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Research Article

BIOSYSTEMATICS INVESTIGATION OF ARCTIUM GENUS FROM ASTERACEAE FAMILY IN NORTHERN IRAN

Mahsa Shokouhi[1], *Arman Mahmoudi Otaghvari[1] and Seyed-Kamal Kazemitabar[2]
[1]*Department of Biology, University of Mazandaran, Babolsar, Iran*
[2]*Department of Crop & Plant Breeding, Sari Agricultural Sciences and Natural Resources University, Sari, Iran*
**Author for Correspondence*

ABSTRACT
This research aims at biosystematic investigation of *Arctium* L. genus in three northern provinces of Iran (Gilan, Mazandaran and Golestan) using the molecular and morphological studies. The genetic diversity among 15 studied populations is done through ISSR Marker. The DNA extraction, CTAB modified by Culling and polymerase chain reaction (PCR) are done with 5 ISSR primers. A total of 53 bands are detected and the level of polymorphism is determined equal to 79.2%. The coding is performed based on the presence or absence of bond and Jaccard similarity coefficient is utilized to determine the rate of similarity between populations. The target dendrogram is drawn using the UPGMA method and cluster analysis. According to the dendrogram, the greatest similarity is between the Farsian (Golestan) and Shaft (Gilan) populations with 80.9% of similarity and the least similarity is between the Masuleh (Gilan) with other populations with 44.7% of similarity. The principal component analysis (PCA) is also performed for molecular data. Furthermore, 25 morphological traits including 9 qualitative traits and 16 quantitative traits are selected for conducting the morphological studies. After coding the principal component analysis (PCA), the cluster analysis is done using the WARD method and ordination for studied populations. The results of morphological studies are fairly consistent with the molecular studies.

***Keywords**: Arctium, ISSR Marker, Morphological Trait, Genetic Diversity*

INTRODUTION
Arctium with the Persian name of burdock, is a herbaceous plant from the Asteraceae family and is known as the medicinal plant in Europe, Asia and North America since hundreds of years ago (Agarwal *et al.*, 2008). There are the compounds such as the vitamins, proteins, carbohydrates, minerals, unsaturated fatty acids and polyphenols in this plant. It is consumed as the edible vegetable in Asia (Chang *et al.*, 2009). Its unripe flowering stem, which is harvested before the flower blooming, and its root are the parts applied in various foods. The leaves of burdock are utilized for treating the burns and its root oil for improving the hair strength and shine (Chan *et al.*, 2011). In Traditional Chinese Medicine (TCM), its leaves, seeds and root are also used to treat the skin diseases. Furthermore, the anti-diabetic, anti-cancer, anti-bacterial and anti-viral properties of burdock are also discovered (Chen, 2012). The abandoned places, road edges, near the streams, and pastures are the places for growing this plant. This genus is native to Eurasia and is now distributed in the North America (Duistermaat, 1997); (Fazeli and Chogha-Mirza, 2011). The morphological characteristics of this plant include the straight and branched stems covered with fluff, heart-shaped leaves, and same-genus heaps and the cluster inflorescences or pistil and almost spherical involucres, bracteoles in several bayonet and narrow rows, akene with cut and slightly compressed tip (Ghahreman, 1994). The number of basic chromosome in this genus is determined n =18 and 2n = 36 (Gross *et al.*, 1980). In Flora Iranica, three studied species are reported in Gilan, Golestan, Mazandaran, Tehran, Khorasan, Kerman, Lorestan and Azerbaijan provinces (Henry (2002); Karimi *et al.*, 2001). In the past, the taxonomists considered only the morphological characteristics in order to classify the plants (Kumar *et al.*, 2009). Note that unlike the morphological traits, the molecular markers are independent of environmental factors and play the significant roles in plant classification (Lee and Sahd, 2011). The genetic diversity in plants has various applications such as the phylogenetic analysis, study and genetic conservation, population genetic and genetic heuristics of plants (Lopez *et al.*, 2010).

The polymerase chain reaction-based (PCR) methods are now widely used due to the numerous advantages such as the speed, easiness, low cost and no need for radioactive probe (Lee and Sahd, 2011). Since it is impossible to do the genome sequencing projects in all plant territories, the use of markers such as SSR, ISSR, AFLP, and RAPD is common in molecular studies (Lopez *et al.,* 2009). This study utilizes the ISSR marker to investigate the genetic diversity of Arctium. This marker has high reproducibility; the low amount of DNA is needed and it helps to detect those with close relationship (Lym and Travnicek, 2012); (Mohammadi *et al.,* 2008). In this technique, the high purity of model DNA is essential as well as optimizing the initial reactions (Mohammadi *et al.,* 2008). In recent years, a few molecular and morphological studies are conducted on Arctium genus. For instance, the new microsatellite markers are separated and characterized for Arctium minus and thus six populations of central and southern Europe and Asia are selected; and the primers are designed through two genomic libraries for this genus with CA and AAG replications and applied in genetic population study (Nike, 2005). In a research entitled the morphological and molecular evidence for Arctium hybridization in Central Europe, 109 individuals of three species, namely, A.lappa, A. minus, and A.tomentosum are studied through 9 morphological traits and 5 RAPD primers. The results of analysis showed three distinct species and also identified the numbers of first generation hybrids among A.lappa species and one of A. minus or A.tomentosum species (Rechinger, 1963-2010). Furthermore, a research entitled the "Phylogeny and evolution of research Arctium-Cousinia complex", investigates the rpS4-trnT-trnL sequences of 138 species including 129 species of *Cousinia* genus. In *Cousinia* genus, the *Cynaroides* and *Hypacanthodes* sub-species are generally 30 species which have much closer relationship with Arctioid clad than Cousinioid clad. The shape and involucres are the main morphological traits of Arctioid clad. The results of morphological and molecular studies are in conflict and there is no proposed taxonomic solution to this conflict (Repplinger *et al.,* 2007). A research is conducted in the field of identifying *A. Lappa* through ITS sequences. Six Arctium populations are collected from southern Taiwan and it is found after analyzing the PCR Product sequence that there is a mononucleotide polymorphism in some populations in base pair position 277 in ITS sequence, and thereby their common origin is proved (Shivakumar *et al.,* 2011). This study is conducted with the aim at investigating the genetic diversity using the ISSR markers among the studied populations of Arctium species as well as its comparison with the results of morphological studies.

MATERIALS AND METHODS

Plant Materials:

The plant samples for conducting the molecular and morphological studies are collected as the herbarium specimens and frozen leaves from three Northern provinces of Iran including Gilan, Mazandaran and Golestan, and then transferred into the laboratory. Nowadays, the wide use of agricultural pesticides has eliminated this weed in agricultural lands, and thus the sample is obtained from the mountains of these three provinces.

DNA Extraction:

After testing different methods including Dellaporta's method and Dellaporta's CTAB combined method, the DNA extraction is finally performed using the changed CTAB method by Culling. The quality and quantity of extracted DNA are measured by electrophoresis methods on Agarose gel 0.7% and spectrophotometer. The dilution of DNA samples is done until the concentration of 60 monograms per micro liter.

Optimization of Polymerase Chain Reaction (per) Conditions:

The polymerase chain reaction (PCR) is done in the volume of 25 micro liter with the amounts of 2 micro liter (60 DNAng/µl), 0.8 micro liter of Primer (10µM), 0.5 micro liter of Dntp (10mM), 2.5 micro liter of PCR buffer (10X), 1.5 micro liter of $Mgcl_2$ (50 Mm), and 0.3 micro liter of Taq Polymerase (5u/µl). The thermal cycles consists of initial denaturation at 95.5°C for 7 min, the secondary denaturation at 94°C for 1 min, the specific annealing temperature per primer for 1 min, the initial expansion at 72°C for 1 min, and the secondary extension at 72°C for 5 min. The electrophoresis of PCR product is done on Agarose gel of 1.5% with a voltage of 90 V for 1.5 hours. The gel is stained to detect the bonds through ethidium

bromide and imaging by Gel Doc instrument. Table 1 shows the coordinates of places for collecting the plant samples.

Table 1: Coordinates of places for collecting the plant samples

No.	Abbreviation	Habitat address	Height (m)	Geographical coordinates
1	Kh	Khushal, Kojur district, Noshahr	1522	N 51.5538 E 36.3465
2	N	Narrab, Cheshmeh Saran District, Azadshahr	1361	N 55.0612 E 37.0098
3	A	Eshkevar, Rahimabad district, Rudsar	524	N 47.1921 E 36.8811
4	G	Jennat Rudbar, Dalkhani forest, Ramsar	1268	N 50.6163 E 36.8094
5	Sh	Eshagh Imamzadeh, Shaft	996	N 47.0066 E 35.6576
6	M	Gilvanrud margin, Masuleh	451	N 47.0320 E 35.6695
7	Ar	Arabkalu district, Galikash	546	N 55.3572 E 37.1667
8	K	Kiaram, Kuhsar district, Galikash	998	N 49.9205 E 41.7140
9	F	Farsian, Kuhsar district, Galikash	893	N 49.9012 E 41.6738
10	D	Dasht-e Nazir, Kojur district, Noshahr	982	N 51.4038 E 36.3983
11	B	Bandpey, Babol	665	N 52.6533 E 36.2568
12	Al	Alasht, Lil district, Savadkuh	924	N 52.9044 E 36.0865
13	Fi	Chamebon, Filband, Amol	1231	N 52.4757 E 36.1955
14	L	Lacan, Rasht	34	N 49.5866 E 37.1946
15	Du	2000-3000 Road, Ramsar	370	N 47.1986 E 37.5475

Morphological Study:
25 morphological traits including 9 qualitative and 16 quantitative traits are selected for conducting the morphological study on 15 populations consisting of 45 individuals. Coding is done based on both lower and higher than the total mean.

RESULTS AND DISCUSSION

Results

In morphological study, the principal component analysis (PCA) is done to identify the most variable morphological traits. The cluster analysis is done through WARD method and the ordination based on the principal component analysis (PCA) in order to determine the affinity of species. Furthermore, the presence or absence of bond for each of five primers is coded in zero and one in order to conduct the molecular studies, and then the matrix of similarity is drawn through zero and one matrix with NTSYSpc 2.02 software and based on Jaccard similarity coefficient. The cluster analysis is done through UPGMA

method. The principal component analysis (PCA) is done for molecular data. It's two and three-dimensional plots are also drawn. Furthermore, the Co-phentic correlation coefficient is calculated, and Jaccard similarity matrix is compared with obtained Co-phentic matrix.

Results of Molecular Studies:

Investigating the genetic diversity of Burdock (Arctium) is done through 5 ISSR primers. From the total of obtained 53 bonds, 79.2% were polymorphs and 20.8% mono-morphs. The bonds are observed at the range of 500-1500 bp. The percentage of polymorphism (POL) for these primers is between 50-100% and the mean polymorphism information content (PIC) is equal to 0.4. The marker index (MI) is measured from multiplying the PIC by POL and it is at the range of 20.5-41. Given the similarity matrix, the maximum similarity is obtained between Farsi and Shaft populations with 80.9% of similarity, and the minimum similarity is found between Masuleh and other population with 447% of similarity. Furthermore, the dendrogram of cluster analysis, shown in Figure 4, indicates four groups at the similarity level of 0.57. The first group is divided into two subgroups. The first subgroup is divided into two sub-groups; and the first sub group contains the subsidiary subgroups including the populations of Khushal, Narrab, Eshkevar, Shaft and Farsi an.

The second sub-group is divided into the subsidiary subgroups including Rudbar and Alasht populations. The second subgroup consists of the subsidiary subgroups including Arabkalu population in a cluster and Kiaram and Dasht-e Nazir in another cluster. The second group is divided into two subgroups including Bandpey population in a subgroup and 2000 population in another sub group. The third group has two sub-groups including Filband and Lakan populations. Masuleh population can be observed in the fourth group. In the principal component analysis (PCA), shown in Figure 7, three first components have a total contribution of 65.53% in diversification. Furthermore, the two and three dimensional plots resulted from the principal component analysis (PCA) are shown in Figures 5 and 6. The Co-phentic correlation coefficient is measured equal to 0.78 based on Jaccard similarity coefficient in order to investigate the adaption of similarity matrix and dendrogram. In fact, the comparison of Jaccard similarity matrix with obtained Co-phentic matrix, which is shown in Figure 8, also shows the low correlation between these two components. The similarity matrix, measured by Jaccard similarity coefficient, and also the polymorphism observed by ISSR11 primer are presented in Figure 3. Furthermore, the information about the primers, and the PIC, POL and MI values for each primer are shown in Table 4.

Results of the Morphological Study:

The principal component analysis (PCA) for morphological data indicates that the first component with the contribution of 31.6 percent includes the bracteole width, bracteole margin, inflorescence length, fruit width, bracteole length, ratio of bracteole length to width, and involucres length. The second component with the contribution of 17.6 percent includes the fruit color characteristics, inflorescence arrangement, inflorescence width, and length of inflorescence main axis. The third component with contribution of 12.7% includes the traits such as the peduncle length, stem surface, type of stem crack, and stem color, ratio of involucres length to width. In general, the first three components play the maximum role in this analysis with 61.9% of contribution. The cluster analysis, which is done based on WARD method on 7 populations of *A. lappa* species, 5 populations of *A. Minus* species and 3 populations of *A. Platylepis* species, is shown in Figure 1. The obtained dendrogram contains two main groups and the first group is divided into two subgroups. The first subgroup has the other subsidiary subgroups including three populations of Arabkalu, Dasht-e Nazir and Filband from *A. lappa* species, and the second subgroup includes the populations of Narrab, Khushal, and Jennat Rudbar from *A. platylepis* species. The second subgroup is divided into two subsidiary sub-group including Shaft and Alasht from A.minus species. The second group is divided into two subgroups. The first subgroup includes the subsidiary groups including the populations of Lakan, Masuleh and Kiaram from A.lappa species and the second group consists of two subsidiary sub-groups. The first sub group includes 2000 and Bandpey populations from A.lappa and A. minus species respectively. The second subsidiary sub-group includes the populations of Farsi an and Eshkevar from A.minus species. The ordination chart based on the principal component analysis (PCA) is shown in Figure 2 and indicates that the differentiation between the populations of *A. platylepis* species

and *A. minus* species is properly done. The gap between the populations of each species is fairly high. Bandpey population from *A. minus* species has a little distance from Kiaram population in *A. lappa* species. Furthermore, three populations of Arabkalu, Dasht-e Nazir, and Filband from *A. lappa* species are fairly close to the populations of *A. Platylepis* species and have greater distance from other populations of this species. The first three components of principal component analysis (PCA) can see in Table 2.

Table 2: Three principle components of PCA using the morphological traits in studied populations and species of *Arctium* genus

Variable	PCl	PC2	PC3
Inf	-0.177	-0.350*	0.030
1. inf	0.290*	-0.161	-0.058
w.inf	0.207	-0.278*	-0.159
l/w.inf	-0.131	0.292	-0.145
Ama.inf	0.211	0.252*	-0.239
pl.inf	0.158	0.096	-0.420*
d.inf	-0.047	0.103	-0.085
Sc	0.012	0.175	-0.326*
Ls	0.018	0.164	0.374*
Dws	0.026	-0.024	0.068
Cs	-0.018	-0.164	-0.374*
p. inf	0.169	-0.121	-0.213
tip.inf	0.049	-0.343	0.017
Lp	0.288*	-0.046	-0.064
Wp	0.295*	0.017	0.157
l/w.p	-0.285*	-0.018	-0.032
Mp	-0.295*	-0.017	-0.157
Fc	0.147	0.361*	-0.108
Fl	0.166	-0.252	-0.178
Fw	0.290*	-0.161	-0.058
l/w.f	-0.177	0.192	-0.190
Iw	0.262	0.138	0.051
Il	0.272*	0.196	0.027
l/w.i	-0.206	0.095	-0.296*
Pl	0.176	0.252	0.211

The index traits in each of 3 principle components are shown by *.

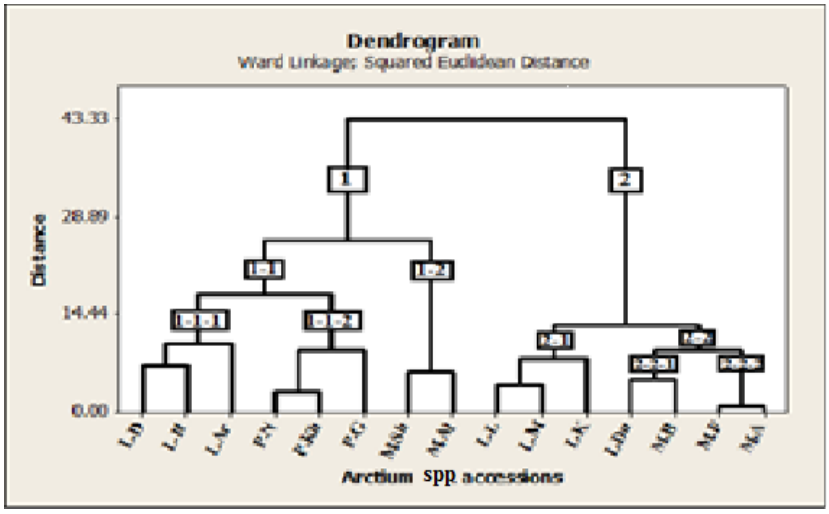

Figure 1: The cluster analysis for morphological traits using the WARD analysis in studied populations of *Arctium* genus

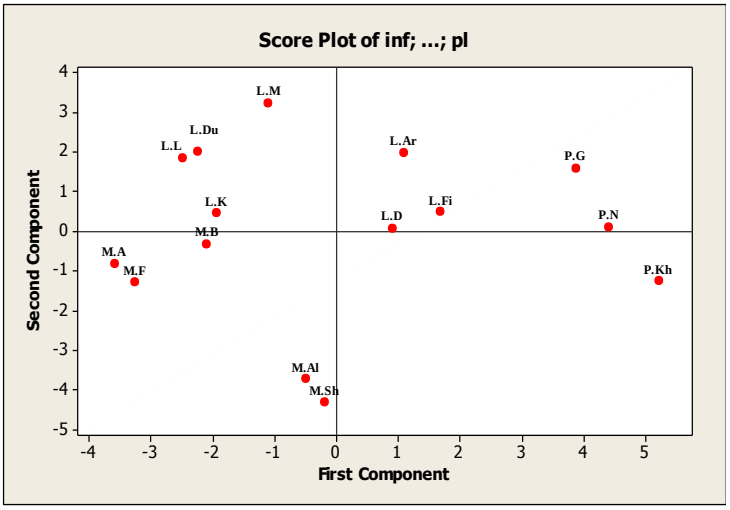

Figure 2: Ordination of species and populations of *Arctium* genus based on the first and second two principal components of morphological traits

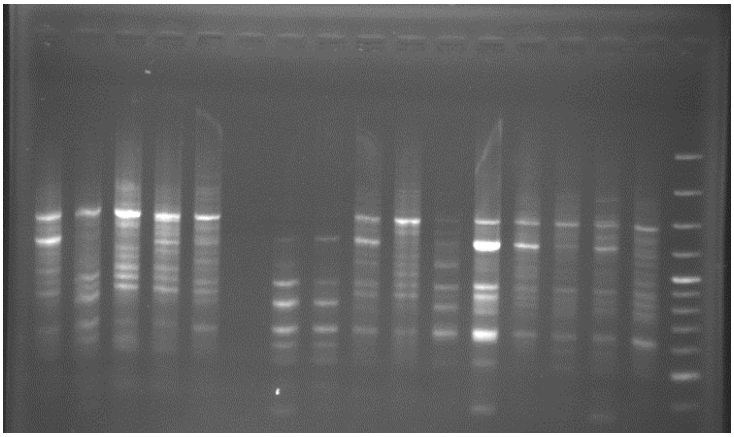

Figure 3: Polymorphism observed with ISSR11 primer in studied populations

Table 3: Similarity matrix of populations and species of *Arctium* genus using the Jaccard similarity coefficient

	KH	N	A	G	SH	MB	AR	K	F	D	B	AL	FI	L	DU
KH	1														
N	0.787879	1													
A	0.706958	0.706958	1												
G	0.644172	0.644172	0.644172	1											
SH	0.706958	0.706958	0.758711	0.644172	1										
MB	**0.447173**	**0.447173**	**0.447173**	**0.447173**	**0.447173**	1									
AR	0.596467	0.596467	0.596467	0.596467	0.596467	**0.447173**	1								
K	0.596467	0.596467	0.596467	0.596467	0.596467	**0.447173**	0.648413	1							
F	0.706958	0.706958	0.758711	0.644172	**0.809524**	**0.447173**	0.596467	0.596467	1						
D	0.596467	0.596467	0.596467	0.596467	0.596467	**0.447173**	0.648413	0.794118	0.596467	1					
B	0.525630	0.525630	0.525630	0.525630	0.525630	**0.447173**	0.525630	0.525630	0.525630	0.525630	1				
AL	0.644172	0.644172	0.644172	0.666667	0.644172	**0.447173**	0.596467	0.596467	0.644172	0.596467	0.525630	1			
FI	0.525630	0.525630	0.525630	0.525630	0.525630	**0.447173**	0.525630	0.525630	0.525630	0.525630	0.540137	0.525630	1		
L	0.525630	0.525630	0.525630	0.525630	0.525630	**0.447173**	0.525630	0.525630	0.525630	0.525630	0.540137	0.525630	0.684211	1	
DU	0.525630	0.525630	0.525630	0.525630	0.525630	**0.447173**	0.525630	0.525630	0.525630	0.525630	0.682927	0.525630	0.540137	0.540137	1

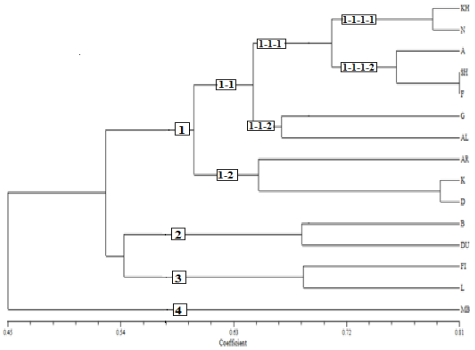

Figure 4: The dendrogram resulted from the cluster analysis of studied populations and species of *Arctium* genus

Table 4: Information about the primers and PIC, POL and MI values for each primer

Primer name	Primer sequence	Total number of bonds	Number of polymorph bonds	Percentage of polymorphism (POL)	Polymorphism information content (PIC)	Marker index (MI)
ISSR-2	5´ GAGAGAGAGAGAGAGAGAC - 3´	-8	4	50	0.41	20.5
ISSR-11	5´ -GAGAGAGAGAGAGAGAC - 3´	14	12	85	0.38	32.3
ISSR-13	5´ -TCTCTCTCTCTCTCTCG- 3´	9	6	66	0.41	27.06
ISSR-14	5´ -TCTCTCTCTCTCTCTCG- 3´	8	6	75	0.39	29.25
ISSR-18	5´ -ATC ATC ATC ATC ATC ATCT- 3´	14	14	100	0.41	41

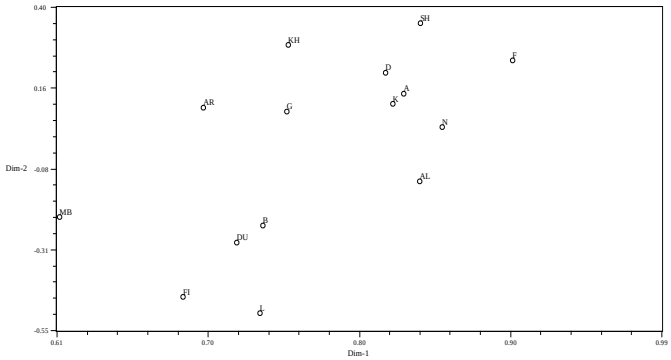

Figure 5: Two-dimensional plot of principal component analysis of populations and species of *Arctium* genus

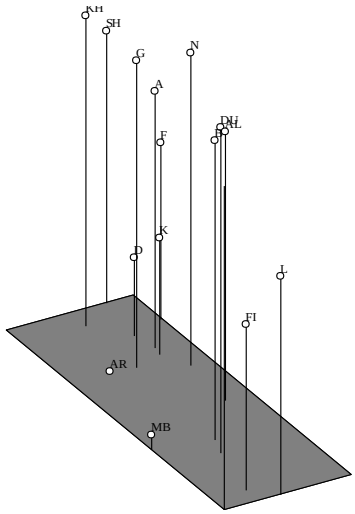

Figure 6: Three-dimensional plot of principal component analysis of populations and species of *Arctium* genus

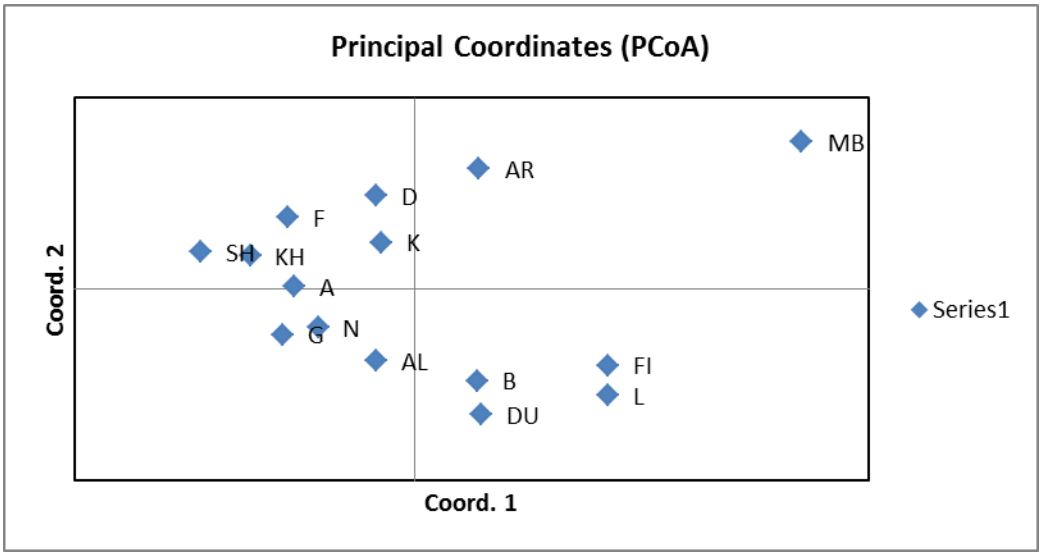

Figure 7: principal component analysis (PCA) for molecular data of species and populations of *Arctium* genus

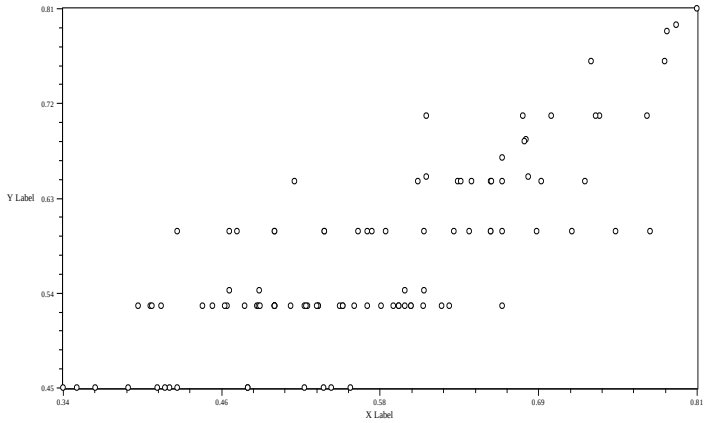

Figure 8: Comparison of Jaccard similarity matrix (X) with obtained Co-phentic matrix (Y)

Discussion

5 primers are utilized in this study and the obtained results can be referred. Two RAPD and ISSR markers are used for determining the genetic relationships between 19 Chickpea varieties and 5 wild breeds, while the acceptable results are obtained in classifying these populations only by 6 ISSR primers while 30 RAPD primers are applied. ISSR is the most obvious marker and the Jaccard similarity coefficient is the best coefficient for investigating the genetic diversity obtained from the obvious markers and they are applied by UPGMA method in this research (Susanna *et al.*, 2003). Given the similarity matrix obtained from the molecular study, the maximum similarity is between the samples of Farsian population from Golestan Province and Shaft population from Gilan province with 80.9% of similarity. Despite the large distance between these two populations, since both species are from Arctium minus species, this result is not unexpected. Masuleh population with 44.7% of similarity has the lowest similarity to other populations. The hybrid population of Masuleh is considered as a possibility in this study. The similar results are obtained in the study on wild and cultivated populations of barberry (*Berberis vulgaris*) in Khorasan Province using AFLP marker. According to the dendrogram of cluster analysis, *Mahonia aquifolium* species is put in a separate cluster. The relationship between this species with Berberis genus

was unclear in the past, but the results of morphological studies introduced this species as a separate genus (Repplinger *et al.*, 2007). Therefore, more botanical studies are essential on Masuleh population for more precise classification. Some of the populations of each province are close and others with distance from each other in dendrogram of cluster analysis; and the high genetic diversity is observed among the studied populations. The calculated Co-phentic correlation coefficient is equal to 0.78% indicating the low efficiency of this method for classification of populations. Furthermore, the morphological study of cluster analysis using the WARD method indicates that *A. platylepis* is properly separated from other species, while two A.lappa and A.minus species are fairly distinct from each other. The comparison of ordination diagram and obtained Dendrogram indicates that three Eshkavar, Bandpey and Farsian populations from *A. minus* species are on Dendrogram in cluster 2 and they are also much closer to one another on ordination diagram. However, Alasht and Shaft populations are in cluster 1 and it is observed with large distance from three other populations of *A. minus* species on ordination diagram. Lakan, 2000-3000 road, Masuleh and Kiaram populations from *A. lappa* species in cluster 2 of Dendrogram are close to each other on the ordination diagram. Arabkalu, Filband and Dasht-e Nazir populations of the same species are in cluster 1. These three populations have larger distance from other four populations on ordination diagram. *A. Platylepis* species is also put in Cluster 1 and the populations of this species are close to each other and with lower distance from *A. Lappa* species and lerger distance than *A. minus* species on ordination diagram. Therefore, the relative separation is observed for populations of each species. Furthermore, the large distance between the populations of each species indicates the little similarity and high dispersion of these populations and it strengthens the possibility of hybridization among *A.lappa* and *A.minus* and *A.Platylepis* species; more research is needed in this regard. Numerous studies have indicated the existence or absence of significant correlation between the similarity matrices obtained from molecular and morphological studied on plant classification. Despite the fact that the amplified DNA fragments in ISSR marker are related for both coding and non-coding regions of genome and this marker is semi-random, the morphological traits are only related to the coding regions of genome; and on the other hand, the morphological traits are influenced by the environmental conditions [22]. It is likely that the average level of concordance between the results of molecular and morphological study is due to the proliferation of non-coding regions by ISSR primers utilized in this study.

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