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Original scientific article

RAPD MARKER TO ASSESS GENETIC DIVERSITY and EXCHANGE GENES IN *Alcea* (Malvaceae)

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In order to comprehend the plant resources' protection and management in every setting, genetic variousness research is required. Because of the minimal number of distinguishing morphological features, for example, leaf sequence and carpel structure, *Alcea* includes a problematic taxonomy.34 *Alcea* species are reported in Iran among them, 15 species are endemic. There are no specifics. *Alcea* genetic variousness was studied using RAPD (Random Amplified Polymorphic DNA). As a result, we gathered and evaluated six species from five Iranian provinces. Seventy plant samples were gathered in total. Our objectives were to 1) determine the genetic variousness of *Alcea* species and 2) determine the genetic variousness of *Alcea* species. 2) Is there a link between a species's genetic makeup and geographical location? 3) Population and taxon genetic configuration. In-plant species, we found considerable variances in quantitative morphological features. *Alcea* species were separated into two categories using NJ clusters and principal component analysis. The unbiased anticipated heterozygozygosity (UHe) of *Alcea rhyticarpa* existed in the scope of 0.12. In *Alcea aucheri*, knowledge of Shannon was increased (0.39). The lowest value, 0.20, was found in Alcea rhyticarpa. In the Alcea species, gene flow (Nm) was quite modest (0.33). The current findings demonstrated RAPD markers and morphometry approaches for studying genetic variousness in *Alcea* species.

Keywords: Alcea, gene flow, isolation, morphometry, Random Amplified Polymorphic DNA (RAPD)

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INTRODUCTION

Alcea L. is one of the biggest Malvaceae genera, with approximately 70 species found mostly in Southeast Asia (ZOHARY 1963a, b, HUTCHINSON 1964, RIEDL 1976, HEYWOOD *et al*. 1978). The genus Alcea has been thought to have evolved in the Turkish Armenian and Kurdistan Highlands (ZOHARY 1963b). RIEDL (1976) identified 39 species in Iran; however, owing to taxonomic rearrangement, the total has decreased to 34, with 15 species being endemic (PAKRAVAN 2008b). *Alcea* species are usually tall-growing hemicryptophytes that grow yearly, biennially, or perennially. The stem is upright, seldom branching from the ground, and in a few instances acaulescent. The leaves are variable in shape from simple to lobed, palmatipartite or palmatisect. The sepals are 5 and connate at base. Petals are pentamerous and variable in color. Mericarps come in various shapes and sizes, each having a sterile upper chamber and a single-seeded bottom chamber. Plants in the Malvaceae family produce mucilage, which contains carbohydrates utilized in medication (AZIZOV *et al*., 2007). *Alcea rosea*, in particular, has been utilized as a diuretic, demulcent, emollient, and aperient, as well as in the cure of burning sensations, skin illness, 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). Nevertheless, differences in the staminal queue and fruit characteristics led to the classification of Alcea and Althaea as two distinct taxa (ALEFELED 1862).

Molecular-phylogenetic information also supports the monophyly as well as 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 complexness of *Alcea* is rather high (ZOHARY 1963a,b, RIEDL 1976, TOWNSEND 1980). *Alcea* has so far suggested two infrageneric categories, each divided into a few informal groupings. Despite the huge total of species, no official subgeneric categorization has yet been established. Some features, such as leaf ordering, mericarp form, comparative length of calyx vs. epicalyx, and indumentum morphology, are more appropriate in *Alcea* taxonomy due to homogeneity and significant plasticity in morphological characters of this genus (particularly in flower and fruit characters) (ESCOBAR GARCIA *et al*. 2012; JIA, *et al*. 2020;

BI, *et al*. 2021).

Because of the minimal total of distinguishing morphological features, for example, leaf ordering and carpel structure, *Alcea* includes a difficult taxonomy (PAKRAVAN, 2008). Because DNA markers are less affected by the surroundings and permit straightforward detection at the DNA level, they have several benefits than morphological features. Genetic diversity studies are usually tapped due to molecular markers. Molecular markers are an excellent method to disentangle phylogenetic association between species and population. Among molecular methods or markers, RAPD (Random Amplified Polymorphic DNA) are sensitive to detect variability among individuals of species. RAPD method is cost-effective and can work with limited sample quantities. In addition to this, RAPD can amplify and target genomic regions with potential and several markers (ESFANDANI-BOZCHALOYI; ZHU, *et al*., 2021; ZHAO *et al.* 2021; YIN, *et al.* 2021; MA, *et al.* 2021; PENG, *et al.* 2021). Taxonomical Systematics studies were conducted in the past to identify the *Alcea* species. According to the best of our knowledge, there is no

existing RAPD data on genetic diversity investigations in Iran. We studied seventy samples. Our objectives include; 1) determining the genetic variousness of *Alcea* species and 2) Is there a link among species and distance from home? 3) Folk and taxon genetic construction 4) Are the *Alcea* species able to exchange genes?

MATERIAL AND METHODS

Plant materials

Six *Alcea* species were collected from different regions of Iran (Table 1). These species were studied via morphological and molecular methods. Seventy plant samples (10-15 per plant species) were examined for morphometry purposes. The number of samples used in the haphazard intensified polymorphic DNA analysis technique was restricted to 70. We focused on the following species sp1= *A. aucheri;* sp2= *A. angulata;* sp3= *A. rhyticarpa*; sp4= *A. sulphurea*; sp5= *A. striata*; sp 6= *A. loftusii*. According to previous references, all the species were identified (ZOHARY 1963a, b, HUTCHINSON 1964, RIEDL 1976).

Table 1. List of the investigated taxa including origin of voucher specimens.

No	Sp.	Locality
Sp1	*Alcea aucheri* (Boiss.) Alef.	Esfahan:Ghameshlou, Sanjab
Sp2	*Alcea angulata* Freyn & Sint.	Tehran, Damavand
Sp3	*Alcea rhyticarpa* (Trautv.) Iljin	Khorasan, Mashhad
Sp4	*Alcea sulphurea* (Boiss.& Hohen.) Alef.	Tehran, Tochal
Sp5	*Alcea striata* (DC.) Alef.	Kermanshah, Islamabad
Sp6	*Alcea loftusii* (Baker) Zohary	Lorestan, Oshtorankuh, above Tihun village

Morphometry

Altogether, 24 quantitative and 13 qualitative qualities of the flowers were studied, calyx length, calyx width, corolla length, corolla color and stamens position. Data were transformed (Mean= 0, variance = 1) prior to ordination. Euclidean distance was implemented to cluster and ordinate plant species (PODANI 2000).

Random Amplified Polymorphic DNA

We extracted DNA from fresh leaves. Leaves were dried. DNA extraction was carried out on the report of the aforementioned protocol (ESFANDANI-BOZCHALOYI *et al.* 2019). DNA quality was checked on an agarose gel to verify the purity. We amplified the DNA with the aid of RAPD primers (Operon technology, Alameda, Canada). These primers belonged to OPA, OPB, OPC, OPD collections. We selected those primers (5) which could show clear bands and polymorphism (Table 2). Overall, the polymerase chain reaction contained 25µl volume. This 25 volume had ten mM Tris-HCl buffer, 500 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of per dNTP; 0.2 µM of a single primer; 20 ng genomic DNA as well as 3 U of *Taq* DNA polymerase (Bioron, Germany). We observed the following cycles and conditions for the amplification. Five minutes initial denaturation step was carried out at 94°C after these forty processes of 1 minute at 94°C were observed. Then 1-minute cycle was at 52-57°C followed by two minutes at 72°C. In the

end, the final extension step was performed for seven to ten minutes at 72°C. We confirmed the amplification steps while observing amplified products on a gel. Each band size was confirmed according to 100 base pair molecular ladder/standard (Fermentas, Germany).

Data analyses

We used an Unweighted pair group method with arithmetic mean (UPGMA) and Ward methods. Multidimensional scaling and main coordinate analysis were used as well as other ways of ordering (PODANI 2000). The morphological difference among species and population was assessed through analysis of variance (ANOVA). PCA analysis (PODANI 2000) was done to find the variation in plant population morphological traits. Multivariate and all the necessary calculations were done in the PAST software, 2.17 (HAMMER *et al*. 2001). To assess genetic diversity, we encoded RAPD bands as present and absent. Numbers 1 and 0 were used to show the presence and absence of bands. It is essential to know the polymorphism information content and marker index (MI) of primers because these parameters serve to observe polymorphic loci in genotypes (ISMAIL *et al*. 2019). Marker index was calculated according to the previous protocol (HEIKRUJAM *et al.* 2015). Other parameters such as the number of polymorphic bands (NPB) and effective multiplex ratio (EMR) were assessed. Gene diversity associated characteristics of plant samples were calculated. These characteristics include Nei's gene variousness (H), data index of Shannon (I), influential alleles total (Ne), and polymorphism percentage (P% =number of polymorphic loci/number of total loci) (SHEN *et al*. 2017). Unbiased expected heterozygosity (UHe), and heterozygosity were assessed in GenAlEx 6.4 software (PEAKALL and SMOUSE 2006). Neighbor-joining (NJ) and networking were studied to fathom genetic distance plant populations (HUSON and BRYANT 2006; FREELAND *et al*. 2011). The Mantel examination was carried out to find the connection between genetic as well as geographical spaces (PODANI 2000). As we were interested in knowing the genetic structure and diversity, we also investigated the genetic difference between populations through AMOVA (Analysis of molecular variance) in GenAlEx 6.4 (PEAKALL and SMOUSE 2006). Furthermore, gene flow (Nm) was estimated through Genetic statistics ($G_{ST)}$ in PopGene ver. 1.32 (YEH *et al*. 1999). We also did STRUCTURE analysis to detect an optimum number of groups. For this purpose, the Evanno test was conducted (EVANNO *et al*. 2005).

RESULTS

Morphometry

Plant species with important ANOVA findings (P0.01) exhibited variations in quantitative morphological features. Principal component results explained 78% variation. First component of PCA demonstrated 46% of the total variation. Leaf morphology and traits such as calyx length, calyx width positively correlated with corolla length, corolla color (>0.7). The second and third components explained floral characters such as corolla length, leaf length and stamens position. The principal component analysis (PCA) and unweighted pair group technique with arithmetic mean (UPGMA) plots revealed symmetrical findings (Figure 1). Generally, plant specimens belonging to different species were separated from each other due to differences in morphology. Morphological characters divided *Alcea* species into two groups, as evident in the UPGMA tree (Figure not included). Populations belonging to *Alcea aucheri* and *Alcea angulata*

were in the first group. On the other hand, the second group consisted of two sub-groups. *Alcea rhyticarpa* formed the first sub-group. *Alcea striata* and *Alcea loftusii formed* the second sub-group. These groups and sub-groups were formed due to morphological differences among the individuals of *Alcea*. Our PCA results also confirmed the application of morphological characters in separating and clustering the species in separate groups (Figure 1). Identical results were also reported in the UPGMA tree (Figure not included).

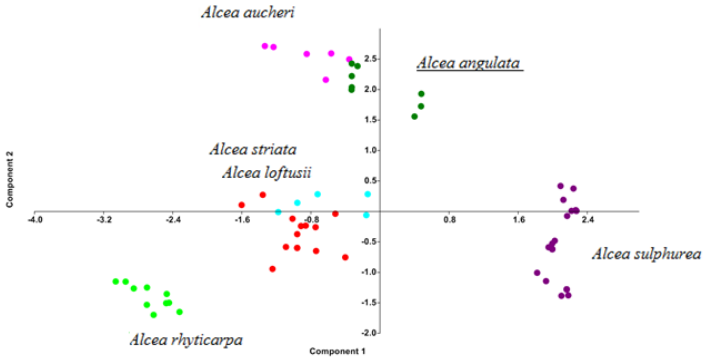

Figure 1. PCA plot morphological characters revealing species delimitation in *Alcea* species

Species Identification and Genetic Diversity

The primers, i.e., OPC-04 and OPA-05, could amplify plant (*Alcea*) DNA. 65 polymorphic bands were generated and amplified. Amplified products varied from 100 to 3000 bp. We recorded the most elevated polymorphic bands for OPA-06. OPD-03 had the lowest polymorphic bands. The average polymorphic bands ranged to 11.8 for each primer. The polymorphic information content (PIC) had values in the range of 0.27 (OPA-05) to 0.54 (OPA-06). Primers had 0.40 average polymorphic information content values.

Table 2. RAPD primers and other parameters.

Primer name	Primer sequence (5'-3')	TNB	NPB	PPB	PIC	PI	EMR	MI
OPA-05	5′-AGGGGTCTTG-3′	16	13	91.22%	0.27	4.26	9.16	3.11
OPA-06	5′-GGTCCCTGAC-3′	17	15	94.66%	0.54	5.28	7.23	4.22
OPD-02	5′-GGACCCAACC-3′	13	13	100.00%	0.36	3.22	10.88	3.15
OPD-03	5′-GTCGCCGTCA-3′	9	7	90.99%	0.43	6.51	9.43	5.33
OPD-05	5′ -TGAGCGGACA-3′	10	10	100.00%	0.44	5.34	11.55	5.22
Mean		13.7	11.8	93.33%	0.40	4.9	9.3	4.8
Total		65	58					

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 CBDP primers.

Marker index (MI) values were 3.11 (OPA-05) to 5.33 (OPD-03), with an norm of 4.8 for each primer. Effective multiplex ratio (EMR) values are useful to distinguish genotypes. In our study, we reported 7.23 (OPA-06) to 11.55 (OPD-05) EMR values. EMR values averaged 9.3 per primer (Table 2). All the necessary genetic features calculated of six *Alcea* species are shown (Table 3). The neutral anticipated heterozygosity (UHe) of *Alcea rhyticarpa* was in the scope of 0.12. *Alcea aucheri* showed a 0.36 UHe value heterozygosity had a mean value of 0.27 in overall *Alcea* species. Shannon information was high (0.39) in *Alcea aucheri*. *Alcea rhyticarpa* showed the lowest value, 0.20. Mean values for Shannon information was 0.29. In Alcea angulate and Alcea loftusii, the experimental number of alleles varied from 0.33 to 0.49. For Alcea angulate and Alcea striata, the influential number of alleles was in the scope of 1.034-1.17.

Table 3. Genetic diversity variables of Alcea

taxon	N	Na	Ne	I	He	UHe	%P
Alcea aucheri	14.000	0.344	1.042	0.39	0.37	0.36	67.22%
Alcea angulate	10.000	0.336	1.034	0.25	0.25	0.29	51.83%
Alcea rhyticarpa	10.000	0.458	1.039	0.20	0.18	0.12	20.11%
Alcea sulphurea	10.000	0.448	1.049	0.28	0.18	0.23	49.38%
Alcea striata	15.000	0.455	1.177	0.277	0.24	0.22	55.05%
Alcea loftusii	10.000	0.499	1.067	0.24	0.19	0.24	49.26%

(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 in populations).

Analysis of Molecular Variance (AMOVA) test highlighted genetic differences among *Alcea* species (P = 0.001). AMOVA showed that 70% of genetic variation was among the species. Relative less variation (30%) was reported within the species. Genetic similarity and dissimilarity assessed through Genetic statistics (GST) showed significant differences i.e., (0.444, P = 0.001) and D_est values (0.356, p = 0.001). The neighbor-joining tree also revealed some major groups (Figure 2). The neighbor-joining tree also repeated the same pattern as indicated in figure 1. In current work, molecular findings also coincided with the traditional taxonomical (morphology) approaches for *Alcea* species.

In the *Alcea* species, gene flow was quite modest (0.33). Genetic identity and phylogenetic distance in the *Alcea* members are mentioned (Table not included). *Alcea striata*, as well as *Alcea loftusii*, were shown to be genetically associated (0.95). Because of their low genetic resemblance (0.73), *Alcea aucheri* and *Alcea angulata* were shown to be different. The mantel examination revealed a link between genetic and geographical spaces (r = 0.66, p=0.0001).

The Evanno test showed ΔK =6 (Figure not included). According to STRUCTURE analysis, *Alcea striata* and *Alcea loftusii* were closely related to common alleles. The rest of the *Alcea* species are genetically differentiated due to different allelic structures (Figure 2). The neighbor-joining plot also showed the same result. K-Means and STRUCTURE studies also supported the Limited gene flow results. We could not find any evidence of significant gene flow among the Alcea species. Because these folks were positioned near each other, this outcome agrees with the grouping we got using Neighbor-Net (Figure not contained). These common alleles form a relatively small percentage of the genomes in these folks, as indicated by

the STRUCTURE plot based on the admixture model. These data concur in indicating a high degree of genetic stratification among Alcea communities.

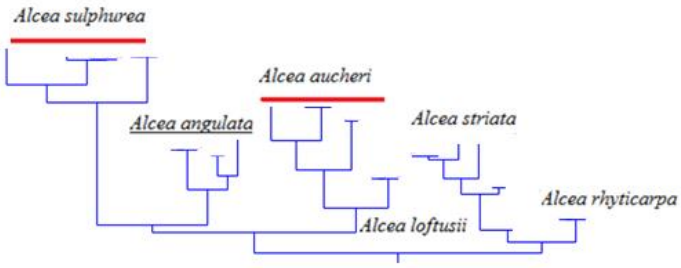

Figure 2. NJ clusters of RAPD data revealing species delimitation in *Alcea* species

DISCUSSION

The *Alcea* is a relatively complex taxonomic group, and several morphological characters make it difficult to identify and classify *Alcea* species (ESCOBAR GARCIA *et al*. 2012). Given the complexity, it is necessary to explore other methods that could complement the traditional taxonomical approach (ERBANO *et al*. 2015). Advent and developments in molecular techniques have enabled plant taxonomists to utilize molecular protocols to study plant groups (ERBANO *et al*. 2015). We examined genetic diversity in *Alcea* species by morphological and molecular methods. We mainly used RAPD markers to investigate genetic diversity and genetic affinity in *Alcea* species. Our clustering and ordination techniques showed similar patterns. Morphometry results clearly showed the utilization or significance of morphological characters in *Alcea* species. PCA plot results also confirmed the application of morphological characters to separate *Alcea* species. The present study also highlighted those morphological characters such as corolla color, leaf shape, leaf length, stamens position, leaf margin could delimit the *Alcea* group. The *Alcea* species highlighted morphological differences. We argue that such a dissimilarity was due to differences in quantitative and qualitative traits. In our study, morphology and micro-morphology of flower and leaf characters in six taxa of *Alcea* species are given in detail for the first time. This research aimed to identify diagnostic traits that may be used to distinguish *Alcea* species in Iran. As earlier stated, morphological features are thought to be a good aid for species designation (ESCOBAR GARCIA *et al*. 2012).

Polymorphic information content (PIC) values are useful to detect genetic diversity. The current study recorded average PIC values of 0.40. This value is sufficient to study genetic diversity in the population (KEMPF *et al*. 2016). High genetic diversity among the *Alcea* population was reported in the present study. Genetic analysis conducted via analysis of molecular variance and STRUCTURE showed genetic differences among the species. Just infrequent research has studied genetic variousness in Alcea yet. KAZEMI *et al*. (2011) found a 93 percent polymorphism level in *A. Rosea* folks in Iran, with a wide range of genetic similarities

(0.31 to 0.75) founded on the RAPD markers study. Utilizing RAPD markers, OZTÜRK *et al*. (2009) evaluated the 18 *Alcea* species' genetic profiles and found a considerable difference (0.13 to 0.69) between them. In the BADRKHANI *et al*. (2014) report, the sequence-related intensified polymorphism (SRAP) marker was used to evaluate the genetic variousness and genetic resemblance links among 14 *Alcea* species gathered from northwest Iran. Seventeen SRAP primer mixtures yielded 104 pieces, with a norm of 5.7 polymorphic pieces for each primer (93%) and 97 (93%) polymorphic fragments. The polymorphism percentage varied from 50% (ME2-EM6) to 100% (ME2-EM6), with a mean polymorphism data scope value of 0.3. The genetic resemblance in *A. sophiae* and *A. flavovirens* was lower (0.17), but the greatest was identified between *A. digitata* and *A. longipedicellata* (0.68). Using UPGMA, two primary clusters were discovered, none of which corresponded to the species' geographical source. On the report of their findings, SRAP markers may be suitable for analyzing genetic variousness in *Alcea*. So far, only morphological information has been used to define Iranian *Alcea* species. However, owing to the tiny total of characteristics, the genus has a difficult taxonomy. In the report of Pakravan's (2008) research on Alcea, just the leaf sequence and carpel configuration are important traits. The only difference between *A. fl avovirens* and A. *glabrata* is the carpel's dimensions and the wing's breadth (PAKRAVAN 2008). Our findings divided these two species into two distinct groups.

ESCOBAR GARCIA *et al*. (2012) used three molecular markers (nrDNA ITS and the plastid spacers psbA-trnH and trnL-trnF) to construct a phylogeny for Alcea as well as examine prior infrageneric taxonomic ideas and its monophyly with Althaea, a genus with which it has been combined often. They also go into morphological divergence and the use of morphological features as phylogenetic relationship indicators.While genetic findings indisputably corroborate the circumscription of *Alcea* deduced from morphology, they are of little value in clarifying interspecific associations, indicating that great species variousness of *Alcea* is attributable to fast and current radiation. Their research establishes the first *Alcea* phylogeny and intends to pave the way for future research into the mechanisms that underpin species radiation in the Irano-Turanian area.

Molecular markers (RAPD) and morphometry analysis were useful to study genetic diversity and population structure in *Alcea* species identification. All the species had distinct genetic differentiation. Present results highlighted isolation and limited gene flow are the main deterministic factors that shape the *Alcea* population. We discovered that the *Alcea* species has increased genetic variousness, indicating that it is able to adjust to altering circumstances because high genetic variousness is connected to species flexibility.

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PRIMENA RAPD MARKERA ZA UTVRĐIVANJE GENETIČKOG DIVERZITETA KOD *Alcea* (Malvaceae)

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Izvod

Da bi se razumela zaštita i upravljanje biljnim resursima u svakom okruženju potrebna su različita genetička istraživanja. Zbog minimalnihog broja različitih morfoloških sxojstva, na pr, delovi lista i struktura ploda *Alcea* ima problematičnu taksonomiju. 34 *Alcea* vrsta su nađene u Iranu od toga 15 su edemične. Genetička različitost *Alcea* je ispitana sa RAPD (Random Amplified Polymorphic DNA). Kao rezultat, prikupljeno je i ispitano šest vrsta iz Iranskih provincija. Ukupno je prikupljeno sedamdeset uzoraka. Ciljevi rada su: određivanje genetičke različitosti *Alcea* vrsta i da li postoji veza između genetskog sastava i okruženja, genetička konfiguracija populacije i taksona, Utvrđena je značajna varijabilnost kvantitativnih morfoloških svojstava. *Alcea* vrste su razdvojene u dve kategorije primenom NJ klastera i PCA. UHe *Alcea rhyticarpa* je 0.12. Kod *Alcea aucheri*, Shannon je bio veći (0.39). Najniža vrednost 0.20, je nađena kod *Alcea rhyticarpa*. Kod Alcea vrsta protok gena (Nm) je bio umeren (0.33).

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