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Molecular identification and genetic relationships among *Alcea* (Malvaceae) species by ISSR Markers: A high value medicinal plant

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Abstract. *Alcea* L. is one of the largest genera of *Malvaceae* family with nearly 70 species worldwide mainly distributed in SW Asia. According to the latest revision of the family, it is represented by 34 species in the Flora of Iran, among them, 15 species are endemic. It is tough to accurate germplasm/ plant recognition by using morphological characteristics because of its propagation, growing and using. We conducted a molecular data analysis on these plant species due to their importance. We examined 156 plants from 14 species in 16 regions that were selected randomly for this investigation. It has been 119 polymorphic bands (94.33%) were resulted from 128 bands of 10 primers in amplification of genomic DNA. ISSR primers have a great capacity to detect polymorphic loci among *Alcea* species, as evidenced by the high average PIC and MI values found. The genetic similarity of 14 species was calculated and ranged between 0.635 to 0.990. Inter-Simple sequence repeats (ISSR) markers research revealed that *Alcea tarica* Pakravan & Ghahreman and *Alcea kopetdaghensis* lljin had the least similarity, while *Alcea semnanica* Pakravan and *Alcea mazandaranica* Pakravan & Ghahreman had the most. The current study attempts to answer three questions: 1) can ISSR markers identify *Alcea* species? 2) what is the genetic structure of these taxa in Iran? and 3) what is the inter-relationship between these taxa? The current study discovered that ISSR markers can be used to identify species.

Keywords: population structure, gene flow, network, genetic admixture.

INTRODUCTION

It is vital to determine the precise boundaries of a species in order to gain a better understanding of any scientific investigations. As a result, in the context of biology, species delimitation is a topic that receives a lot of attention (Collard and Mackill 2009; Wu *et al.* 2013). However, establishing the criterion that could be used to resolve species borders is a contentious issue (Esfandani-Bozchaloyi *et al.* 2018a, 2018b, 2018c, 2018d). (Pandey *et al.* 2008).

Furthermore, the analysis of wild population genetic structure and the study of intra-specific levels of genetic diversity are critical for the creation of successful conservation measures. The Malvaceae family includes the perennial herb *Alcea* L., which has its primary centers of diversity in the Western Mediterranean Basin and the Middle East (Zohary 1963a, b, Hutchinson 1973, Riedl 1976, Heywood *et al*. 1978). In Europe, there are only a few species of *Alcea* (Escobar *et al*. 2009). The Flora of Iran has 34 species, 15 of which are endemic, according to the most recent revision of the family (Pakravan 2008) *Alcea* species are usually tall-growing hemicryptophytes that grow annually, biennially, or perennially. The stem is erect, rarely branching at the base, and occasionally acaulescent. The leaves might be simple, lobed, palmatipartite, or palmatisect in shape. The sepals are five in number and are connate at the base. Petals are pentamerous and come in a variety of colors. Mericarps come in a variety of shapes and sizes, each with a sterile upper chamber and a single seeded bottom chamber. (Ghahreman *et al*. 2001, Pakravan & Ghahreman 2006, Pakravan 2006, 2008).

The mucilage that containing the plants of the Malvaceae family are sources of carbohydrates, which are used in medicine (Azizov *et al*. 2007). The species of this family, especially *Alcea rosea* has been used as diuretic, demulcents, emollient, aperients, and in the treatment of burning sensation, skin disease, and constipation (Shaheen *et al*. 2010).

Delimitation of *Alcea* and *Althaea* ganera has been a challenging task in taxonomic history of Malvaceae. *Alcea* has been traditionally included in *Althaea* based on epicalyx characteristics (Bentham & Hooker 1862, Baker 1890, Candolle 1837, Edlin 1935, Willdenow 1800). However, characteristics of staminal column and fruit features led to consider *Alcea* and *Althaea* as two separate taxa (Alefeled 1862; Boissier 1867; Iljin 1949).

Molecular-phylogenetic data also support the monophyly and distinctness (as suggested by morphological data) of *Alcea* but they are of limited use in determining relationships between species and species delimitations (Escobar Garsia *et al*. 2012). The taxonomic complexity of *Alcea* is remarkable (Zohary 1963a,b, Riedl 1976, Townsend 1980). *Alcea* has so far proposed two infrageneric classifications, each of which is divided into a few informal groups. Despite the fact that it has a significant number of species, no formal subgeneric categorization has been established. Due to uniformity and pronounced plasticity in morphological characters of this genus (especially in flower and fruit characters), some traits such as leaf sequence, mericarp shape, relative length of calyx versus epicalyx, and indumentum morphology are more applicable in taxonomy of *Alcea* (Escobar Garcia *et al*. 2012). For researching genetic diversity, molecular markers are a useful tool. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Mutation (ISSM) are two sophisticated genetic markers. For diversification assessments, ISSR markers have been routinely used (Pharmawati *et al*. 2004). The RAPD approach is rapid, simple, and does not require any prior sequence awareness. Then uses a single primer of any nucleotide sequence, the approach detects nucleotide sequence polymorphism (Moreno *et al.* 1998). A single 16-18 bp. long primer consists of a repeating sequence attached at the 3' or 5' end of 2-4 arbitrary nucleotides is used to amplify DNA for ISSR markers. The method is faster, easier, and less affordable than RAPD, and it is more repeatable (Esfandani-Bozchaloyi *et al*. 2017a, 2017b, 2017c, 2017d; Collard and Mackill 2009; Wu *et al*. 2013). The current study used new gene-targeted molecular markers, namely ISSR markers, to assess the genetic diversity and connections among different *Alcea* species. We conducted a genetic research of 156 collected specimens of 14 *Alcea* species because this is the first study on the usage of ISSR markers in the *Alcea* genus.

We 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 *Alcea* species in Iran?

MATERIALS AND METHODS

Plant materials

A total of 156 individuals were sampled representing 16 geographical populations belonging to 14 *Alcea* species in East Azerbaijan, Lorestan, Kermanshah, Mazandaran, Esfahan, Tehran, Khorasan, Semnan, Fars, Golestan Provinces of Iran during July-Agust 2016-2019 (Table 1). We utilized 156 botanical accessions (three to twelve samples of each group) from 16 different populations with various eco-geographic attributes for ISSR analysis which were extracted and stored in -20 until further use. More information about geographical distribution of accessions are in Table 1 and Fig. 1.

During several field excursions to the all part of Iran as well as survey to the several herbaria {Herbarium of Iranian Research Institute of Plant Protection (IRAN), Herbarium of Tehran University (TUH), Herbarium of Shahid Beheshti University (SBUH), and some Herbaria of Natural Resources Research Centers in most provinces of Iran such as: East and West Azerbaijan], some new

Table 1. Voucher details of *Alcea* species in this study from Iran.

No	Sp.	Locality	Latitude	Longitude	Altitude (m)
Sp1	*Alcea aucheri* (Boiss.) Alef.	Esfahan:Ghameshlou, Sanjab Kermanshah, Islamabad	38°52'37"	47°23'92"	1144
Sp2	*Alcea angulata* Freyn & Sint.	Tehran, Damavand	32°50'03"	51°24'28"	1990
Sp3	*Alcea rhyticarpa* (Trautv.) Iljin	Khorasan, Mashhad	29°20'07"	51°52'08"	1610
Sp4	*Alcea sulphurea* (Boiss.& Hohen.) Alef.	Tehran, Tochal	38°52'37"	47°23'92"	1144
Sp5	*Alcea striata* (DC.) Alef.	Kermanshah, Islamabad Esfahan, Semirom	33°57'12"	47°57'32"	2500
Sp6	*Alcea loftusii* (Baker) Zohary	Lorestan, Oshtorankuh, above Tihun village	34°52'37"	48°23'92"	2200
Sp7	*Alcea gorganica* (Rech. f., Aellen & Esfand.) Zohary	Golestan, Gorgan	38°52'37"	47°23'92"	1144
Sp8	*Alcea popovii* Iljin	Tehran, Chalous	35°50'03"	51°24'28"	1700
Sp9	*Alcea mazandaranica* Pakravan & Ghahreman	Mazandaran Province, Kelardasht, Rodbarak	36°14'14"	51°18'07"	1807
Sp10	*Alcea tarica* Pakravan & Ghahreman	Tehran, Damavand	32°36'93"	51°27'90"	2500
Sp11	*Alcea ghahremanii* Pakravan & Assadi	East Azerbaijan, Arasbaran	37°07'02"	49°44'32"	48
Sp12	*Alcea kopetdaghensis* lljin	Khorasan, Koppeh Dagh	28°57'22"	51°28'31"	430
Sp13	*Alcea iranshahrii* Pakravan, Ghahreman & Assadi	Fars, Estahban	30°07'24"	53°59'06"	2178
Sp14	*Alcea semnanica* Pakravan	Semnan, Damghan	28°57'22"	51°28'31"	288

Figure 1. Map of Iran shows the collection sites and provinces where 14 *Alcea* species were obtained for this study; sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii* ; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica.*

information were obtained. The specimens were identified using the identification keys and descriptions of the *Alcea* species in the relevant floras [Taxonomical Studies in *Alcea* of South-western Asia (Zohary 1963a, b), Flora Orientalis (Boissier 1967), Flora Palestina (Zohary 1972), Flora Iranica (Riedl 1976), Flora of Iraq (Townsend *et al.* 1980), and The Taxonomic Revision of *Alcea* and *Althaea* in Turkey (Uzunhisarcikli & Vural 2012).

DNA extraction and ISSR Assay

In every one of the tested populations, fresh leaves were also used in random from one to twelve plants. Silica gel powder was used to dry them. To extract genomic DNA, the CTAB activated charcoal procedure was applied (Esfandani-Bozchaloyi *et al.* 2019). A 0.8 percent agarose gel was used to test the purity of the isolated DNA. 22 primers from the UBC (University of British Columbia) series were evaluated for DNA amplification for the ISSR study. Based on band reproducibility, ten primers were chosen for ISSR study of genetic diversity (Table 2).

PCR reactions were carried in a 25µl volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 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 following program was used to perform the amplifications and reactions in a Techne thermocycler (Germany): 94°C for 5 minutes, then 40 cycles of 1 minute at 94°C, 1 minute at 52-57°C, and 2 minutes at 72°C.

A final extension step of 7-10 minutes at 72°C finished the reaction. Running the amplification results

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

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
ISSR-1	DBDACACACACACACACA	15	13	93.84%	0.66	4.66	11.33	4.67
ISSR-2	GGATGGATGGATGGAT	12	11	94.91%	0.48	5.21	12.50	5.65
ISSR-3	GACAGACAGACAGACA	16	14	95.74%	0.67	5.66	9.57	5.37
ISSR-4	AGAGAGAGAGAGAGAGYT	13	12	92.31%	0.54	8.21	10.23	4.55
ISSR-5	ACACACACACACACACC	17	17	100.00%	0.47	7.32	11.55	4.18
ISSR-6	GAGAGAGAGAGAGAGARC	11	10	96.89%	0.43	6.56	9.34	7.17
ISSR-7	CTCTCTCTCTCTCTCTG	13	12	95.81%	0.34	4.21	6.78	5.59
ISSR-8	CACACACACACACACAG	12	12	100.00%	0.47	3.37	9.55	3.45
ISSR-9	GTGTGTGTGTGTGTGTYG	11	9	93.89%	0.53	6.56	8.34	6.11
ISSR-10	CACACACACACACACARG	11	11	100.00%	0.59	4.22	10.11	4.33
Mean		12.8	11.9	94.33%	0.55	5.32	10.66	5.7
Total		128	119					

Note: 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 CAAT box- derived polymorphism (CBDP) primers.

over a 1 percent agarose gel and staining with ethidium bromide revealed the amplification products. A 100-bp molecular size ladder was used to assess the fragment size (Fermentas, Germany).

Data analyses - Molecular analyses

The collected ISSR bands were coded as binary characters (presence = 1, absence = 0) and utilized to analyze genetic diversity. The UPGMA (Unweighted paired group using average) ordination methods were utilized to sort the plant specimens into groups (Podani 2000). To quantify the capability of each primer to distinguish polymorphic loci amongst these genotypes, two measures, polymorphism information content (PIC) and marker index (MI), were utilized to assess its discriminatory ability (Powell *et al.* 1996). MI is calculated for each primer as MI = PIC × EMR, where EMR is the product of the number of polymorphic loci per primer (*n*) and the fraction of polymorphic fragments (β) (Heikrujam *et al.* 2015). For each primer, the effective multiplex ratio (EMR) and the number of polymorphic bands (NPB) were computed. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (P% =number of polymorphic loci/number of total loci) were determined (Weising *et al*, 2005, Freeland *et al.* 2011). Shannon's index was calculated by the formula: H' = -Σpiln pi. Rp is defined per primer as: Rp = ∑ Ib, were "Ib" is the band informativeness, that takes the values of 1-(2x [0.5-p]), being "p" the proportion of each genotype containing the band. GenAlEx 6.4 software is used to analyze the percentage of polymorphic loci, the mean loci by accession and population, UHe, H', and PCA (Peakall & Smouse 2006). Neighbor Joining (NJ) clustering and Neighbor-Net networking were based on Nei's genetic distance between populations (Freeland e*t al*. 2011, Huson & Bryant 2006). The Mantel test was used to see if there was a link between the analyzed populations' geographical and genetic distances (Podani 2000). The comparison of genetic divergence or genetic distances, estimated by pairwise FST and related statistics, with geographical distances by Mantel test is one of the most popular approaches to evaluate spatial processes driving population structure. The Mantel test, as originally formulated in 1967,

$$Z_m = \sum_{i=1}^{n} \sum_{j=1}^{n} g_{ij} \times d_{ij}$$

Where *gij* and *dij* are, respectively, the genetic and geo-graphic distances between populations i and j, considering populations. Because Zmis given by the sum of products distances its value depends on how many populations are studied, as well as the magnitude of their distances. The Zm-value can be compared with a null distribution, and Mantel originally proposed to test it by the standard normal deviate (SND), given by SND =Zm/var(Zm)1/2 (Mantel 1967). These analyses were done by PAST ver. 2.17 (Hammer *et al.* 2012), DARwin ver. 5 (2012) software. To show genetic differences between the populations, the AMOVA (Analysis of molecular variance) test (with 1000 permutations) was utilized, which was implemented in GenAlex 6.4

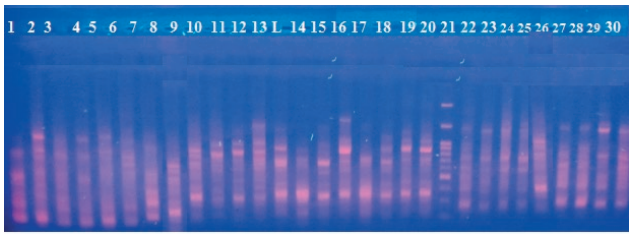

Figure 2. Electrophoresis gel of *Alcea* species from DNA fragments produced by ISSR-5 and ISSR-3; sp1,14= *A. aucheri;* sp2,15= *A. angulata;* sp3,16= *A. rhyticarpa*; sp4,17= *A. sulphurea;* sp5,18= *A. striata*; sp 6,19= *A. loftusii*; sp7,20= *A. gorganica*; sp8,21= *A. popovii;* sp9,22= *A. mazandaranica*; sp10,23: *A. tarica*; sp11,24: *A. ghahremanii*; sp12,25= *A. kopetdaghensis*; sp13,26= *A. iranshahrii*; sp14,27= *A. semnanica.*

(Peakall & Smouse 2006). This approach considers the equal amount of gene flow among all populations. The genetic structure of populations was studied by Bayesian based model STRUCTURE analysis (Pritchard *et al.* 2000), and maximum likelihood-based method of K-Means clustering of GenoDive ver. 2 (2013). Data were evaluated as dominating markers for STRUCTURE analysis (Falush *et al.* 2007). Under the correlated allele frequency model, we used the admixture ancestry model. After a 105 burn-in period, a Markov chain Monte Carlo simulation was ran 20 times for each value of K. Using the delta K value, the Evanno test was run on the STRUCTURE result to determine the right number of K. (Evanno *et al.* 2005)

RESULTS

Species identification and genetic diversity

To examine genetic links among *Alcea* species, ten ISSR primers were tested; all of the primers yielded replicable polymorphic bands in all 14 *Alcea* species. Figure 2 depicts the ISSR amplification induced by the ISSR-5 primer. Across 14 *Alcea* species, a total of 119 amplified polymorphic bands were produced. The amplified fragments were between 100 and 3000 bp in length. With an average of 11.9 polymorphic bands per primer, ISSR-5 had the most and lowest number of polymorphic bands, with 17 and 9 respectively. The average PIC of the 10 ISSR primers was 0.55, ranging from 0.34 (ISSR-7) to 0.67 (ISSR-3). The MI of the primers ranged from 3.45 (ISSR-8) to 7.17 (ISSR-6) on average, with an average of 5.7. ISSR primers had an EMR ranging from 6.78 (ISSR-7) to 12.50 (ISSR-2), with an average of 10.66 per primer (Table 2). The primers with the highest EMR values were thought to be more useful in separating the genotypes. The genetic parameters for all 14 *Alcea* species amplified with ISSR primers were calculated (Table 3). Unbiased expected heterozygosity (*H*) ranged from 0.15 (*Alcea popovii*) to 0.39 (*Alcea aucheri*), with a mean of 0.28. Shannon's information index (I) showed a similar pattern, with the greatest value of 0.39 in *Alcea aucheri* and the lowest value of 0.10 in (*Alcea popovii*) , with a mean of 0.27. The number of alleles (Na) observed in *Alcea rhyticarpa* ranged from 0.201 to 0.645 in *Alcea kopetdaghensis.* The effective number of alleles (Ne) in

Table 3. Genetic diversity parameters in the studied *Alcea* species.

SP		N	Na	Ne	I	He	UHe	%P
Sp1	*Alcea aucheri*	5.000	0.462	1.095	0.398	0.48	0.39	76.55%
Sp2	*Alcea angulata*	8.000	0.399	1.167	0.322	0.398	0.344	65.77%
Sp3	*Alcea rhyticarpa*	8.000	0.201	0.095	0.23	0.27	0.22	42.23%
Sp4	*Alcea sulphurea*	5.000	0.341	1.058	0.24	0.27	0.20	53.75%
Sp5	*Alcea striata*	5.000	0.455	1.077	0.277	0.24	0.22	55.05%
Sp6	*Alcea loftusii*	8.000	0.499	1.067	0.24	0.23	0.24	49.26%
Sp7	*Alcea gorganica*	6.000	0.555	1.020	0.22	0.25	0.28	43.53%
Sp8	*Alcea popovii*	10.000	0.431	1.088	0.20	0.22	0.25	41.53%
Sp9	*Alcea mazandaranica*	3.000	0.255	1.021	0.25	0.28	0.22	47.15%
Sp10	*Alcea tarica*	9.000	0.261	1.024	0.292	0.23	0.23	43.15%
Sp11	*Alcea ghahremanii*	12.000	0.287	1.253	0.266	0.254	0.28	51.99%
Sp12	*Alcea kopetdaghensis*	3.000	0.645	1.062	0.24	0.224	0.213	44.73%
Sp13	*Alcea iranshahrii*	8.000	0.499	1.067	0.24	0.281	0.24	49.26%
Sp14	*Alcea semnanica*	12.000	0.287	1.233	0.271	0.284	0.292	51.91%

Abbreviations: N = number of samples, Na= number of different alleles; Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations.

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

Source	df	SS	MS	Est. Var.	%	*ΦPT*
Among Pops	29	1601.364	45.799	15.194	67%	
Within Pops	122	454.443	1.905	2.884	33%	67%
Total	151	2033.807		17.020	100%	

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

these species ranged from 0.095 (*Alcea rhyticarpa*) to 1.253 (*Alcea ghahremanii*). The AMOVA test revealed a substantial genetic difference (P = 0.001) between the species investigated. It was discovered that 67 percent of overall variance was found across species, whereas 33 percent was found within species (Table 4). Significant Nei's GST (0.245, P = 0.001) and D est (0.765, P = 0.001) values further indicated the genetic difference of these species. In comparison to within-species genetic diversity, these findings demonstrated a larger distribution of genetic variety within *Alcea* species.

Species identification and inter-relationship

Because the results of other clustering and ordination approaches were similar, PCA plot and UPGMA clustering are provided here (Figure 3-4). Plant samples from different species were put together and formed various groups in general. This study demonstrates that the examined species were divided into several groups based on molecular characteristics. We didn't find any intermediate forms in the specimens we looked at. The dendrogram based on ISSR data was constructed by UPGMA analysis, grouping all of the *Alcea* species into two major clusters (Fig. 4). The first major cluster divided into two minor clusters of which the first minor cluster again divided into two sub-minor clusters. The first sub-minor cluster consisted of *A. aucheri; A. rhyticarpa* and *A. striata.* The second sub-minor cluster was represented by *A. angulata; A. sulphurea*. The second major cluster divided in to two minor clusters of which the first minor cluster consisted of *A. loftusii, A. gorganica* and *A. popovii*. The second sub-minor cluster was represented by *A. kopetdaghensis*, *A. mazandaranica*, *A. tarica*, *A. ghahremanii*, *A. iranshahrii*; and *A. semnanica*. This is consistent with the AMOVA and genetic diversity metrics previously reported. Genetically, the species are distinct from one another. ISSR molecular markers can be employed to taxonomist *Alcea* species, according to these findings.

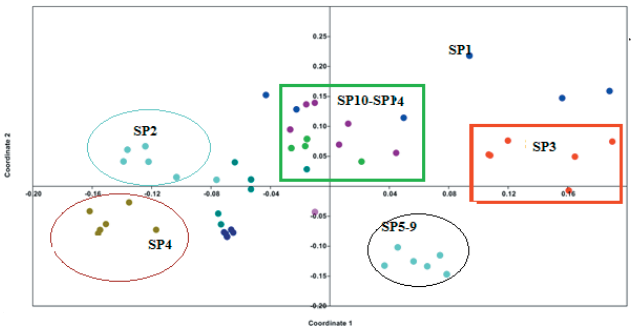

Figure 3. PCA plots of based on ISSR data revealing species delimitation in *Alcea* species; sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii* ; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica.*

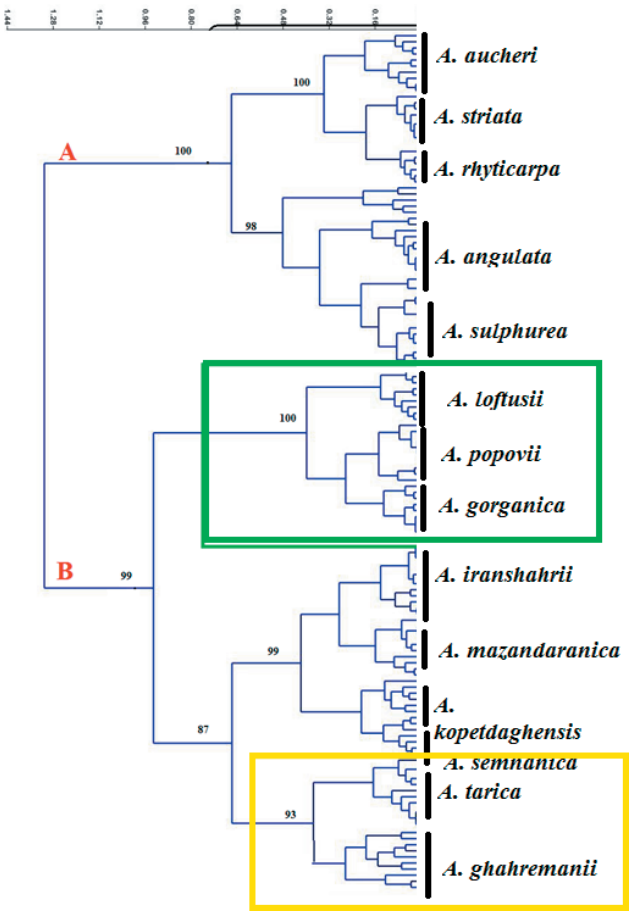

Figure 4. Dendrogram generated using the unweighted pair group method with arithmetic average (UPGMA) analysis showing relationships among different *Alcea* species using ISSR data.

Table 5. The matrix of Nei genetic similarity (*Gs*) estimates using ISSR molecular markers among 14 *Alcea* species. sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii*; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica*

sp1	1.000														sp1
sp2	0.887	1.000													sp2
sp3	0.891	0.744	1.000												sp3
sp4	0.738	0.787	0.842	1.000											sp4
sp5	0.705	0.742	0.745	0.775	1.000										sp5
sp6	0.778	0.891	0.744	0.936	0.838	1.000									sp6
sp7	0.599	0.702	0.808	0.875	0.836	0.862	1.000								sp7
sp8	0.754	0.785	0.676	0.829	0.733	0.800	0.709	1.000							sp8
sp9	0.757	0.741	0.758	0.816	0.740	0.785	0.676	0.725	1.000						sp9
sp10	0.737	0.890	0.722	0.719	0.853	0.741	0.758	0.834	0.746	1.000					sp10
sp11	0.807	0.799	0.755	0.812	0.774	0.990	0.722	0.768	0.800	0.721	1.000				sp11
sp12	0.782	0.744	0.636	0.834	0.750	0.799	0.755	0.720	0.785	0.635	0.839	1.000			sp12
sp13	0.702	0.757	0.703	0.778	0.691	0.744	0.636	0.829	0.741	0.750	0.799	0.642	1.000		sp13
sp14	0.751	0.774	0.732	0.790	0.750	0.797	0.812	0.774	0.990	0.675	0.727	0.728	0.684	1.000	sp14

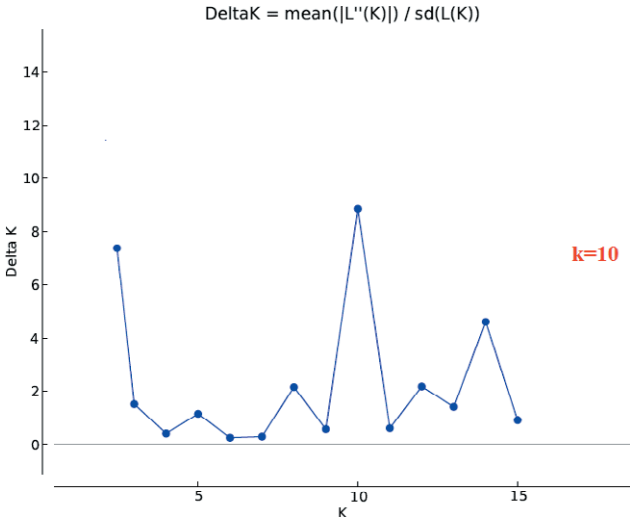

Figure 5. Evanno test produced ΔK = 10 of ISSR data in *Alcea* species.

degree of genetic resemblance (0.99). Between *Alcea tarica* and *Alcea kopetdaghensis*, there was the least genetic affinity (0.63). The low Nm value (0.47) implies little gene flow or ancestrally shared alleles between the species investigated, as well as considerable genetic divergence between and within *Alcea* species.

To determine the ideal number of genetic groups, we used STRUCTURE analysis followed by the Evanno test. In the species analyzed, we employed the admixture model to show interspecific gene flow and/or ancestrally shared alleles.

STRUCTURE analysis followed by Evanno test produced ΔK = 10 (Fig. 5). The STRUCTURE plot (Fig. 6) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and/ or gene flow among *Alcea* species. This plot revealed that Genetic affinity between *Alcea aucheri* and *A. sulphurea* (similarly colored, No. 1, 4), as well as *A. gorganica*; *A. popovii* and *A. semnanica;* (No. 7,8,14)

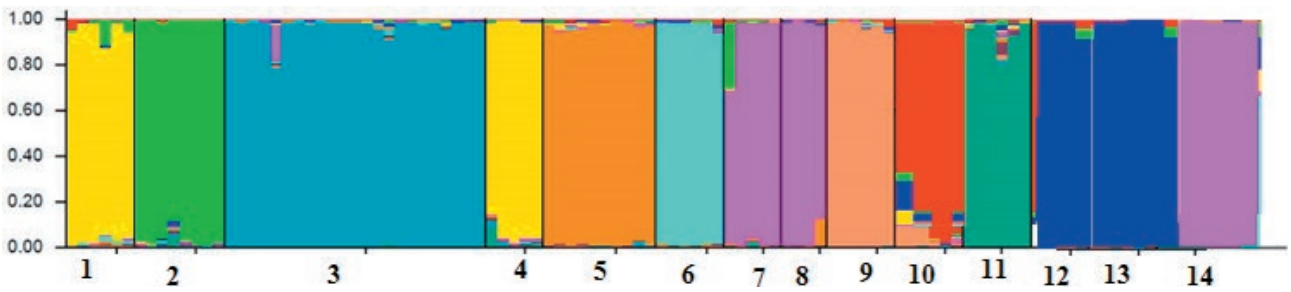

Figure 6. STRUCTURE plot of ISSR data in *Alcea* species. sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii*; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica.*

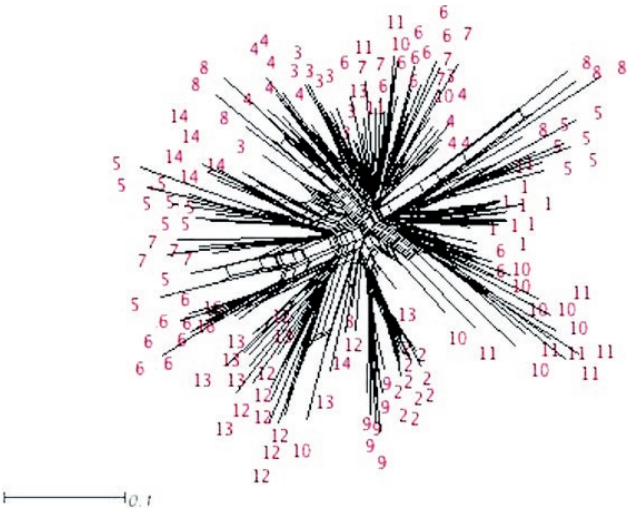

Figure 7. Neighbor-Net of ISSR data in *Alcea* species. sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea;* sp5= *A. striata*; sp 6= *A. loftusii*; sp7= *A. gorganica*; sp8= *A. popovii;* sp9= *A. mazandaranica*; sp10: *A. tarica*; sp11: *A. ghahremanii*; sp12= *A. kopetdaghensis*; sp13= *A. iranshahrii*; sp14= *A. semnanica*

due to shared common alleles. This is in agreement with UPGMA dendrogram presented before. The other species are distinct in their allele composition. The NeighborNet diagram (Fig. 7) also revealed almost complete separation of the studied species within the network, supporting the AMOVA results. Populations 1, 2 and 12,13 are distinct and stand separately from the other populations at a great distance. Populations 6 and 7 and populations 10 and 11 show a closer genetic affinity and are placed close to each other.

DISCUSSION

In the biology of long-term evolution of a group of animals or species, genetic diversity plays a crucial role. The foundation for a taxon's presence, development, and evolution. To recognize the taxonomy, origin, and evolution of a taxon, it is necessary to investigate its genetic diversity. In addition, such study could provide a theoretical foundation for the conservation, expansion, exploitation, and breeding of germplasm resources (Lubbers *et al*. 1991). The current study provided fascinating information about genetic variability, genetic stratification, and morphological difference in Iran's north and west. The degree of genetic variability within a species is significantly connected with its reproduction method; the higher the degree of open pollination/cross breeding, the greater the genetic variability in the taxon under study (Meusel *et al.* 1965). A primer's PIC and MI features aid in establishing its efficacy in genetic diversity analysis. The ability of a marker technique to resolve genetic variability, according to Sivaprakash *et al.* (2004), may be more directly connected to the degree of polymorphism. PIC values ranging from zero to 0.25 indicate relatively low genetic variation among genotypes, 0.25 to 0.50 indicate a mid-level of genetic diversity, and ≥ 0.50 indicate a high level of genetic diversity (Tams *et al.* 2005). The PIC values of the ISSR primers in this study ranged from 0.34 to 0.66, with a mean value of 0.55, indicating that ISSR primers have a good level of competence in detecting genetic diversity among *Alcea* species. In the *Alcea* taxon, all ten primer pairs demonstrated good polymorphism. For the species under investigation, a total of 128 alleles were discovered. The total number of polymorphic bands per primer varied from 9 to 17, and the average allele number in loci was 11.9. Occurrence of high polymorphism could be explained for species in different climatic zones with varying selection pressure during the course of evolution (Mishra *et al.* 2011).

In most studies, population size is limited to several vegetative accession (Meusel *et al.* 1965; Uotila 1996). This population could be showed genetic drift, whose effect are observed in the high level of F_{IS} and low level of genetic diversity. The isolation of the population and absence the gene flow led to fragmentation of the *Alcea* populations. Between genetic diversity parameters and population size were showing positive correlations that confirmed various studies (Leimu *et al.* 2006). There are two reasons for the positive correlation between genetic diversity and population size (Leimu *et al.* 2006). 1- A positive connection may confirmed the existence of an extinction vortex, in which declining population reduces genetic variety, resulting in inbreeding depression.

Plant fitness separates populations depending on habitat quality changes, which is the second cause. Low levels of genetic variation, according to Booy *et al.* (2000), can impair plant fitness and limit a population's capabilities to react to environmental changes by selection and adaptation.

Genetic diversity (33%) was obtained within populations, whereas 67% of genetic variation obtained between the evaluated populations. The breeding system in plant species is one of the primary elements controlling the distribution of genetic variation (Duminil 2007). Couvet (Booy *et al.* 2000) shown that one migrant each generation is insufficient to ensure long-term persistence of tiny populations, and that the number of migrants is determined by family background characteristics and population genetics (Vergeer *et al.* 2003). For the lack of distinctions across isolated groups, there are two explanations. The initial theory proposed that genetic variety

in and between populations demonstrates gene flow patterns, resulting in group splitting (Dostálek *et al.* 2010). Geographically close communities are far more successfully associated via gene flow than populations segregated by considerable distance, according to the next objective. Merely a few research have investigated into Alcea's genetic diversity thus far. Kazemi *et al.* (2011) found a 93 percent polymorphism ratio with strong genetic resemblance (0.31 to 0.75) within *A. rosea* species in Iran using RAPD identifiers analysis. Utilizing RAPD markers, Oztürk *et al.* (2009) evaluated the genetic profiles of 18 *Alcea* species and found wide difference (0.13 to 0.69) throughout them. According to Badrkhani *et al.* (2014), the sequence-related amplified polymorphism (SRAP) identifier was used to evaluate the genetic diversity and genetic similarity links among 14 *Alcea* species were collected from the northwest of Iran. Seventeen SRAP primer pairings produced 104 segments, with an average of 5.7 polymorphic fragments per primer. The percentage of polymorphism spanned from 50% (ME2-EM6) to 100% (ME2-EM6), with an average polymorphism information content value of 0.3. The genetic similarity between *A. sophiae* and *A. flavovirens* was the lowest (0.17), while the highest was identified between *A. digitata* and *A. longipedicellata* (0.68). Using UPGMA, two primary clusters were discovered, neither of which corresponded to the species' geographical origin. According to their findings, SRAP markers may be suitable for analyzing genetic diversity in *Alcea*. So far, only morphological data has been used to define Iranian *Alcea* species. However, due to the very small number of characteristics, the genus has a challenging taxonomy. According to Pakravan's (2008) study on *Alcea*, only the leaf sequence and carpel structure are valuable traits.

Escobar Garcia *et al.* (2012) with using three molecular markers (nrDNA ITS and the plastid spacers *psbA-trnH* and *trnL-trnF*), showed that a phylogeny of *Alcea* and test previous infrageneric taxonomic hypotheses as well as its monophyly with respect to *Althaea*, a genus with which it has often been merged. They also go into morphological variation and the use of morphological features as phylogenetic association indicators. While molecular findings indisputably corroborate the circumscription of *Alcea* deduced from morphology, they are of limited usefulness in clarifying interspecific relationships, implying that Alcea's great species diversity is attributable to swift and early radiation. Their research establishes the first *Alcea* phylogeny and intends to pave the way for future research into the processes that underpin species radiation in the Irano-Turanian region.

In conclusion, the results of this study showed that to evaluate the genetic diversity of the *Alcea* genus in the Irano-Turanian region, a main center of species diversity for many medium-sized to large genera that remains greatly understudied. ISSR-derived primers were more successful than those produced from all other molecular markers. In addition, *Alcea* species were clearly distinguished from one another in the dendrogram and PCA, demonstrating that the ISSR approach is more effective in identifying *Alcea* species.

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