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GENE FLOW AND POPULATION STRUCTURE IN *ALLOCHRUSA* (CARYOPHYLLOIDEAE, CARYOPHYLLACEAE) WITH THE USE OF MOLECULAR MARKERS

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Allochrusa Bunge is a genus of subfamily Caryophylloideae Rabeler & Bittrich contains about 8 species distributed in Turkey, Central Asia, Afghanistan, Caucasus, Transcaucasus and Iran. Three species of *Allochrusa versicolor, A. bungei* and *A. persica* occur in Iran show some degree of morphological overlaps that make the species delimitation difficult. Till present time, there has been no detailed information available on morphological and genetic structure of these species in the country. The aims of the present study are: 1) to find the diagnostic value of SCoT markers in delimitation of *Allochrusa* species, 2) to find the genetic structure of these taxa in Iran, and 3) to investigate the species inter-relationship. For this study, 97 randomly collected plants from 7 geographical populations in three *Allochrusa* species were used. We encountered extensive within species genetic and morphological diversity. SCoT molecular markers could delimit the studied species. AMOVA and STRUCTURE analysis revealed that the species of *Allochrusa* are genetically differentiated. The Mantel test showed correlation between genetic distance and geographical distance of the populations studied. Genetic affinity of the studied species has been discussed.

Keyword: Allochrusa, Endemics, Population structure, SCoT markers, Species delimitation

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INTRODUCTION

Species delimitation is essential since species is regarded as the basic unit of analysis in nearly all biological disciplines, such as ecology, biogeography, conservation biology, and macroevolution.

Caryophyllaceae is one of the largest angiosperm family. It contains 86 genera and about 2200 species. These species are distributed mainly in Mediterranean region (HEYWOOD, 1998; HARBAUGH *et al*., 2010; GREENBERG and DONOGHUE, 2011; PIRANI *et al*., 2014; HERNÁNDEZ- LEDESMA *et al*., 2015). There are 3 subfamilies among Caryophyllaceae: Alsinoideae Burnett, Caryophylloideae Arn., and Paronychioideae A.St. (MADHANI *et al*., 2018). Genus *Allochrusa* Bunge (BOISSIER, 1867: 559) belonging to family Caryophyllaceae, subfamily Caryophylloideae. *Allochrusa* having about 8 species which is distributed in Turkey, Central Asia, Afghanistan, Caucasus, Transcaucasia and Iran (BOISSIER, 1867; SCHISCHKIN, 1936; CULLEN, 1967; SCHIMAN-CZEIKA, 1988). According to Flora Orientalis by Bunge (BOISSIER, 1867: 559) *Allochrusa* include: three species in Iran [*A. versicolor* Boissier (1867: 559), *A. bungei* Boissier (1867: 560), *A. persica* Boissier (1867: 560)].

SCHISCHKIN (1936) classified *Acanthophyllum* into two subgenera [*Euacanthophyllum* (BOISSIER, 1867: 561) SCHISCHKIN (1936: 783) and *Allochrusa* (Bunge in BOISSIER, 1867: 559) Schischkin (1936: 799)], recognizing two sections in subg. *Allochrusa* with six species. SCHIMAN-CZEIKA (1988) reported four *Allochrusa* species, three of which recorded from Iran, in which two are endemics.

Acanthophyllum Meyer (1831: 210) in a broad sense comprises 80–90 perennial subshrubby species which are distributed mainly in the Irano-Turanian region (see e.g., BITTRICH, 1993; GHAFFARI, 2004; PIRANI *et al.*, 2014). According to the phylogenetic study by Pirani *et al.* (2014), *Acanthophyllum* s.lat. includes 11 sections. Molecular phylogenetic analysis supports that *Allochrusa* belongs to a clade together with *Acanthophyllum* but because of significant morphological differences, *Allochrusa* has been treated as a separate genus (Pirani *et al.*, 2014). *Acanthophyllum*, with a predominantly cushion habit and spiny leaves, should include taxa formerly assigned to *Allochrusa*, *Ochotonophila* Gilli, *Scleranthopsis* Rech.f. and part of *Diaphanoptera* Rech.f (PIRANI *et al*., 2014).

According to MADHANI *et al*. (2018) the *Acanthophyllum* s.l. clade includes some other unarmed plants such as members of *Allochrusa*, and therefore, recognition of neither *Gypsopgila herniarioides* nor *Allochrusa* spp. under separate genera are supported by molecular data (see also PIRANI *et al*., 2014). Their results revealed that for both markers (ITS) and the chloroplast gene *rps16* does not allow *Allochrusa* to be separated from *Acanthophyllum*. The species of the genus *Allochrusa* were considered once as members of *Acanthophyllum* subg. *Allochrusa* (SCHISCHKIN, 1936) and molecular phylogenetic studies by MADHANI *et al.* (2018) corroborate the taxonomic treatment performed by PIRANI *et al.* (2014) and contradict the treatment by HERNÁNDEZ-LEDESMA *et al*. (2015) where it was recognized provisionally at the generic level. According to this concept, it is necessary to resurrect the generic name *Acanthophyllum* for some taxa treated as *Allochrusa* in recent taxonomic surveys (MADHANI *et al.*, 2018)

According to the available literature i.e. (BOISSIER, 1867; SCHISCHKIN, 1936; CULLEN, 1967; SCHIMAN-CZEIKA, 1988) from the morphological point of view, the members of this genus are identified by the character of leaves, which is narrow but not rigid and spiny, flowers in a

paniculate or corymbose inflorescence, calyx tubular, 5-nerved, membranous between the nerves, petals 5, ovules 4–5 on a hemispherical placenta, styles 2, capsule 1-seeded, rupturing irregularly at the apex. Seed are reniform, strongly curved.

Allochrusa lutea Falat. & Mahmoodi is a recently recorded species in flora of Iran its natural habitat is limited to the NW of Iran. They have found out that the more similar species is *A. persica* from which *A. lutea* differ by shape of ovary and number of ovules, petals color and the ratio of inflorescence length to stem length (MAHMOODI and FALATOURY, 2016).

With the progress in plant molecular biology, numerous molecular marker techniques have been developed and used widely in evaluating genetic diversity, population structure and phylogenetic relationships. In recent years, advances in genomic tools provide a wide range of new marker techniques such as, functional and gene targeted markers as well as develop many novel DNA based marker systems (ESFANDANI-BOZCHALOYI *et al.* 2017 a; 2017b; 2017c; 2017d). Start codon targeted (SCoT) polymorphism is one of the novel, simple and reliable gene-targeted marker systems. This molecular marker offers a simple DNA-based marker alternative and reproducible technique which is based on the short conserved region in the plant genes surrounding the ATG (WILLIAMS *et al.* 1990; WELSH and MCCLELLAND, 1990; WEISING *et al.* 1995) translation start codon (COLLARD and MACKILL, 2009). This technique involves a polymerase chain reaction (PCR) based DNA marker with many advantages such as low-cost, high polymorphism and extensive genetic information (HU and QUIROS, 1991; WILDE *et al.* 1992; DEMEKE *et a1.* 1996; GRANDO *et al.* 1996).

There are no attempt to study genetic diversity, ecological adaptation and intra- and interspecific differentiation on *Allochrus* of Iran. Therefore, we performed molecular study of 3 collected species of *Allochrusa*. The project try to answer the following questions: 1) Is there infra- and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa? 4) Is there any gene exchange between *Allochrusa* species in Iran? Therefore, it is important to delimit the identified species for performing further detailed molecular studies.

MATERIALS AND METHODS

Plant materials

A total of 97 individuals were sampled representing 7 natural populations (6-10 samples from each populations), in East Azerbaijan Provinces of Iran (Table 1). Different references were used for the correct identification of species (*Allochrusa versicolor*, *A. bungei* and *A. persica*), (BOISSIER, 1867; SCHISCHKIN, 1936; CULLEN, 1967; SCHIMAN-CZEIKA, 1988). Details of sampling sites are mentioned in Table 1. Vouchers were deposited at the herbarium of ParsAbad Moghan Branch, Islamic Azad University, ParsAbad Moghan, Iran (IAUH).

DNA extraction and SCoT assay

Fresh leaves were used randomly from 5-8 plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (DOYLE and DOYLE, 1990). The quality of extracted DNA was examined by

running on 0.8% agarose gel. A total of 22 SCoT primers developed by COLLARD and MACKILL (2009), 6

Table 1. Location and herbarium accession numbers of the studied populations of A. bungei, A. persica and A. versicolor collected by Mehri in Iran.

Sp.	Population	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
A. bungei	1	East Azarbayejan, Tabriz to Sperkhan to Sahand	36°43'20.25"	48°20'32.07"	1450-2000	IAUH 3455
A. bungei	2	East Azarbayejan, Nematabad, near Tabriz	36°44'22.38"	48°14'35.88"	1400	IAUH 7896
A. bungei	3	East Azarbayejan, between Marand and Jolfa	36°65'86	48°38'65"	1800	IAUH 6899
A. versicolor	4	East Azarbayejan, Marand-Khoy	36°36'39	48°83'93"	1300	IAUH 4187
A. versicolor	5	West Azarbayejan, 10 km from Gharaziaeddin to Marand, 8 km from Babolabad	36°87'77	48°90'10"	955	IAUH 4629
A. persica	6	East Azarbayejan, Tabriz to Sperkhan to Sahand	36°19'22	48°34'88"	1500	IAUH 4567
A. persica	7	East Azarbayejan, Tabriz, Nematabad,	36°30'97	48°90'10"	1200	IAUH 6309

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

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
SCoT-14	ACGACATGGCGACCACGC	15	15	100.00%	0.36	5.86	8.55	3.45
SCoT-15	ACGACATGGCGACCGCGA	10	9	84.99%	0.33	3.91	8.43	3.85
SCoT-16	CCATGGCTACCACCGGCC	19	19	100.00%	0.44	3.34	10.55	2.44
SCoT-17	CATGGCTACCACCGGCCC	20	20	100.00%	0.37	4.88	9.56	4.85
SCoT-18	ACCATGGCTACCACCGCG	15	14	93.74%	0.47	4.66	5.56	3.67
SCoT-19	GCAACAATGGCTACCACC	13	12	92.31%	0.34	5.21	8.60	3.55
Mean		9.2	8.7	91.22%	0.32	4.3	8.67	3.8
Total		92	87					

TNB - the number of total bands, NPB: the number of polymorphic bands, PPB (%): the percentage of polymorphic bands, PI: polymorphism index, EMR, effective multiplex ratio; MI, marker index; PIC, polymorphism information content for each of CBDP primers

PCR reactions were carried in a 25µl volume containing 10 mMTris-HCl buffer at pH 8; 50 mMKCl; 1.5 mM $MgCl_2$; 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: 5min initial denaturation step 94°C, followed by 36 cycles of 1min at 95°C; 1 min at 50-52°C and 1 min at 72°C. The reaction was completed by final extension step of 5-10 min at 72°C. The amplification products were observed by running on 1% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Molecular analyses

SCoT bands were coded as binary characters (presence = 1, absence = 0) and used for the study of genetic diversity. For grouping of the plant specimens, PCA (Principal components analysis) biplot was used among the studied populations (PODANI, 2000). PAST version 2.17 (HAMMER *et al.*, 2012) was used for multivariate statistical analyses of SCoT data.

Using two parameters, polymorphism information content (PIC) and marker index (MI), the discriminatory capacity of the primers used was evaluated to characterise the ability of each primer to detect polymorphic loci among the genotypes (POWELL *et al.*, 1996). For each primer, MI was calculated as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (n) and the polymorphic fragment fraction (β) (HEIKRUJAM *et al*., 2015). For each primer, both the number of polymorphic bands (NPB) and the effective multiplex ratio (EMR) were determined. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were determined (WEISING *et al*., 2005; FREELAND *et al*., 2011). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering. Mantel test checked the correlation between geographical and genetic distance of the studied populations (PODANI, 2000). These analyses were done by PAST ver. 2.17 (HAMMER *et al*., 2012), DARwin ver. 5 (2012) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (PEAKALL and SMOUSE 2006), and was used to show genetic difference of the populations.

The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (PRITCHARD *et al*., 2000). For STRUCTURE analysis, data were scored as dominant markers (FALUSH *et al*., 2007). We used the admixture ancestry model under the correlated allele frequency model. A Markov chain Monte Carlo simulation was run 20 times for each value of K after a burn-in period of 10^5. The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (EVANNO *et al*., 2005). Gene flow was determined by (i) Calculating Nm an estimate of gene flow from Gst by PopGene ver. 1.32 (1997) as: Nm = 0.5(1 - Gst)/Gst.

RESULTS

Species delimitation and inter-relationship

Six SCoT primers were screened to study genetic relationships among *Allochrusa* species; all the primers produced reproducible polymorphic bands in all three *Allochrusa* species. A total of 92 amplified polymorphic bands were generated across three *Allochrusa* species (Table 2). The size of the amplified fragments ranged from 100 to 3000 bp.

Genetic diversity parameters determined in three studied species (Table 3). The highest value of percentage polymorphism (63.91%) was observed in *A. bungei* which shows high value for gene diversity (0.38) and Shanon' information index (0.27). Population of *A. persica* has the lowest value for percentage of polymorphism (44.38%) and the lowest value for Shanon, information index (0.13), and He (0.28).

Table 3. Genetic diversity parameters based on SCoT data in the studied *Allochrusa* species. (N = number of samples, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Species	N	Na	Ne	I	He	UHe	%P
A. bungei	12.000	0.347	1.199	0.271	0.384	0.392	63.91%
A. versicolor	5.000	0.358	1.457	0.234	0.30	0.31	56.50%
A. persica	6.000	0.299	1.029	0.131	0.28	0.23	44.38%

Population genetic differentiation

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 53% of total variation was among species and 47% was within species (Table 4). Pair-wise FST values showed significant difference among all studied species. Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.56, P = 0.01) and D_est values (0.456, P = 0.01). These results indicate that the geographical populations of *Allochrusa* species are genetically differentiated from each other. Nm analysis by Popgene software also produced mean Nm = 0.76, that is considered very low value of gene flow among the studied species. This is in agreement with other analysis.

Table 4. Analysis of molecular variance (AMOVA) of the studied species.

Source	df	SS	MS	Est. Var.	%	*Φ*PT
Among Pops	23	1601.36	88.789	17.134	53%	
Within Pops	110	274.443	2.805	2.858	47%	53%
Total	133	1855.80		19.060	100%	

df: degree of freedom; SS: sum of squared observations; MS: mean of squared observations; EV: estimated variance; *Φ*PT: proportion of the total genetic variance among individuals within an accession, (P < 0.001).

Nei's genetic identity and the genetic distance determined among the studied species are presented in Table 5. The results showed that the highest degree of genetic similarity (0.88) occurred between *A. versicolor* and *A. bungei* and then between *A. persica* and *A. bungei* (0.84). As we did not encounter any intermediate plants particularly in the areas of overlap, we consider these species to have high degree of ancestral shared alleles.

Mantel test with 5,000 permutations showed a significant correlation (r = 0.48, p = 0.0001) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Allochrusa* species studied.

Different clustering and ordination methods produced similar results therefore, only PCA plot of and UPGMA tree based on Nei's genetic distance are presented here (Fig. 1, 2). In general, plant samples of each species were grouped together and formed a separate group. These results show that SCoT marker can delimit *Allochrusa* species. In the studied specimens we did not encounter intermediate forms.

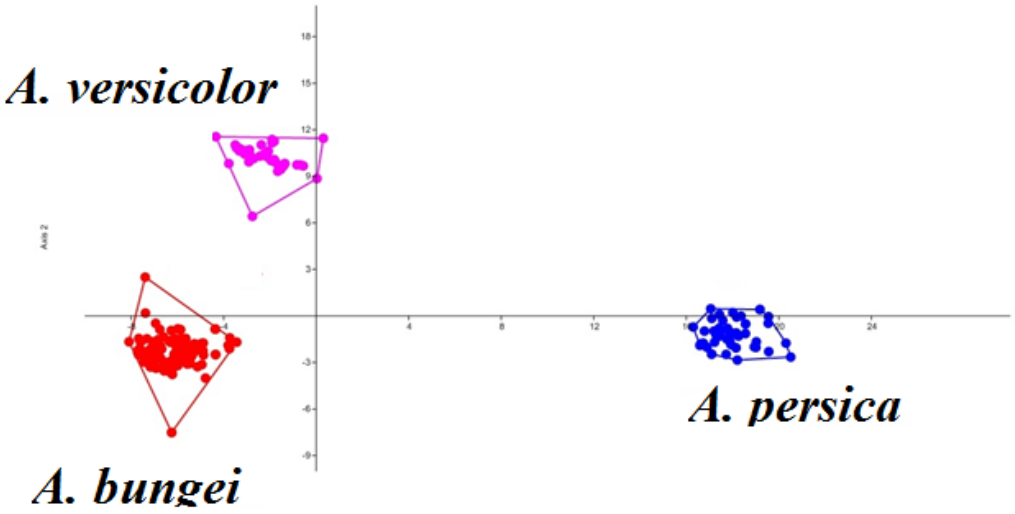

Fig. 1. PCA plot based on SCoT data in the studied *Allochrusa* species

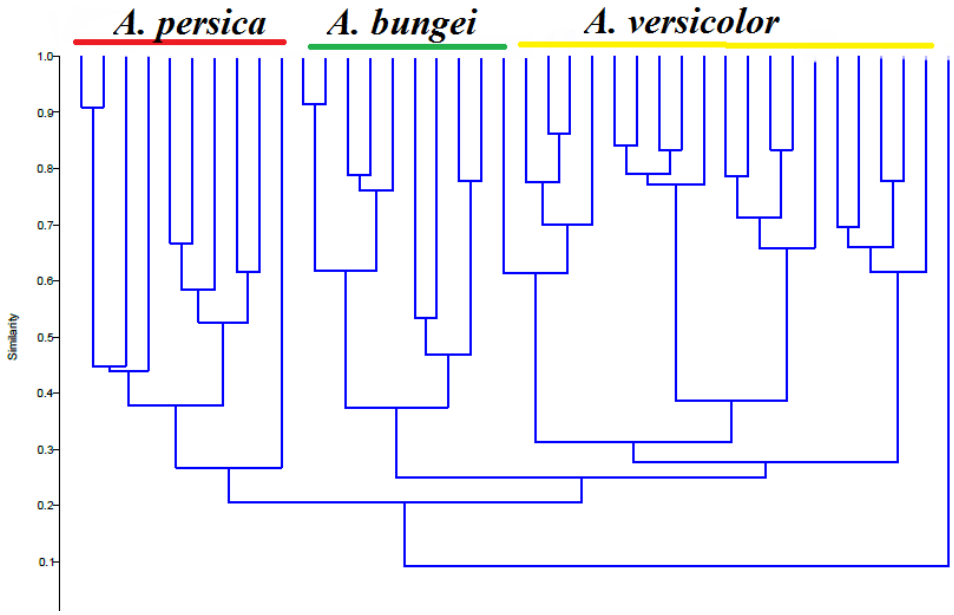

Fig. 2. UPGMA dendrogram of the studied populations based on SCoT data.

Table. 5. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) among the study Allochrusa species

Species	*A. bungei*	*A. versicolor*	*A. persica*
A. bungei	*****	0.8822	0.8405
A. versicolor	0.0176	*****	0.8031
A. persica	0.0145	0.0033	*****

UPGMA dendrogram based on Nei's genetic distance (Fig. 2) separated the species into distinct groups. This indicates that SCoT molecular markers can be used in *Allochrusa* species delimitation. This is in agreement with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other.

We performed STRUCTURE analysis followed by the Evanno test to identify the optimal number of genetic groups. We used the admixture model to illustrate interspecific gene flow and/or ancestrally shared alleles in the species studied.

STRUCTURE analysis followed by Evanno test produced ΔK = 3 (Fig. 3). The STRUCTURE plot (Fig. 4) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and/ or gene flow among *Allochrusa* species.

Therefore, we do have at least 3 genetic groups in the studied species: 1- specimens with red segments. These are accessions of *A. bungei*; 2- specimens having blue colored, these are accessions of *A. versicolor* and specimens having green colored, these are accessions of *A. persica*. This is in agreement with UPGMA dendrogram presented before.

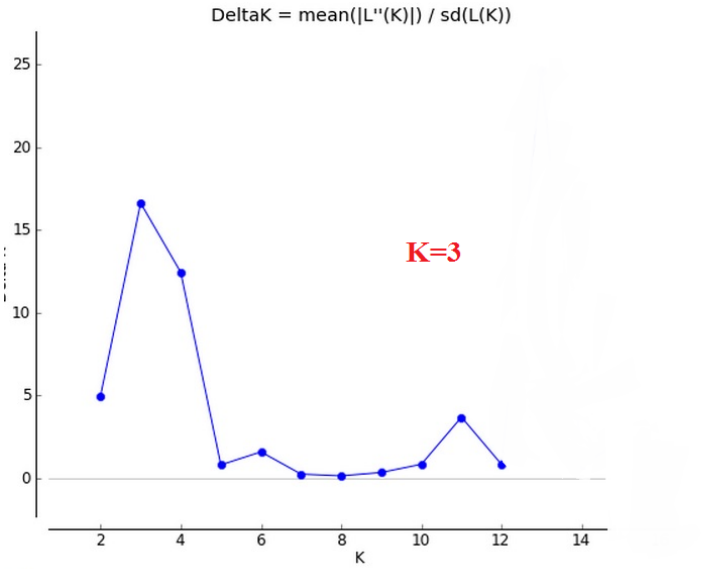

Fig. 3. Delta k plot of Evann's test based on STRUCTURE analysis

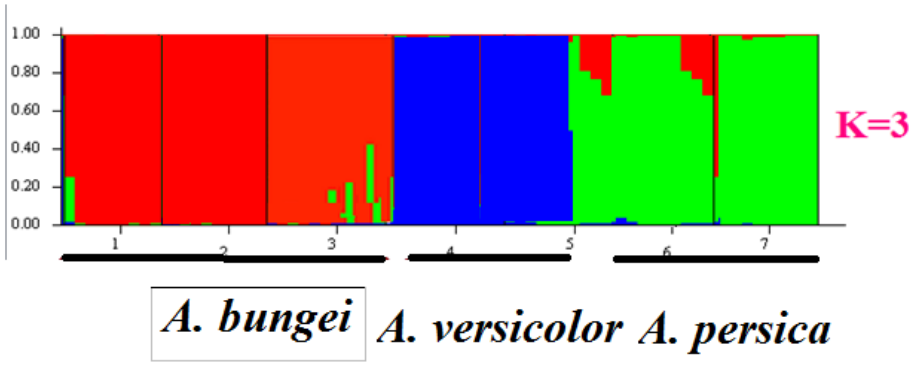

Fig.4. STRUCTURE plot of *Allochrusa* species showing interspecific genetic variability and admixture.

The low Nm value (0.76) indicates limited gene flow or ancestrally shared alleles between the species studied and supports genetic stratification as indicated by K-Means and STRUCTURE analyses. Population assignment test also agreed with Nm result and could not identify significant gene flow among members of the studied species. As evidenced by STRUCTURE plot based on admixture model, these shared alleles comprise very limited part of the genomes in species studied and all these results are in agreement in showing a high degree of genetic stratification in species studied.

DISCUSSION

Species protection programs targeting large areas should focus on the most valuable populations to deliver tangible effects. The initiated actions require a thorough understanding of species biology and ecology as well as knowledge of the levels and distribution of genetic diversity (NEEL and ELLSTRAND, 2003; ESFANDANI-BOZCHALOYI *et al.*, 2018a; 2018b; 2018c; 2018d). Genetic diversity is an important consideration in species conservation because it influences a population's ability to adopt to a changing environment (REED and FRANKHAM, 2003; JUMP *et al*., 2009; KIRK and FREELAND, 2011). Dwindling populations of rare and endangered plant species are often characterized by low levels of genetic diversity (FRANKHAM, 1996; LEIMU, 2006). The above can be attributed to the fact that genetic diversity within an endangered population is lost due to relatively faster genetic drift, which is exacerbated by limited gene flow. This can lead to inbreeding depression and higher homozygosity, which results in reduced adaptive potential. Genetic diversity is often correlated with plant fitness, and more genetically diverse populations are also more fit.

The species delimitation in complex groups and in those that the species have different degree of morphological overlap is a tedious and difficult task. In these situations, it is suggested to use different and combined approaches like morphological, molecular, cytological, etc. to determine the species boundaries (CARSTENS *et al*., 2013). In the last few decades the use of molecular markers as tools for species and subspecies delimitation has drastically increased (5). The basic premise for the use of molecular markers for species delimitation is that the "species tree" should be inferred from a "gene tree".

The present study revealed that a SCoT molecular marker can separate the species. We have no report on these species that considered as endemic to the northern-west of Iran, and therefore we studied the species growing in 1 provinces of the country considering 7 geographical populations.

The main achievements are:

Despite its macro and micromorphological similarities to *A. bungei* and *A. versicolor* is clearly recognized as a separate taxon. The main differences are stem and calyx indumentum, pedicle size, shape of calyx teeth, petal apex and limb shape are significant to separating species. The results are in conformity with (MAHMOODI and FALATOURY, 2016) findings. Also, they showed *Allochrus lutea* is close to *A. persica* from which differs in having 8–9 ovules (instead of 4 ovules), elliptic ovary (not obconic), yellow petals (not white or pink), retuse petal apex (not obtuse) and having flowers only on upper half of the stem. Nevertheless, *A. persica* & *A. lutea* are similar in habit, leaves shape and indumentum.

SCHIMAN-CZEIKA (1988), in Flora Iranica, described *A. bungei* as asubshrubs, covered with glandular hairs; petals pink but *A. persica* is perennial herbs with thick woody caudex, without distinctive glandular hairs; petals white with purple striae on the claw and sometimes at the base of limb, that confirms our result is in agreement with the results of SCHIMAN- CZEIKA (1988) and SHISHKIN (1936).

AMOVA and STRUCTURE analysis revealed that the species of *Allochrusa* are genetically differentiated but have some degree of shared common alleles.

The Nm value obtained based on SCoT data, revealed very limited amount of gene flow among the studied species that was also supported by STRUCTURE analysis as *Allochrusa* species mostly had distinct genetic structure. Reticulation analysis also showed some degree of gene flow for SCoT molecular. We did not observe any intermediate forms in our extensive plant collection. To conclude, the present study revealed the use of SCoT molecular markers in *Allochrusa species* delimitation.

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PROTOK GENA I STRUKTURA POPULACIJE KOD *ALLOCHRUSA* (CARYOPHYLLOIDEAE, CARYOPHYLLACEAE) POMOĆU MOLEKULARNIH MARKERA

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Izvod

Allochrusa Bunge je rod podfamilije Cariophilloideae Rabeler & Bittrich koji sadrži oko 8 vrsta rasprostranjenih u Turskoj, Centralnoj Aziji, Avganistanu, Kavkazu, Zakavkazju i Iranu. Tri vrste *Allochrusa versicolor*, *A. bungei* i *A. persica* se javljaju u Iranu i pokazuju određeni stepen morfoloških preklapanja koja otežavaju razgraničenje vrsta. Do danas nisu postojale detaljne informacije o morfološkoj i genetskoj strukturi ovih vrsta u zemlji. Ciljevi ove studije su: 1) pronalaženje dijagnostičke vrednosti SCoT markera u razgraničenju vrsta *Allochrusa*, 2) pronalaženje genetske strukture ovih taksona u Iranu i 3) istraživanje međusobnog odnosa vrsta. Za ovu studiju je korišćeno 97 nasumično sakupljenih biljaka iz 7 geografskih populacija tri vrste *Allochrusa*. Našli smo na opsežne genetske i morfološke diverzitete vrsta. Molekularni markeri SCoT mogli bi da ograniče proučavanu vrstu. Analiza AMOVA i STRUKTURE otkrila je da su vrste *Allochrusa* genetski diferencirane. Mantel test pokazao je korelaciju između genetske i geografske udaljenosti proučavanih populacija. Analiyiran je I genetski afinitet proučavanih vrsta.

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