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Delimiting species using DNA and morphological variation in some *Alcea* (Malvaceae) species based on SRAP markers

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Abstract. Species identification is fundamentally important within the fields of biology, biogeography, ecology and conservation. The genus *Alcea* (Malvaceae) includes approximately 70 species of mainly Irano-Turanian distribution and is considered one of the most challenging genera of the Middle East, due to its uniformity and pronounced plasticity in morphological traits. In spite vast distribution of many *Alcea* species that grow in Iraq, there are not any available report on their genetic diversity, mode of divergence and patterns of dispersal. Therefore, we performed molecular (SRAP marker) and morphological studies of 80 accessions from 10 species of *Alcea* that were collected from different habitats in Iraq. The aims of present study are: 1) can SRAP markers identify *Alcea* species, 2) what is the genetic structure of these taxa in Iraq, and 3) to investigate the species inter-relationship? The present study revealed that combination of morphological and SRAP data can identify the species.

Keywords: *Alcea,* SRAP, Morphology, Species Identification.

1. INTRODUCTION

Alcea L. (Malvaceae) is considered one of the most complicated and challenging genera of the Middle Eastern flora (Iljin, 1949; Zohary, 1963b; Riedl, 1976; Townsend, 1980; Pakravan, 2001).

The Irano-Turanian floristic region (Takhtajan, 1986) stretches from central Anatolia to the highlands of central Asia and is a main center of diversity for many medium- to large-sized genera.

A well-suited system for investigating radiations in the Irano-Turanian region is the genus *Alcea*. It includes approximately 70 species, which are

mainly of Irano-Turanian distribution with extensions into the Caucasus and the eastern Mediterranean (Zohary, 1963b). Riedl (1976) has reported 39 species in Iran, but the number has been reduced to 34 due to taxonomic rearrangement among them, 15 species are endemic (Pakravan 2008b). *Alcea* species are mainly annual, biennial or perennial, mostly tall-growing hemicryptophytes. The stem is erect and rarely branched from the base or acaulescent in a few cases. The mucilage that containing the plants of the Malvaceae family are sources of carbohydrates, which are used in medicine (Azizov *et al*., 2007).

Any classifications are hampered by uniformity in many morphological and ecological traits (flower, inflorescence and fruit structure, habitat and life form) combined with a pronounced plasticity in the morphological characters considered important for species identification (indumentum, leaf shape and degree of division, calyx and epicalyx morphology, flower colour; Zohary, 1963b, c). Due to this plasticity, accurate species identification requires character state combinations of the sequence of change of leaf morphology along the main stem (hereafter leaf sequence), relative proportions of calyx and epicalyx lobes and (mature) mericarp morphology, which are rarely found together on herbarium specimens. Additionally, due to political tensions in the Middle East and the Caucasus, *Alcea* has received only limited attention in recent studies of Malvaceae (La Duke & Doebley, 1995; Alverson & al., 1998; Nyffeler & al., 2005; Tate & al., 2005; Escobar García & al., 2009). So far, only two infrageneric classification systems have been suggested. Boissier (1867) defined two sections, *Pterocarpae* Boiss. and *Apterocarpae* Boiss., distinguished by winged versus unwinged mericarps. Zohary (1963b, c) proposed nine informal groups based on overlapping character combinations (leaf shape and degree of leaf division, mericarp morphology, relative dimensions of calyx and epicalyx, indumentum).

Previous study on species delimitation and species relationship performed in this genus. Badrkhani et al (2014) sequence-related amplified polymorphism (SRAP) marker was employed to assess the genetic diversity and genetic similarity relationships among 14 species of *Alcea* collected from northwest of Iran. Two main clusters were detected using UPGMA, which did not

correspond to geographical origin of the species. Their study indicates that SRAP markers could be good candidates for assessing genetic variation in *Alcea*.

Escobar García et al. (2012) examines the phylogeny of *Alcea* using three molecular markers (nrDNA ITS and the plastid spacers *psbA-trnH* and *trnL-trnF*), their results show that while molecular data unambiguously support the circumscription of *Alcea* inferred from morphology, they prove to be of limited utility in resolving interspecific relationships, suggesting that *Alcea*s high species diversity is due to rapid and recent radiation.

Literature revealed that studies are mainly dealing with taxonomy, seed and pollen morphology, stem and leaf anatomy (Arabameri and Khodayari 2019; Escobar García et al. 2012) of *Alcea* species and also, genetic diversity of *Alcea* species have been reported in only some studies by (Badrkhani et al (2014)) but there is no attempt to study genetic diversity, ecological adaptation and intra- and inter-specific differentiation along with morphometric studies on of Iraq. Therefore, we performed morphological and molecular study of 80 collected specimens of 10 of *Alcea* species. 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 Iraq?

2. MATERIALS AND METHODS

2.1. Plant materials

We performed morphological and molecular analysis of 10 *Alcea* species growing in Iraq. For morphometric studies we used 80 plant specimens (5-15 samples from each species) and for SRAP analysis, we used 80. Different references were used for the correct identification of species (Zohary, 1963b; Riedl, 1976; Townsend, 1980; Pakravan, 2001). Details of sampling sites are mentioned (Table 1).

2.2. Morphological studies

In total 36 morphological quantitative characters were studied (supplementary Table 2). Data obtained were standardized (Mean= 0, variance = 1) and used to estimate Euclidean distance for clustering and ordination analyses (Podani 2000).

2.3. Dna extraction and srapassay

Fresh leaves were used randomly from 5-11 plants in each of the studied species. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA. The SRAP analysis was performed as described by Li and Quiros (2001). Ten SRAP

Table 1. *Alcea* species and populations, their localities and voucher numbers.

Sp.
A. sachsachanica Iljin
A. flavovirens (Boiss. & Buhse) Iljin
A. rechingeri (Zohary) I. Riedl
A. arbelensis Boiss. & Hausskn.,
A. koelzii I. Riedl,
A. hyrcana (Grossh.) Grossh.
A. peduncularis Boiss. & Hausskn.
A. glabrata Alef.
A. tabrisiana (Boiss. & Buhse) Iljin
A. persarum Bornm.

primer combinations (PCs) were used (Table 3); these were synthesized by Bioneer (Daejeon, Korea). 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 amplifications' reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, followed by 40 cycles of 1 min at 94°C; 1 min at 52-57°C and 2 min at 72°C. The reaction was completed by final extension step of 7-10 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).

2.4. Data analyses

2.4.1. Morphological studies

Morphological characters were first standardized (Mean = 0, Variance = 1) and used to establish Euclidean distance among pairs of taxa (Podani 2000). For grouping of the plant specimens, The UPGMA (Unweighted paired group using average) ordination methods were used (Podani 2000). ANOVA (Analysis of variance) were performed to show morphological difference among the populations while, PCA (Principal components analysis) biplot was used to identify the most variable morphological characters among the studied populations (Podani 2000). PAST version 2.17 (Hammer et al. 2012) was used for multivariate statistical analyses of morphological data.

Table 2. Morphological characters in studied species.

No	Characters
1	Plant height (mm)
2	Length of stem leaves petiole (mm)
3	Length of stem leaves (mm)
4	Width of stem leaves (mm)
5	Length / Width of stem leaves (mm)
6	Number of segment stem leaves (mm)
7	Length of basal leaves petiole (mm)
8	Length of basal leaves (mm)
9	Width of basal leaves (mm)
10	Length / Width of basal leaves (mm)
11	Number of segment basal leaves
12	Calyx length (mm)
13	Calyx width (mm)
14	Calyx length/ width (mm)
15	Petal length (mm)
16	Petal width (mm)
17	Petal length / width (mm)
18	Fruit length (mm)
19	Mericarp length (mm)
20	Mericarp width (mm)
21	Mericarp length/width (mm)
22	Seed length (mm)
23	Seed width (mm)
24	Seed length/ width (mm)
25	Stipules length (mm)
26	Stipules width (mm)
27	Stipules length/ width (mm)
28	Bract length (mm)
29	Bract width (mm)
30	Bract length / width (mm)
31	Pedicel length (mm)
32	Peduncle length (mm)
33	Rostrum length (mm)
34	Style length (mm)
35	Stamen filament length (mm)
36	Number of flowers per inflorescence

(flower, inflorescence and fruit structure, habitat and life form) combined with a pronounced plasticity in the morphological characters considered important for species identification (indumentum, leaf shape and degree of division, calyx and epicalyx morphology, flower colour.

2.4.2. Molecular analyses

SRAP bands obtained were coded as binary characters (presence = 1, absence = 0) and used for genetic diversity analysis. Parameter like Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism were

determined (Freeland et al. 2011). Nei's genetic distance among populations was used for Neighbor Joining (NJ) clustering and Neighbor-Net networking (Freeland et al. 2011, Huson & Bryant, 2006). 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) and SplitsTree4 V4.13.1 (2013) software. AMOVA (Analysis of molecular variance) test (with 1000 permutations) as implemented in GenAlex 6.4 (Peakall and Smouse 2006), and Nei's Gst analysis as implemented in GenoDive ver.2 (2013) (Meirmans & Van Tienderen 2004) were used to show genetic difference of the populations. Moreover, populations' genetic differentiation was studied by G'ST est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008).

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). For STRUCTURE analysis, data were scored as dominant markers (Falush et al. 2007). The Evanno test was performed on STRUCTURE result to determine proper number of *K* by using delta *K* value (Evanno et al. 2005). In K-Means clustering, two summary statistics, pseudo-F, and Bayesian Information Criterion (BIC), provide the best fit for k (Meirmans 2012). 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. This approach considers equal amount of gene flow among all populations. (ii) Population assignment test based on maximum likelihood as performed in Genodive ver. in GenoDive ver. 2. (2013). The presence of shared alleles was determined by drawing the reticulogram network based on the least square method by DARwin ver 5. (2012).

RESULTS

3.1. Species identification and inter-relationship

Morphometry

ANOVA showed significant differences (P <0.01) in quantitative morphological characters among the species studied. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 76% of the total variation. In the first PCA axis with 41% of total variation, such characters as seed outline, seed length, stipules length, shape of petal, peduncle and pedicel hair, stem hair, Stipules length/ width bract and leaf hair have shown the highest correlation (>0.7). Length of bract and peduncle, length of petal, sepal hair, number of flowers per inflorescence were characters influencing PCA axis 2 and 3 respectively.

Different clustering and ordination methods produced similar results. Therefore PCA plot of morphological characters are presented here (Fig. 1). In general, plant samples of each species, were grouped together and formed separate cluster. This result show that morphological characters studied can differentiate the *Alcea* species in two different major clusters or groups. In the studied specimens we did not encounter intermediate forms. The PCA plot of morphological characters (Fig. 1) separated the species into distinct groups with no intermixing. This is in agreement with UPGMA tree.

3.2. Species identification and genetic diversity

All SRAP primer produced polymorphic bands. Genetic diversity parameters determined in the studied species (Table 3) revealed that *A. rechingeri* had the highest level of genetic polymorphism (49.13%), while the lowest level of genetic polymorphism (17.22%) occurred in *A. sachsachanica*. *A. glabrata* also had the highest values for effective number of alleles (Ne = 1.264) and Shannon information index (I =0.235).

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed

that 60% of total variation was among species and 40% was within species. Pair-wise FST values showed significant difference among all studied species (Table 4). Moreover, genetic differentiation of these species was demonstrated by significant Nei's GST (0.89, P = 0.01) and D_est values (0.587, P = 0.01).

NJ tree based on Nei,s genetic distance (Fig. 2), showed that *A. flavovirens*; *A. arbelensis*; *A. persarum* are separated from the other studied species and join the others with a great distance. This dendrogram showed close genetic affinity between *A. koelzii* and *A. hyrcana*. Similarly, *A. rechingeri* and *A. peduncularis* were placed close to each other, to which, *E. litvinovii* was joined with some distance. In general, this indicates that SRAP molecular markers can be used in *Alcea* species differentiation. This is in agreement with AMOVA and genetic diversity parameters presented before. The species are genetically well differentiated from each other. The Nm analysis by Popgene software also produced mean Nm= 0.78, that is considered very low value of gene flow among the studied species.

Mantel test with 5000 permutations showed a significant correlation (r = 0.24, p=0.0002) between genetic

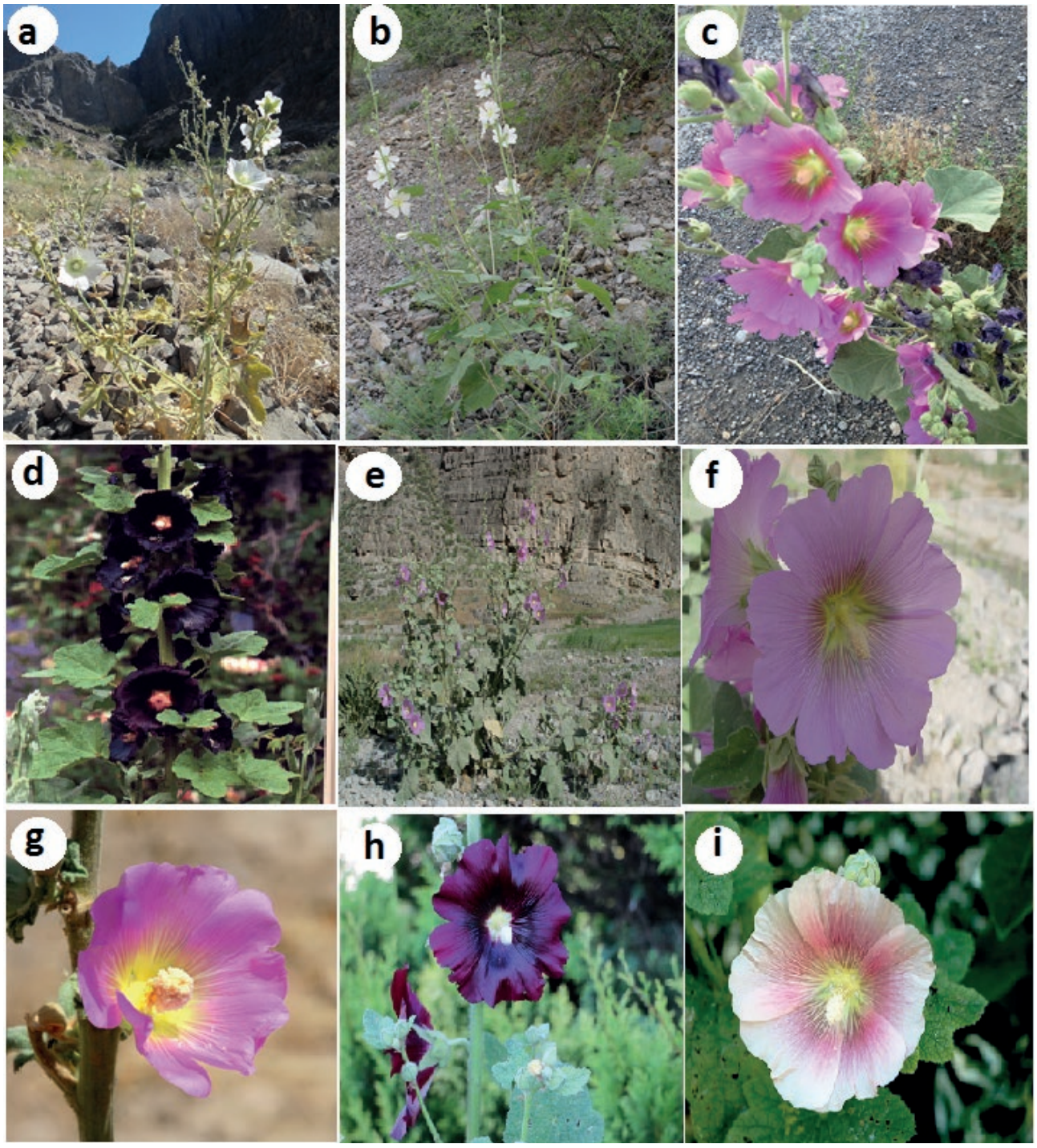

Figure 1. *Alcea* species:a, i: *A. flavovirens*; b: *A. sachsachanica*; c: *A. rechingeri*;d, h: *A. arbelensis*; e: *A. koelzii*; f, g: *A. hyrcana*

distance and geographical distance, so isolation by distance (IBD) occurred among the *Alcea* species studied.

Nei's genetic identity and the genetic distance determined among the studied species (Table is not included). The results showed that the highest degree of genetic similarity (0.83) occurred between *A. koelzii* and *A. hyrcana*. The lowest degree of genetic similarity occurred between *A. arbelensis* and *A. persarum* (0.64).

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

Pop	N	Na	Ne	I	He	UHe	%P
A. sachsachanica Iljin	13.000	0.178	1.017	0.016	0.002	0.019	17.22%
A. flavovirens (Boiss. & Buhse) Iljin	10.000	0.276	1.061	0.053	0.036	0.044	29.68%
A. rechingeri (Zohary) I. Riedl	17.000	0.355	1.145	0.234	0.288	0.211	49.13%
A. arbelensis Boiss. & Hausskn.,	10.000	0.301	1.009	0.211	0.154	0.177	43.23%
A. koelzii I. Riedl,	7.000	0.677	1.087	0.093	0.057	0.099	23.66%
A. hyrcana (Grossh.) Grossh.	10.000	0.699	1.156	0.143	0.094	0.205	27.96%
A. peduncularis Boiss. & Hausskn.	4.000	0.376	1.054	0.055	0.035	0.021	21.83%
A. glabrata Alef.	5.000	0.452	1.264	0.235	0.039	0.044	24.90%
A. tabrisiana (Boiss. & Buhse) Iljin	5.000	0.269	1.021	0.023	0.011	0.023	22.15%
A. persarum Bornm.	8.000	0.548	1.013	0.029	0.012	0.019	19.68%

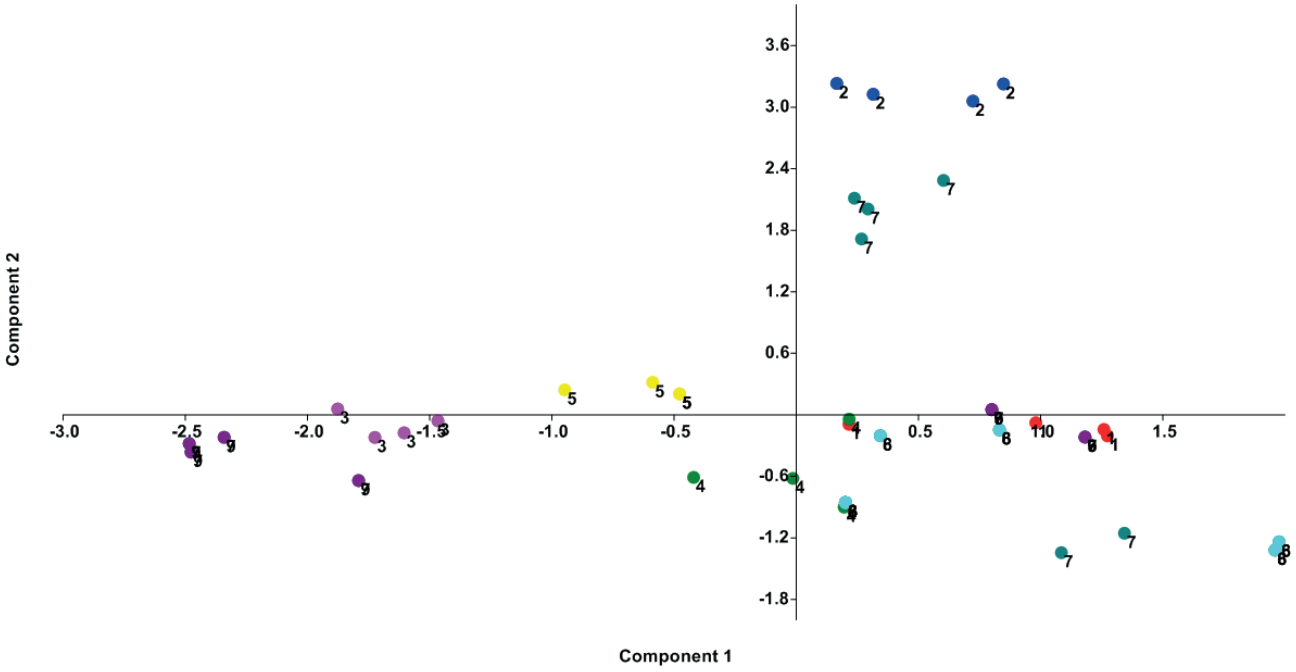

Figure 2. PCA plots of morphological characters revealing species delimitation in *Alcea* species

3.3. The species genetic structure

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 or / and ancestrally shared alleles in the species studied.

STRUCTURE analysis followed by Evanno test produced ΔK =10. The STRUCTURE plot (Fig. 3) 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 *A. rechingeri* and *A. arbelensis* (similarly colored), as well as *A. koelzii* and *A. hyrcana* (similarly colored) due to shared common alleles. This is in agreement with Neighbor joining dendrogram presented before. The other species are distinct in their allele composition and differed genetically from each other.

The low Nm value (0.78) 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

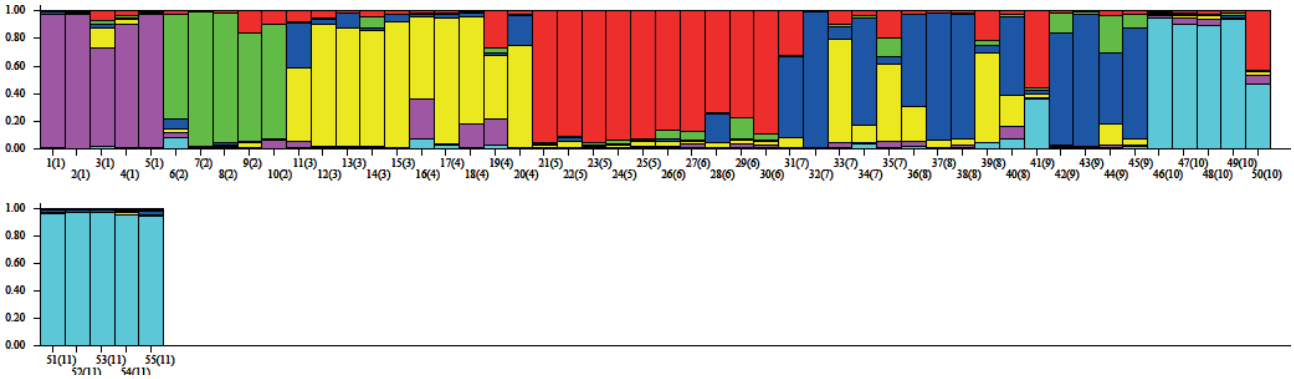

Figure 3. STRUCTURE plot of *Alcea* species based on SRAP data.

the studied species. However, reticulogram obtained based on the least square method (Figure not included), revealed some amount of shared alleles between species 2 and 1,3,5 and between 9 and 1,3-5 also between 3 and 1, 2, 9-10. 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 high degree of genetic stratification in species studied

4. DISCUSSION

4.1. Species identification and taxonomic consideration

Species delimitation is important in different biological disciplines, like ecology, biogeography, and plant conservation (Mayr 1982). Species delimitation is done by tree-based and non-tree-based approaches (Wiens 2007). In the first method, species form distinguishing clades (phylogenetic species concept), whereas in non-tree-based method, the species are recognized on the basis of gene flow assessments (biological species concept; Pérez-Losada et al. 2005).

Wiens & Penkrot (2002), proposed to use DNA data, morphological data and character data for species delimitation while, Knowles & Carstens (2007) addressed how molecular data (i.e., gene trees from DNA sequence data) can be used in species delimitation. The latter authors used coalescent simulations to test the species limits and incorporated data from multiple loci. They showed the importance of population genetics in species delimitation. Similarly, Medrano et al. (2014), applied population genetics methods to the species delimitation problem in *Narcissus* Linnaeus (1753: 289) (Amaryllidaceae J.St.-Hil. nom. cons.) by the help of amplified fragment length polymorphism (AFLP) molecular markers.

In the present study we used morphological and molecular (SRAP) data to evaluate species relationship in *Alcea*. Morphological analyses of the studied *Alcea* species showed that they are well differentiated from each other both in quantitative measures (the ANOVA test result) and qualitative characters (The PCA plot result). In addition, PCA analysis suggests that characters like bract length, stipule length, bract shape, calyx shape, petal shape, length and width of stem-leaf, length and width of petal, peduncle and pedicel hair, mericarp hair density, mericarp surface could be used in species groups delimitation. This morphological difference was due to quantitative and qualitative characters.

4.2. Genetic structure and gene flow

This is the first study on the use of SRAP markers for genetic diversity, species delimitation and determining genetic relationships among *Alcea* species in Iraq. Alarcón, et al., (2012) showed that SRAP technique along with proper statistical tools could be successfully applied to assess the genetic diversity and phylogenetic analysis among *Alcea* species in Iraq. Our results clearly demonstrated that SRAP markers can be used in genetic diversity study as well as genetic identification of *Alcea*. Moreover, our results indicate a very high efficiency of the SRAP markers in the identification and delimitation of *Alcea* species. Similar efficiency of the SRAP markers has also been reported by other authors (Alarcón, et al., 2012; Li, *et al*, 2021; Sun, *et al.* 2021; Xu, *et al*, 2021; Zhang, *et al.* 2022).

AMOVA and STRUCTURE analysis revealed that the species of *Alcea* are genetically differentiated but have some degree of shared common alleles. Several trends in pollination mechanism can be observed in *Alcea* with gradual transition between them. Based on RAPD markers analysis, Kazemi *et al.* (2011) showed

93% polymorphism level with high variation in genetic similarity (0.31 to 0.75) within *A. rosea* populations in Iran. Öztürk *et al*. (2009) analyzed genetic profile of 18 *Alcea* species using RAPD markers and reported wide differentiation (0.13 to 0.69) among them. According to Badrkhani *et al* (2014) sequence-related amplified polymorphism (SRAP) marker was employed to assess the genetic diversity and genetic similarity relationships among14 species of *Alcea* collected from northwest of Iran. Seventeen SRAP primer combinations generated 104 fragments, of which 97 (93%) were polymorphic, with an average of 5.7 polymorphic fragments per primer. Percentage of polymorphism ranged from 50% (ME2-EM6) to a maximum of 100%, and mean polymorphism information content value obtained was 0.3. The lowest genetic similarity (0.17) was observed between *A. sophiae* and *A. flavovirens*, while the highest was found between *A. digitata* and *A. longipedicellata* (0.68). Two main clusters were detected using UPGMA, which did not correspond to geographical origin of the species. Their study indicates that SRAP markers could be good candidates for assessing genetic variation in *Alcea*. Iranian *Alcea* species have only been characterized with morphological data, so far. However, the genus has a complicated taxonomy due to small number of characters. Based on study of Pakravan (2008) on *Alcea*, only examination of the leaf sequence and configuration of the carpels would represent valuable characters. For example, *A. fl avovirens* and *A. glabrata* differ only in the size of the carpel and width of wing (Pakravan 2008). Our results grouped these two species into two different clusters.

The methods we used are indirect estimation of gene flow and if it is identified to occur among species may be either due to ancestral shared alleles or ongoing gene flow. The Nm value obtained based on SRAP data, revealed very limited amount of gene flow among the studied species that was also supported by STRUCTURE analysis as *Alcea* species mostly had distinct genetic structure. Reticulation analysis also showed some degree of gene flow for SRAP. We did not observe any intermediate forms in our extensive plant collection, but morphological variability within each species did occur to some extent. To conclude, the present study revealed the use of SRAP molecular markers along with morphological characters in *Alcea* species identification. Some degrees of interspecific genetic admixture occur in *Alcea* species, but the studied species are strongly differentiated during the speciation process and invasion in new habitats. Genetic drift, strong inbreeding and local adaptation are effective evolutionary forces operating in *Alcea* species and population divergence and adaptation.

Plant species identification is of central importance in phylogenetic systematics, evolution, biogeography and biodiversity. It is significant to infer patterns and mechanisms of speciation and hybridization, the evolutionary process by which new biological species arise and gene flow between closely related phylogenetic species can occur (Al-Quran 2008; Bi, *et al.,* 2021; Duan, *et al.,* 2022; Guo, *et al*, 2021; Guo, *et al,* 2022).

Isolation by distance, local adaptation and gene flow are different mechanisms responsible for species differentiation and genetic diversity (Freeland et al. 2011, Frichot et al. 2013).

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