	East Azarbaijan : Kandavan, Hilevar village, 1600 m, Nouroozi, 8600187-HSBU.
14	*C. haussknechtii*	38°49'56.61"	47° 3'15.91"	East Azarbaijan : Ahar - kaleibar , 1200 m , Nouroozi , 8600190-HSBU.
15	*C. haussknechtii*	38°49'20.98"	45°49'16.88"	East Azarbaijan : Jolfa , kiamaki , Daran village , 1455 m , Nouroozi , 8600188-HSBU.
16	*C. haussknechtii*	38°10'58.80"	44°48'11.91"	West Azarbaijan : Urmia-Salmas , 1380 m , Nouroozi , 8600191-HSBU.
17a	*C. haussknechtii*	37°42'19.04"	48°28'11.95"	Ardabil : Ardebil - khalkhal , 1615 m , Nouroozi , 2010505-HSBU.
17b	*C. haussknechtii*	37°53'29.04"	48°23'36.84"	Ardabil : Ardebil - khalkhal , 1650 m , Nourooz , Nouroozi , 8600189-HSBU.
18	*C. haussknechtii*	38°23'28.89"	48°26'42.05"	Ardabil : Ardebil-Namin , 1353 m , Naji , 2010510-HSBU.

Morphological studies

Morphological characters studied included quantitative and qualitative characters that are given in Table 2.

Table 2. Morphological characters studied.

1	Pappus length
2	Capitulum length (With floret)
3	Involucre length
4	Involucre width
5	Length of the involucre bract
6	Width of the involucre bract
7	Length the terminal spine in Involucre Bract
8	Length of the middle lobe of leaf
9	Width of the middle lobe of leaf
10	Length of spine in the middle lobe leaf
11	Length of the terminal spine in Leaf
12	Length of the spine in leaf margin
13	Corolla length
14	Corolla tube length
15	Lamina length
16	Length of the shortest corolla lobe
17	Length of the longest corolla lobe
18	Ratio of length to width of involucre
19	Ratio of length to width of involucre bract
20	Ratio of lamina to corolla tube
21	Ratio of corolla tube to corolla
22	Ratio of lamina to corolla
23	Ratio of tallest to shortest corolla lobe
24	Ratio of length to width of middle lobe in leaf
25	Ratio of length to width of leaf
26	Ratio of middle lobe spine to terminal spine in leaf
27	Cover of the upper surface of leaf
28	Cover of the lower surface of leaf
29	Stem cover
30	Position of the last leaf to capitulum
31	Cover of the involucre bract

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 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)9T 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: 10 Min initial denaturation step 94°C, 30 S at 94°C; 1 Min at 57°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).

The ISSR analysis performed was tested for marker reproducibility/genotyping errors, by repeating the experiment 3 times and scoring only the sharp and consistent bands obtained (POMPANON *et al*., 2005). Moreover, the initial DNA material was obtained from 10 randomly collected leaves in each replication. Although we did not calculate error rate for the studied samples, we performed Hickory test for ISSR data analysis that is Bayesian approach based method and run the program for 3 times for consistency. Moreover, we used no DNA sample in ISSR-PCR for each primer *per se*.

Data analyses

Morphometry

The analysis of variance (ANOVA) test was used to reveal significant morphological difference among the studied populations. For grouping of the plant specimens, UPGMA (Unweighted Paired Group with Arithmetic Average) and multidimentional scaling (MDS) were used. Morphological data were standardized (mean = 0, variance = 1) for these analyses (PODANI, 2000). Principal components analysis (PCA) was used to identify the most variable morphological characters among the studied populations.

Genetic diversity and population structure

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). The genetic diversity parameters like, Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (FREELAND *et al*., 2011, WEISING *et al*., 2005), were determined for each species. Nei's genetic distance was used for clustering. Neighbor Joining (NJ) clustering and Neighbor-Net method of networking were used for grouping after 100 times bootstrapping/ permutations (FREELAND *et al*., 2011; HUSON and BRYANT, 2006). The Mantel test was performed to check correlation between geographical and the genetic distances of the studied populations (PODANI, 2000). PAST ver. 2.17 (HAMMER *et al.*, 2012) and, DARwin ver. 5 (2012) programs were used for these analyses.

AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (PEAKALL and SMOUSE, 2006), and Nei' Gst analysis of GenoDive ver.2 (2013) (MEIRMANS and VAN TIENDEREN 2004), were used to reveal significant genetic difference among

the studied species (SHEIDAI *et al*., 2014).

The population genetic differentiation was studied by G'st_est = standardized measure of genetic differentiation (HEDRICK, 2005), and D_est = Jost measure of differentiation (JOST, 2008). In order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program, Hickory (ver. 1.0) (HOLSINGER and LEWIS, 2003), was used to estimate parameters related to genetic structure (theta B value) (TERO *et al*., 2003).

PCoA plot produced after 5000 permutation (as performed in GeneAlex) and Bayesian based model STRUCTURE analysis (PRITCHARD *et al.*, 2000), was used to study the genetic structure of populations. For STRUCTURE analysis, data were scored as dominant markers (FALUSH *et al*., 2007). The Evanno test (EVANNO *et al.*, 2005) was used to identify optimum k genetic groups (SHEIDAI *et al*., 2014).

Gene flow

Gene flow was determined by different approaches. 1- Calculating Nm an estimate of gene flow from Gst by PopGene version 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst. This approach considers equal amount of gene flow among all populations. 2- STRUCTURE analysis based on admixture model and Bayesian approach (PRITCHARD *et al.*, 2000), and 3- population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013).

RESULTS

Cirsium aduncum

ANOVA test revealed significant morphological difference for quantitative characters ($P < 0.01$) among the studied populations in *Circium aduncum*. Moreover, MDS plot separated almost all plant populations indicating significant difference for all morphological characters studied (Fig. 1).

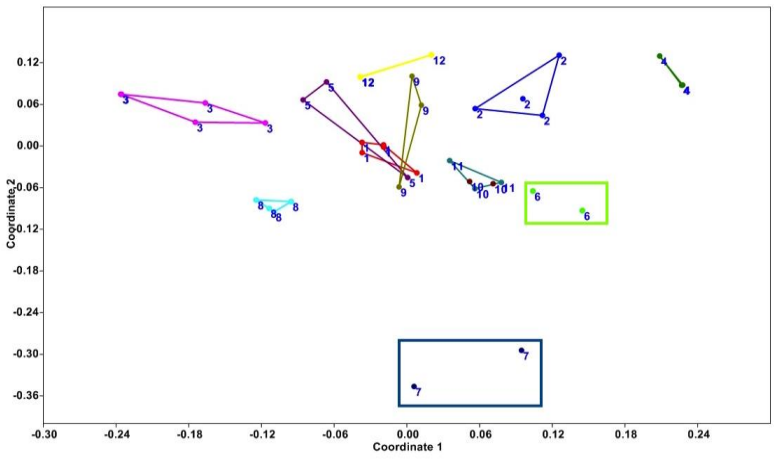

Fig. 1. MDS plot of *Circium aduncum* populations based on morphological characters. (Populations' No. are according to Table 1).

PCA analysis revealed that the first 3 axes comprised about 82% of total variance and morphological characters 12 (r = -.72), 27 and 28 (r = 0.99), (in the first axis), as well as characters 7 (r = -0.72), and 26 (6 = 0.60) (in the second axis), were identified as the most variable characters. These results revealed morphological discontinuities among geographical populations of *C. aduncum*.

Genetic diversity parameters determined in 12 studied populations of *Cirsium aduncum* is presented in Table 3. Marand-Tabriz population (population 5) had the highest value for Ne (1.24), Shannon Information Index (I = 0.20), gene diversity (He = 0.13) and genetic diversity due to population (Hs = 0.227). Tabriz-Ahar population (population 2) had the highest value for percentage of polymorphism (41.18%).

Table 3. Genetic diversity parameters in populations of Cirsium aduncum. (Population numbers are according to Table 1).

Pop	Na	Ne	I	He	UHe	%P	Hs
Pop1	0.753	1.204	0.177	0.120	0.130	31.76%	0.160
Pop2	0.929	1.202	0.198	0.127	0.146	41.18%	0.218
Pop3	0.894	1.214	0.196	0.128	0.142	40.00%	0.184
Pop4	0.471	1.071	0.057	0.039	0.047	9.41%	0.063
Pop5	0.859	1.245	0.201	0.138	0.165	34.12%	0.227
Pop6	0.435	1.083	0.071	0.049	0.065	11.76%	0.118
Pop7	0.388	1.100	0.085	0.058	0.078	14.12%	0.141
Pop8	0.659	1.178	0.142	0.098	0.112	23.53%	0.131
Pop9	0.741	1.199	0.172	0.116	0.139	30.59%	0.204
Pop10	0.600	1.132	0.124	0.082	0.098	23.53%	0.157
Pop11	0.424	1.083	0.071	0.049	0.065	11.76%	0.118
Pop12	0.565	1.141	0.117	0.080	0.096	20.00%	0.133

Na = No. of alleles, Ne = No. of effective alleles, He = Gene diversity, Uhe = Unbiased gene diversity, %P = Percentage of polymorphism, and Hs = Genetic diversity due to populations. Populations 13-18 are: Hilevar village, Ahar-Kalibar, Jolfa, Daran village, Urmeyah, Salmas, Ardebil, Khalkhal, and Ardebil, Namin.

AMOVA test revealed significant molecular difference (P = 0.01) among the studied populations. It showed that 54% of total genetic variability occurred due to among populations genetic difference, while 46% was due to within population genetic variability. Pairwise Fst values obtained for the studied populations were significant (P = 0.01). This indicated genetic divergence of all studied populations. High Hickory theta B value (0.40) obtained supported AMOVA result. This was supported by significant G'st (0.63, P = 0.01) and D_st (0.65, P = 0.01) values obtained as well as by Hickory (Theta = 0.20) test. Nei, genetic identity and distance were determined among the studied populations. The results showed that the highest value of genetic identity (0.86) occurred between populations No. 10 and 11 (0.85). The lowest value of genetic identity occurred between populations No. 4 and 5 as well as 4 and 8 (r = 0.61).

The grouping of the populations by NJ tree and Network plot produced similar results. Therefore, NeighborNet plot is only presented here (Fig. 2). Populations 1, 9, 10, 11 and 12 showed higher degree of genetic affinity and were place close to each other. Populations 4, 5 and 8 were placed far from each other due to their genetic difference.

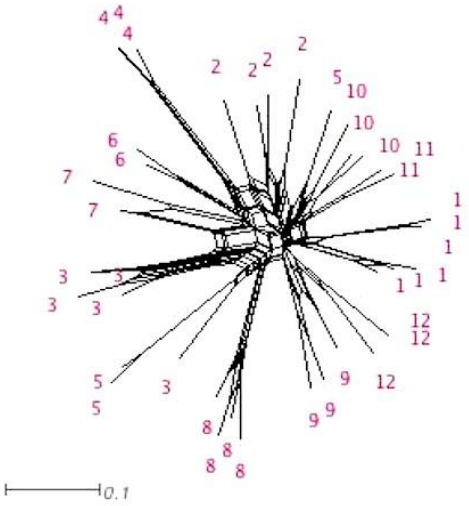

Fig. 2. NeighborNet diagram of *Cirsium aduncum* populations. (Populations 1-12 are according to Table 1).

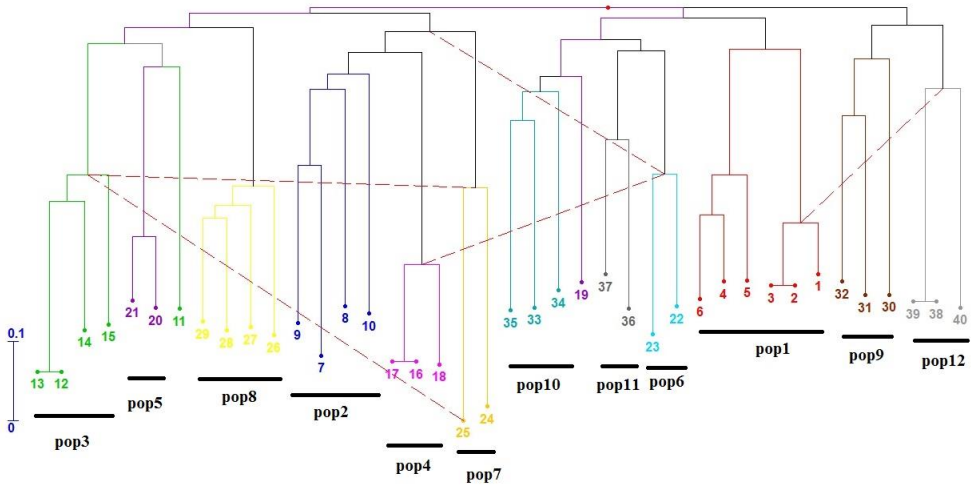

Fig. 3. Reticulogram of *Cirsium aduncum* populations. (Populations 1-12 are according to Table 1, dash lines = reticulation).

The reticulogram obtained (Fig. 3) revealed some degree of gene flow between population 1 and 12, 4 and 6, 3 and 7. These are either due to ancestral gene flow or due to ongoing genetic admixture among the studied populations.

The Evanno test produced the best number of genetic groups as K = 8. Therefore, STRUCTURE analysis was performed for K = 8. The STRUCTURE plot obtained (Fig. 4) revealed more detailed information about genetic admixture of the studied populations. This plot showed that although each population had some private alleles (differently colored segments) they had few shared alleles that were obtained from the other populations. Intra-population genetic variability was observed in most of these populations. For example, plant numbers 8- 10 of the population 2, had some alleles from population 6 (similarly colored segments). Plants of population 8 had some shared alleles with population 9, due to genetic admixture.

POPGENE analysis revealed that the mean Nm value for 85 loci for the studied populations was 0.22. However, 17 ISSR loci were highly exchanged (Nm value >1.0) among populations. The Mantel test produced significant correlation (P = 0.01) between genetic distance and geographical distance of the studied populations. Therefore, the populations that are in closer vicinity had the chance for gene flow between each other.

Consensus tree was obtained from morphological and genetic trees (Fig. 5) Most of the studied populations were morphologically and genetically separated from each other (except populations 1, 3, and 10). Populations 2 and 4 were placed far from the other studied populations due to their genetic and morphological differences. These two populations had short and equal-sized lateral branches. Moreover, the color of plants in this population was dark green compared to the light green color of the plants of other populations. Population 4 also had the highest value for the ratio of longest to shortest corolla lobe.

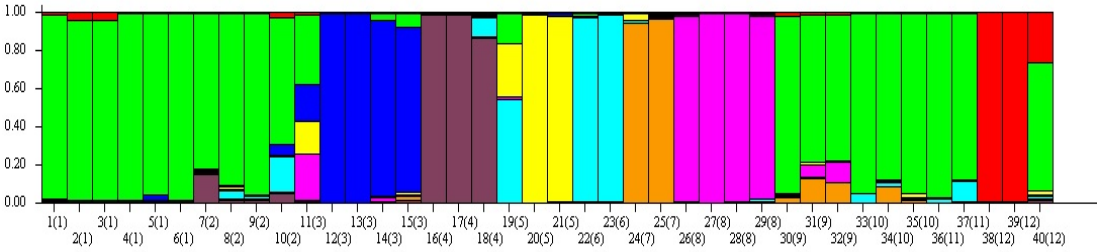

Fig. 4. STRUCTURE plot of *C. aduncum* populations. (Populations 1-12 are according to table 1).

Plants of population 12 were also placed in a cluster far from the other studied populations and were differentiated by their allelic composition and having the highest or the lowest values of morphological characters 4, 24 and 26.

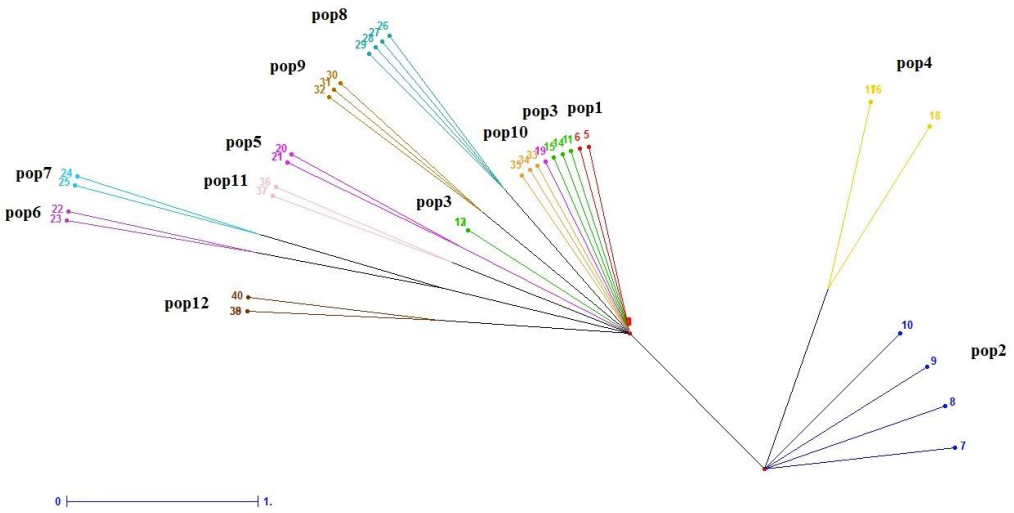

Fig. 5. Consensus tree of morphological and genetic trees in *C. aduncum*.

C. haussknechtii

ANOVA test revealed significant difference in quantitative morphological characters among the studied populations. Moreover, MDS plot of all morphological characters separated these populations from each other, indicating their morphological differences.

Genetic diversity parameters determined in the studied populations of *Cirsium haussknechtii* is presented in Table 4. The highest value for Ne (1.164) occurred in population 13 (Hilevar village), while the highest value for Shannon Information Index (I = 0.202), gene diversity (He = 0.135) and percentage of polymorphism (37.65%) occurred in population 17 (Ardebil, Khalkhal).

Table 4. Genetic diversity parameters in populations of C. haussknechtii. (Population numbers are according to Table 1).

Pop	Na	Ne	I	He	UHe	%P	Hs
Pop13	0.753	1.164	0.153	0.101	0.112	29.41%	0.155
Pop14	0.741	1.161	0.144	0.096	0.109	27.06%	0.151
Pop15	0.576	1.125	0.107	0.073	0.097	17.65%	0.176
Pop16	0.329	1.000	0.000	0.000	0.000	0.00%	0.000
Pop17	0.776	1.228	0.202	0.135	0.150	37.65%	0.198
Pop18	0.471	1.103	0.087	0.059	0.071	15.29%	0.102

Na = No. of alleles, Ne = No. of effective alleles, He = Gene diversity, Uhe = Unbiased gene diversity, %P = Percentage of polymorphism, and Hs = Genetic diversity due to populations. Populations 13-18 are: Hilevar village, Ahar-Kalibar, Jolfa, Daran village, Urmeyah, Salmas, Ardebil, Khalkhal, and Ardebil, Namin.

AMOVA revealed significant molecular difference (P = 0.01) among the studied populations. It also showed that 54% of total genetic variability was due to among populations genetic difference, while 46% was due to within population genetic variability. Pairwise Fst values were obtained for all populations that were all significant (P = 0.01). This indicated genetic divergence of all studied populations. High value of Hickory test theta B (0.35) supported AMOVA result. The populations' genetic differentiation was evidenced by significant differentiation indices obtained (G'st = 0.64, P = 0.01, and D_st = 0.23, P = 0.01) values obtained as well as by Hickory (Theta = 0.20) test.

Nei' genetic identity determined among populations revealed the highest value of genetic similarity between populations 13 and 14 (Hilevar village and Kalibar respectively) (0.86), followed by populations 13 and 17 (0.85). The lowest value of genetic similarity occurred between pop 15 and 16 (0.61), followed by pop 5 and 4 (0.68).

Reticulogram of ISSR data and NeighborNet diagram produced similar results. Therefore, reticulogram is presented here (Fig. 6). It produced four major clusters. Plants of population 13 (Hilevar village, plants No. 1-5) formed the first major cluster. Plants of populations 14 (Kalibar, plants No. 6-9) and 15 (Daran village, plants No. 10 and 11) formed two sub-clusters of the second major cluster. Similarly, plants of populations 16 (Salmas, plants No. 12 and 13) and 18 (Namin, plants No. 19-21) formed two sub-clusters of the third major cluster. Finally, plants of population 17 (Khalkhal, plants No. 14-18) formed the last major cluster. This plot showed the occurrence of gene flow among most of the studied populations in *Cirsium haussknechtii*.

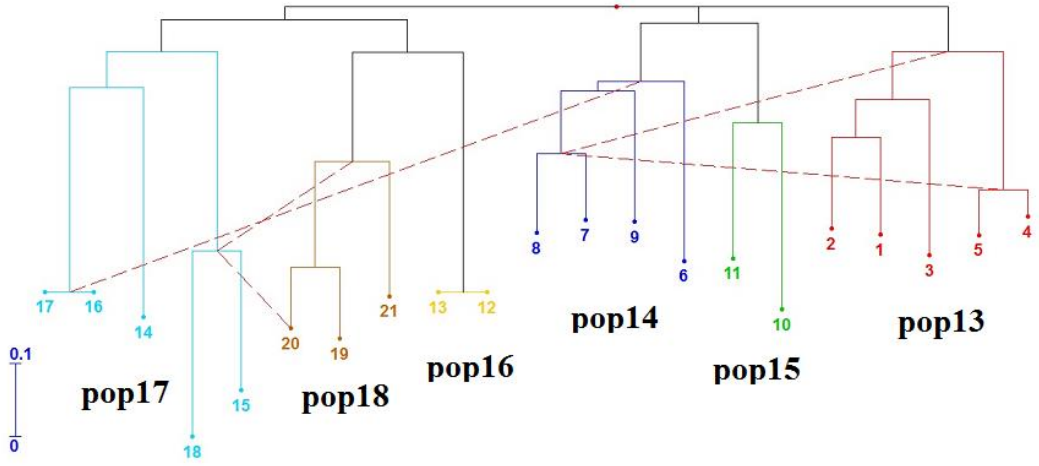

Fig. 6. Reticulogram of *Cirsium haussknechtii* populations. (Population codes are according to Table 1).

Evanno test produced the optimum number of genetic groups as K = 5. The STRUCTURE plot revealed more detailed information about genetic structure and genetic admixture of the studied populations. For example, plant number 3 of population 13 (Hilevar village), had some alleles that were possibly obtained from the other geographical populations.

Similarly, plant numbers 6 and 9 from population 14 (Kalibar) had some alleles obtained from population 15 (Daran village).

A high degree of intra-population genetic variability was observed among plants of population 17 (Khalkhal). Plant numbers 14 and 15 differed greatly from the other plants in this population.

Consensus tree was obtained from morphological and genetic data (Fig. 7) Most of the studied populations were morphologically and genetically separated from each other. Plants of population 13 were placed in a cluster far from the other studied populations and were differentiated by their allelic composition and having the highest or the lowest values of morphological characters 2, 10 and 12.

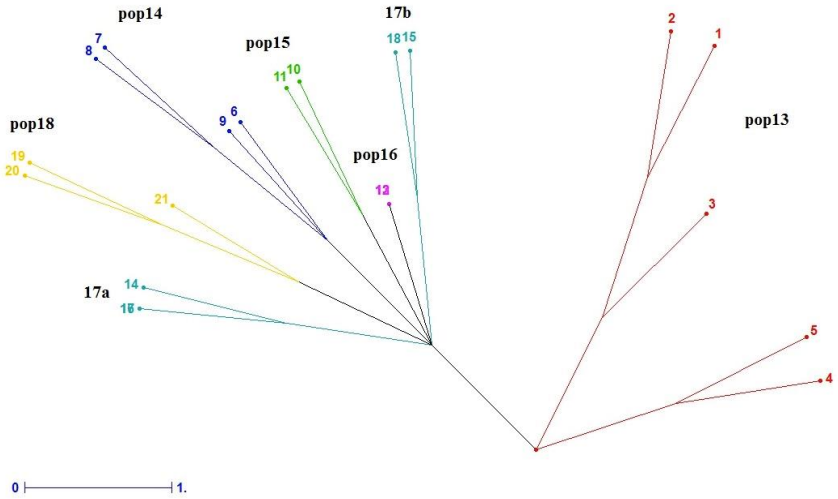

Fig. 7. Consensus tree of morphological and genetic trees in *Cirsium haussknechtii* populations.

Similarly, plants of populations 17 that were collected from two locations (named 17a and 17b), were placed in two different clusters and separated based on their allelic composition and morphological characters 4, 8, 9, 11, and 16 in location 17a, and having morphological characters 3, 8, 9, 11, and 16. Therefore, they differed from each other for morphological characters 3 and 4 as well as in their allelic contents. Other populations also formed separate clusters and contained their specific alleles and morphological characters.

Inter-specific gene flow

During our plant collection we encountered great morphological variability in populations of *Cirsium aduncum* and *C. haussknechtii*. In fact many plant specimens from one species had morphological characters overlapping the other species. Since some of these populations are in close vicinity, and inter-specific hybridization is well known in *Circum*, we investigated the possible occurrence of inter-specific gene flow between these two species.

PCoA plot of morphological characters in the studied populations of both species is presented in Fig. 8. It revealed some degree of morphological admixture among populations of the two studied species. (Red colored dots = *C. aduncum* and green colored dots = *C. hauskenchtii*).

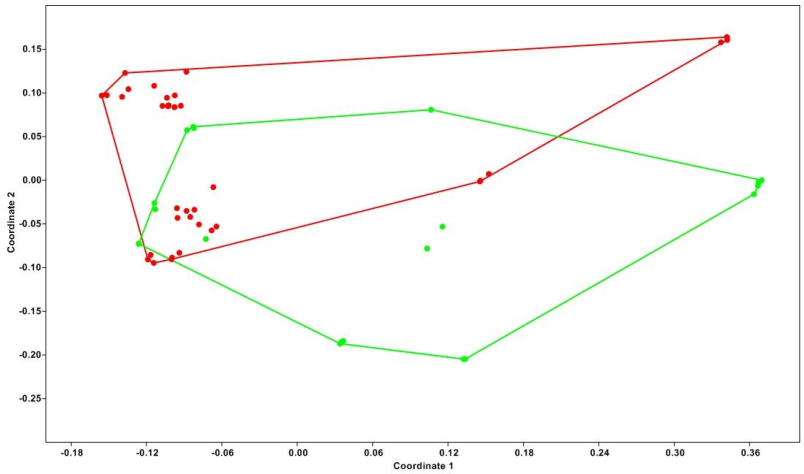

Fig. 8. PCoA plot of morphological characters in *C. aduncum* and *C. haussknechtii* showing morphological overlap of few plants.

Similarly, the PCoA plot of genetic data also revealed some degree of genetic admixture between the two species. Some populations were placed in overlapped (intermixed) region in both morphological and molecular plots. For example, population No. 5 (Tabriz, East Azarbaijan) of *C. aduncum* was placed close to population No. 14 (Ahar, East Azarbaijan) of *C. haussknechtii*. Similarly, population No. 8 (Ardabil) of *C. aduncum* was placed close to population No. 15 (East Azarbaijan : Jolfa) of *C. haussknechtii*. Some degree of overlap occurred also between population No. 2 (East Azarbaijan, Tabriz-Ahar) of *C. aduncum* with population No. 14 (Ahar, East Azarbaijan) of *C. haussknechtii*. These populations are geographically almost in close contact and all grow in East Azarbayejan province. In fact, the Mantel test produced significant correlation (P = 0.01) between genetic distance and geographical distance of the studied populations. Therefore, the populations that are in closer vicinity had the chance for gene flow between each other.

To study possible genetic admixture between the studied species, we used STRUCTURE analysis and maximum likelihood method (population assignment test.

Triangle plot of STRUCTURE program revealed that some members of species *C. aduncum* are genetically intermixed with plants of *C. haussknechtii* (Fig. 9). Finally, the assignment test (Table 5) identified some of the studied plants to be inferred from the other species. For example, plant number 7 of the population 2 was inferred to be from population 17. Similarly, three plants from population 4 were inferred to be from populations 13 and 17, etc.

These results strongly support inter-specific introgression between *Circium haussknechtii* and *C. aduncum*.

Table 5. Population assignment test for both studied Circium species. (Population numbers are according to Table 1).

Individual	Current	Inferred	Lik_max
7	Pop002	Pop017	-92.23
9	Pop002	Pop001	-80.512
16	Pop004	Pop013	-123.921
17	Pop004	Pop013	-123.921
18	Pop004	Pop017	-121.217
20	Pop005	Pop017	-96.543
22	Pop006	Pop013	-81.728
23	Pop006	Pop013	-87.597
24	Pop007	Pop017	-80.411
25	Pop007	Pop017	-69.013
26	Pop008	Pop017	-85.874
27	Pop008	Pop017	-97.441
28	Pop008	Pop017	-78.959
29	Pop008	Pop017	-78.23
30	Pop009	Pop017	-73.578
31	Pop009	Pop017	-94.581
32	Pop009	Pop013	-85.076
33	Pop010	Pop017	-58.191
46	Pop014	Pop001	-73.609
50	Pop015	Pop001	-101.685
59	Pop018	Pop003	-84.43

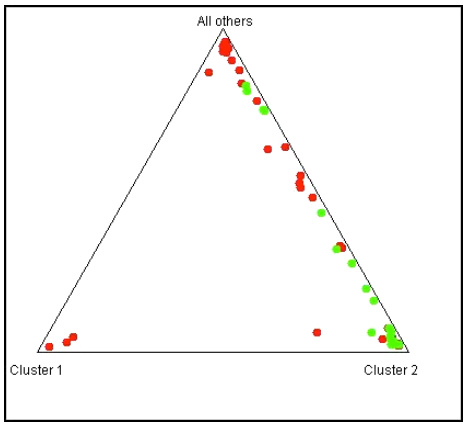

Fig. 9. Triangle plot of STRUCTURE analysis revealing genetic admixture between *C. aduncum* and *C. hauskenchtii*.

DISCUSSION

Genetic diversity parameters as well as AMOVA revealed high level of within species genetic variability in the studied *Cirsium* species and populations. AMOVA showed that 54% of total genetic variability was due to among populations genetic difference, while 46% was due to within population genetic variability. These populations also showed some degree of among population of gene flow.

Genetic diversity is important for continuity of plant species and adaptation to environmental conditions (ÇALIŞKAN, 2012). The occurrence of gene flow between different geographical populations introduces new genes to the local populations and adds to the genetic variability of these populations (HOU and LOU, 2011; SHEIDAI *et al*., 2014). Similar studies performed in *Cirsium arvense*, Canada thistle (JUMP *et al*., 2003; BODO SLOTTA *et al*., 2010; SEIF *et al*., 2012), *Cirsium hillii* (FREELAND *et al*., 2010) and *C. pyramidale* (SHEIDAI *et al*., 2013) produced similar results. The studied populations exhibited greater within-population diversity (60%) than expected for a reported clonally reproducing species. However, total diversity of sampled locations in North America (0.183) was less than previously reported for European locations (0.715) (JUMP *et al*., 2003), indicating that different *Cirsium* species and populations differ in the range of genetic diversity in different regions of the world.

The high rate of gene flow we obtained here is in agreement with the results of other studied *Circium* species (JUMP *et al*., 2003; BODO SLOTTA *et al*., 2010). In these taxa, many individuals from geographically distant regions clustered together, indicating long-distance translocation of prologues and obligate out-cross nature of *Circium* species.

It is a known fact that in plant species that form geographical populations, as geographical isolation increases, a reduction in both seed dispersal and pollen flow will result in decreased gene flow between populations (JUMP *et al*., 2003). The resulting genetic isolation may lead to pronounced geographical structuring in genetic variation within a species as population differentiation increases. Both genetic drift and inbreeding are likely to be of increased importance in isolated populations, with the result that genetic diversity may be reduced toward the species periphery (JUMP *et al*., 2003). The present finding also showed isolation by distance in both studied *Circium* species.

Population isolation and genetic differentiation observed in two *Circium* species studied are also known to persist in other *Circium* species, like *Circium dissectum* (meadow thistle). "*C. dissectum* populations showed high levels of genetic differentiation and strong isolation by distance using microsatellite genetic markers. Both microsatellite genetic markers and morphological traits revealed geographical structuring between populations. Plants in Ireland showed higher levels of morphological differentiation compared to Britain. *C. dissectum* showed strong, early acting inbreeding depression when plants were selfed and a trend towards outbreeding depression when genetically distant populations were crossed (VERE, 2007)".

The present study revealed some degree of inter-specific introgression and also the occurrence of plants with overlapping morphological characters due to this phenomenon. Inter-specific hybridization is an important evolutionary mechanism that brings about two genomes of divergent but related species together. It can produce new genetic and phenotypic traits that can help the species ecological adaptation (FREELAND *et al*., 2011). Inter-specific hybridization occurs frequently in various plants groups but is under influence of different factors like, the genetic architecture of the species involved, the fitness of the hybrid and genotype-environment interaction (FREELAND *et al*., 2011; HARRISON and LARSON, 2014).

Introgression (or "introgressive hybridization") is the incorporation of alleles from one species into the gene pool of a second, divergent entity species via hybridization and back-cross (HARRISON and LARSON, 2014). "Introgression is a relative term; alleles at one locus introgress with respect to alleles at other loci. Therefore, some portion of the gene pool of each of the hybridizing taxa must remain constant and uncontaminated such that we can actually recognize that 2 distinct gene pools exist. The genes that define the 2 gene pools and make them distinct are those that comprise the species boundary" (HARRISON and LARSON, 2014).

The genus *Circium* is well known for the occurrence of interspecific hybridization and the presence of extensive morphological variability. Such great morphological variation of the *Cirsium* is also partly due to its highly variable germplasm interacting with different environmental conditions. The occurrence of frequent hybrids in the genus *Cirsium* indicates lack of breeding barriers and, therefore, new forms may arise in nature, due to interspecific hybridization (BUREŠ *et al*., 2004).

The genus is considered to be taxonomically complex due to the variability and intergradation of diagnostic characters among taxa mostly due to inter-specific hybridization (DABYDEEN 1980; WAGENITZ, 1987; BUREŠ *et al*., 2004). In fact, several hybrids have been described from Caucasus and adjoining regions of Asia minor (WAGENITZ, 1987; CHARADZE, 1998; BUREŠ *et al*., 2004). Hybrids are usually fertile they and can often produce introgressive hybrids with the parental species or triple hybrids with other taxa (WAGENITZ, 1987). It is stated that great diversity in *Cirsium* taxa is due to rapid evolutionary diversification (KELCH and BALDWIN, 2003). High degree of genetic and morphological variability observed in *C. aduncum* and *C. hauskenchtii* as well as identification of potential inter-specific hybrids can be used in conservation of these medicinal plants in the country.

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SAVREMENA INTER-VRSNA SPECIFIČNA HIBRIDIZACIJA IZMEĐU *Cirsium aduncum* and *C. haussknechtii* (Asteraceae): EVIDENCIJA REZULTATA MOLEKULARNIH I MORFOLOŠKIH ISTRAŽIVANJA

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Izvod

Cirsium aduncum Fisch. & C.A.Mey. Ex DC. i *C. haussknechtii* Boiss., (Asteraceae) su različite medicinske biljke koje uspevaju u različitim regionima Irana. Nema saznanja o genetičkoj strukturi populacija, protoku gena unutar i između vrsta kao i prisustvu zona sa hibridima u Iranu..U cilju obezbeđenja podataka potrebnih za konzervaciju ove dve medicinski značajne vrste vršena su ispitivanja populacione genetike i morfometričkih osobina kod 18 geografski različitih populacija ovih vrsta. ANOVA i MDS analizama utvrđene su značajne morfološke razlike izmeđi ispitivanim popolacijama dok je MDS plot analiza pokazala morfološka preklapanja kod biljaka ove dve vste. AMOVA test je potvrdio znaačajne genetičke razlike među ispitivanim populacijama. MANTEL test je pokazao pozitivne značajne korelacije između genetičke i geografske udaljenosti i pojavu izolacije uslovljene udaljenošću. Dodatni test populacije i struktura genetičkih podataka je potvrdila među vrsnu introgresiju između ispitivanih vrsta.

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