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Seed Protein Analysis as a Tool for Taxonomy of *Alcea* (Malvaceae) in Iran

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Abstract

The genus *Alcea* L. consists of over 50 species, which are primarily distributed in the Irano-Turanian region but have also spread into the Caucasus and the Eastern Mediterranean. Due to the high phenotypic plasticity observed in this genus, species identification requires a combination of traits that are often not all present in a single herbarium specimen. In this study, the electrophoresis of seed proteins is investigated in 24 species and 4 varieties of the genus *Alcea.* Plant samples were collected from 18 different provinces. This study aimed to apply the seed protein pattern in *Alcea* species to determine the boundary between *Alcea* species by using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The observed protein bands provided a basis for comparing the species. A total of 7 common bands were found among all species, which can be considered characteristic markers of the genus *Alcea.* Similarity coefficients and Jaccard indices were used to create a similarity matrix, and a cluster analysis was performed using the Ward method with SPSS software. The results showed that *A. aucheri* was closely related to *A. arbelensis, A. koelzii, A. rechingerii, A. kurdica,* and *A. schirazana* based on seed protein storage. A close relationship was observed between *A. arbelensis* and *A. rechingerii*, with a 90% protein similarity. Additionally, 92% protein similarity was found between *A. gorganica* and *A. popovi*. In the cluster analysis, the species were grouped into 7 clusters, which were nearly identical to the morphological grouping of the species. The seed electrophoresis results were compared with previous molecular phylogenetic studies. We can conclude that seed protein analysis is more useful in determining the relationship of closely related species and subspecies within the genus *Alcea*.

Keywords: *Alcea*, Electrophoresis, Iran, SDS-PAGE, Storage proteins

Introduction

The Malvaceae family was initially recognized as a separate family by de Jussieu in the 18th century (Judd & Manchester,1997). In the early 20th century, the Malvaceae family was divided into subfamilies such as Malvoideae (Hutchinson, 1967; Judd & Manchster, 1997; Alverson et al., 1999; Takhtajan, 1980). In the late 20th and early 21st centuries, advancements in genetic

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technologies, the family relationships within Malvaceae were reassessed using DNA analysis. These changes led to the merger of the Tiliaceae and Sterculiaceae families into Malvaceae (Kubitzki & Bayer, 2003; Stevens, 2001, 2014; APG III, 2009 & APG IV, 2016; Le Péchon & Gigord, 2014; Walker & Eggli, 2023; Hanes et al., 2024).

Currently, the Malvaceae family includes four main subfamilies: Malvoideae, Bombacoideae, Sterculioideae, and Matisioideae. This classification is based on genetic evidence and morphological characteristics (Colli-Silva et al. 2025).

The genus *Alcea* L., a prominent genus within the Malvaceae family, encompasses more than 50 species. These species are primarily distributed in the Irano-Turanian region, although they have also spread into the Caucasus and the Eastern Mediterranean (Zohary, 1963). Among the 33 species that grow in Iran, he majority are located in the western regions (Pakravan 2008). Most species of *Alcea* are tall hemicryptophytes with palmate to simple or lobed leaves, covered with stellate or branched hairs (Pakravan, 2008). The flowers have five sepals and 5 to 9 epicalyxes, and they are large and colorful. Zohary suggested nine informal species groups for *Alcea* based on leaf shape, epicalyx, and mericarp characters (Zohary, 1963a, b). *Althea* L. (the sister group of *Alcea* [Tate et al., 2005; Escobar et al., 2009]) is similar to *Alcea*, but distinguished from *Alcea* by having flowers smaller than 30 mm, a cylindrical stamen tube, and one chambered carpel (Escobar et al., 2012). *Alcea* is one of the most challenging genera in Central Asia (Iljin, 1949; Zohary, 1963b; Riedl, 1976; Townsend, 1980). Any classification in this genus encounters similarities in the characteristics of various organs. Due to the high phenotypic plasticity observed in this genus, species identification requires a combination of traits, such as leaf shape, the ratio of the calyx to epicalyx, and the shape of mature mericarps, which are often not all present in a single herbarium specimen.

There have been few morphological studies on this genus, including the classification of the subgenus by Bossier (1867), Zohary (1963), Riedl (1976), and Pakravan (2001, 2003, 2005, 2006a, 2006b, 2008). Studies on pollen (Arabameri et al., 2023), fruit, and seed (Özbek & Uzunhisarcıklı, 2023) have been attempted to assist in the classification of *Alcea* species. Research on seed proteins, which serves as a valuable approach for determining species relationships, has thus far been limited to the family level within the Malvaceae family (Ibrahim et al., 2023). They analyzed seed proteins in 49 species of 34 genera, and the results contributed to establishing the family classification. This research confirmed the effectiveness of utilizing seed proteins as a reliable approach for the classification of taxa within the Malvaceae subfamilies.

Several phylogenetic studies have been conducted on the Malvaceae family and the *Althea* genus (Escobar et al. 2009); however, the only comprehensive molecular phylogenetic analysis was conducted by Escobar et al. (2012), focusing on the *Alcea* genus. Using three molecular markers (nrDNA, the plastid spacers *psbA-trnH* and *trnL-trnF*), they confirmed the monophyly of the *Alcea* and distinguished it from the *Althea*.

Given that seed proteins are considered valuable molecular markers at the protein level for plant classification and have not been studied within the genus *Alcea*, this study aims to utilize the seed protein patterns in *Alcea* species to ascertain the accurate taxonomic positioning of the species and subsequently aid in their classification. Lastly, the study will investigate the alterations that have occurred within the intraspecific divisions observed in specific species.

Material and methods

Seed collection and protein extraction

Seeds of 24 species and four varieties were collected from 18 provinces (Table 1). A minimum of two to three individuals from the accessions of each species were utilized for the analysis. Since the number of individuals with ripe seeds in each population was small and the seeds were also light in weight, fewer individuals were examined despite multiple collections.

0.5 gram of each seed were ground using liquid nitrogen in a cold environment, and the protein extract was prepared using seed powder in a Tris-Glycine buffer (pH = 7.2) at a ratio of 1:6 (including 30 grams of Tris, 144g of Glycine, 10g of SDS, 70cc of water, 15µg of Temed, and 30 g of Acrylamide, 0.8 g of Bis-acrylamide). The extract was centrifuged for 45 minutes at 1500g.

Electrophoresis

SDS-PAGE electrophoresis was performed on polyacrylamide gels following the method of Laemmli (1976). After injecting the protein extract, the gels were transferred to an electrophoresis tank and subjected to a 5mA current at room temperature. Due to the large number of samples, electrophoresis was conducted on three separate gels, each containing 18 columns. To determine the molecular weight of unknown proteins, a standard solution (including Bovine serum albumin, egg serum albumin, Pepsin, trypsinogen, β-lactat albumin, Lysozyme) was used (Table 2). Protein concentration was measured using the Bradford method (Bradford, 1976).

Gel staining

Following electrophoresis, the gels were washed several times with distilled water and then stained with Coomassie Blue solution (containing 0.25 grams of Coomassie Blue, 125 mL of methanol, 25 mL of glacial acetic acid, and 100 mL of water) overnight (Smith, 1984). After staining, excess dye was removed by destaining the gels in a solution of acetic acid and methanol for 15hours.

Statistical analysis

The number and location of protein bands, and their Rm values, were determined. Cluster analysis based on the Jaccard and similarity coefficient using the Ward method (Podani, 2000) was performed using SPSS software. Based on these coefficients, the similarity percentage was calculated, and a matrix was created.

Results and Discussion

Overall, 37 protein bands were observed in different species of *Alcea* through protein electrophoresis. By comparing the protein bands and measuring the RM, it can be observed that Bands 1, 15, 22, and 30 are present in all species, so these bands may serve

Table 1. Voucher details of *Alcea* species

Species	Location	Collector & Number	Voucher
A. angulata Freyn & Sint	Tehran: 87 Km from Tehran to Firuzkuh, 35.6053276, 52.4592907	Pakravan & Darrehshuri 26433	TUH
	Tehran: 3 Km to Robat-Karim on the road from Saveh to Tehran, 35.5345503, 51.1035845	Pakravan & Darrehshuri 26391	TUH
A. arbelensis Boiss. & Hausskn.	Fars: 45 Km to Yasuj from Esfahan, Tange-Tizab, 30.3690210, 51.7875348	Pakravan & Darrehshuri 26406	TUH
	Kermanshah: 4 Km from Sahneh to Kangavar, 34.4466768, 47.7419318	Pakravan & Hayelmoghadam 26449	TUH
A. aucheri (Boiss.) Alef.	Fars: Noorabad to Kazerun road, research institute, 29.5696222, 51.7416341	Sardabi & Latifian 42157	TARI
A. glabrata Alef.	Tehran: Ghazvin road, 31 Km from Takestan to Buin Zahra, 35.8731861, 49.5497782	Pakravan & Hayelmoghadam 26384	TUH
A. glabrata Alef. var. microcarpa	Tehran: 30 Km from Karaj to Challus, 36.2006439, 51.3617076	Pakravan & Darrehshuri 26372	TUH
	Tehran: Kan, near the river, 35.8005378, 51.2589569	Pakravan & Darrehshuri 26397	TUH
A. gorganica (Rech. f., Aell. & Esfand.) Zoh.	Golestan: Golestan National Park, 450 m, 37.4443110, 56.1424860	Akhani 11819	W
	Khorassan: Between Bojnurd and Maraveh tappeh, 38.0817152, 56.4403929	Assadi & Mozaffarian 35605	TARI
A. popovii Iljin	Golestan: 5 Km from Galikesh to Golestan National Park, 250 m, 37.3267723, 55.4897674	Mozaffarian & Maasoumi 79112	TARI
A. mazandaranica	Mazandaran: Kelardasht, Roodbarak, 1650 m, 36.4821203, 51.1279873	Mozaffarian 45495	TARI
A. kurdica (Schlecht.) Alef.	KurdestanSanandaj, Abidar Park, 35.3099883, 46.9701690	Pakravan & Hayelmoghadam 26401	TUH
A. kurdica (Schlecht.) Alef. var. *laxiflora* (Riedle) Pakravan	Kermanshah: Between Khamseh and Bisotun, 34.4374004, 47.4986567	Pakravan & Hayelmoghadam 26443	TUH
A. rechingeri (Zohary) Riedl	Kermanshah: Tagh-e Bostan, 34.3857435, 47.1344034	Pakravan & Hayelmoghadam 26439	TUH
A. shirazana Alef.,	Fars: Between Ardakan & Komeh, 30.3318827, 51.9687954	Pakravan & Darreh shuri 26408	TUH
A. koelzii I. Riedl	Markazi: Between Arak and Salafchegan, 34.2975075, 50.2345917	Ghahreman 10098	TUH
	Kohgiluyeh: Yasuj, 30.6661864, 51.6230154	Ghahreman 10095	
A. tiliacea (Bornm. Zohary	Khorassan: Neyshabur, upper Mirab, 1600-1900 m, 36.0934766, 58.7878643	Assadi & Mozaffarian 36099	TARI
	Khorassan: 14 Km from Kashmar to Neyshabur, 1400-1500 m, 32.4214271, 58.5088468	Assadi & Maasoumi 35622	TARI

A. sulphurea (Boiss. & Hohen.) Alef.,	Tehran: Tehran: Fasham, 35.9368816, 51.5048014	Pakravan & Darreh shuri 26433	TUH
	Tehran: Damavand, 3 Km from Darbandsar to Fasham, 35.9891013, 51.4861346	Pakravan & Darreh shuri 26378	TUH
A. striata (DC.) Alef.,	Bushehr: Kuh-e Haft-Chah, 1600 -2000 m, 27.6834441, 52.4473810	Mozaffarian 74085	TARI
A. tabrisiana (Boiss. & Buhse) Iljin	Tehran: 37 Km from Tehran to Ghazvin, 36.1188777, 50.3946687	Pakravan & Hayelmoghadam 26455	TUH
A. wilhelminae Riedl	Azerbaijan: Ghuschi pass, between Salmas and Urumiyeh, 38.0130092, 44.9547488	Pakravan & Hayelmoghadam 26418	TUH
A. wilhelminae Riedl var. *lineariloba (*Riedl)Pakravan	Azerbaijan: Ghuschi pass, 38.0131846, 44.9402488	Pakravan & Hayelmoghadam 26389	TUH
A. sachsachanica Iljin,	Azerbaijan: Ghushchi pass, 38.0131846, 44.9402488	Pakravan & Hayelmoghadam 26390	TUH
A. mozaffarianii Ghahreman, Pakravan & Assadi	Ardebil: Road of Ardebil to Khalkhal, 2000-2500 m, 37.8482705, 48.3729127	Mozaffarian & Maasoumi 78245	TARI
A. iranshahri Pakravan, Ghahreman & Assadi	Fars: Kuh-e Dena, Bijan pass, 2500 m, 30.8629268, 51.4947690	Assadi & Mozaffarian 31162	TARI
A. flavovirens (Boiss. & Buhse) Iljin	Hamedan: Avaj pass 35.5365856, 49.1372051	Pakravan & Hayelmoghadam 26388	TUH
A. flavovirens (Boiss. & Buhse) Iljin var. *albiflora* Zohary	Azerbaijan: 81 Km from Tabriz to Mianeh, 37.3053915, 47.1045185	Pakravan & Hayelmoghadam 26387	TUH
A, ghahremanii Pakravan, Maasoumi & Assadi	Azerbaijan: Mianeh, 1700 m, 37.4760113, 47.6628803	Attar & Dadjoo 18044	TUH
A. transcaucasica (Iljin) Iljin	Kurdestan: 101 Km from Marivan to Paveh, 35.2731969, 46.1621723	Runemark & Assadi 27434	TARI
A. calverti (Boiss.) Boiss. var. *albiflora* Zohary	Kohgiluyeh &Boyerahmad: Between Yasuj & Dehdasht, Kuh-e Saverz, 2300-3200 m, 30.7059248, 51.1285056	Assadi & Abuhamzeh 46386	TARI
A. tarica Pakravan	Tehran: Road Firuzkuh to Damavand, Tar Lake, 35.7300093, 522224757	Pakravan & Darreh shuri 26380	TUH

Table 2. Molecular weight and logarithm of molecular weight of proteins used in SDS-PAGE

Proteins	Molecular Weight (kDa)	Logarithm of molecular weight
Albumin bovine	66000	4.820
Albumin egg	45000	4.652
Pepsin	34700	4.540
Trypsinogen (Bovine Pancreas)	24000	4.380
B-Lactalbumin	18400	4.265
Lysozyme	14300	4.155

as the genus markers. Band 32 was observed in the *A. kurdica* group (such as: *A. arbelensis* Boiss. & Hausskn., *A koelzii, A. rechingerii, A. kurdica* (Schlecht.) Alef., *A. schirazana* Alef.), as well as in *A. striata* (DC.) Alef. and *A. iranshahrii* Pakravan, Ghahreman & Assadi (Fig. 2). Based on the common bands, the closeness of the taxa can be inferred. In this study, *A. wilhelminae* Riedl and *A. wilhelminae* var. *leariloba* (Riedl) Pakravan share 33 bands (Fig.1), indicating a very close relationship between these two taxa. Therefore, based on this closeness, the reduction of *A. leariloba* at the varietal level of *A. wilhelminae* (Pakravan 2008) is confirmed. Additionally, *A. gorganica* (Rech. f., Aell. & Esfand.) Zoh., and *A. popovii* Iljin share 35 bands, indicating a high degree of similarity between these two species (Fig. 1). Furthermore, *A. glabrata* Alef. and *A. glabrata* var. *microcarpa* (Zohary) Pakravan & Ghahreman share 33 bands (Fig. 1).

Using the similarity matrix table derived from the protein data (Table 3), the proximity of the above species can be expressed in a better way. As shown in Figure 1, within the *A. kurdica* species group, *A. arbelensis* and *A. koelzii* have a similarity of 0.68%, but the similarity between *A. arbelensis* Boiss. & Hausskn. and *A. rechingerii* (Zohary) Riedl is higher (0.90%). In this group, the similarity between *A. kurdica* var. *laxiflora* (Riedle) Pakravan and *A. kurdica* is 0.68%. Additionally, the similarity between *A. kurdica* and *A. arbelensis* is 0.75%. This degree of similarity among the species confirms their placement in the same species group. The similarity percentage between *A. gorganica* and *A. popovi* is 0.92%, which confirms a decline of *A. popovi* as a variety of *A. gorganica*.

In the *A. flavovirens* group, a high similarity of 0.71% is observed between *A. glabrata* and var. *microcarrpa*. Based on this percentage of similarity, the classification of var. *microcarpa* as a variety of *A. glabrata is* supported.

In the dendrogram obtained from cluster analysis (Fig. 3), the placement of *A. aucheri* (Boiss.) Alef, along with *A. kurdica* var. *laxiflora*, *A. rechingerii*, *A. koelzii I. Riedl* and *A. arbelensis* do not consistently align with the species grouping based on morphological traits. Considering the phylogenetic tree in the previous study (Escobar et al., 2012), our results somewhat agree with the phylogenetic tree of *Alcea* species based on molecular data. In the phylogenetic studies

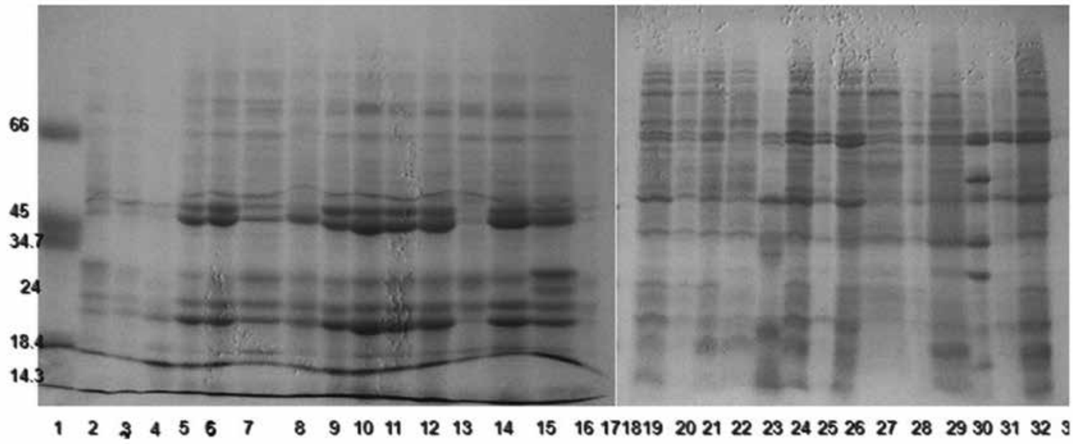

Fig. 1. SDS-PAGE polyacryl amid gel electrophoresis of seed proteins extracted of *Alcea* species studied: sequences of taxa from left to right: 1: Marker, 2, 3, 5: *A. angulata*; 4: *A. transcaucasica*; 6: *A.popovii*; 7: *A. gorganica*; 8,9: *A. wilhelminae* var. *leariloba*; 10: *A. ghahremanii*; 11: *A. flavovirens*; 12: *A. flavovirens* var. *albiflora*; 13: *A. sachsachanica*; 14, 15: *A. wilhelminae*; 16: *A. tabrisiana*; 17: *A. tarica*; 18, 28, 31,32: *A. glabrata*; 19: *A. rhyticarpa* var. *tiliacea*; 21: *A. calverti*; 21, 22: *A. striata*; 23, 26: *A. glabrata* var. *microcarpa*; 24, 25, 33: *A. angulata*; 27: *A. tarica*; 29, 30: *A. sulphurea*

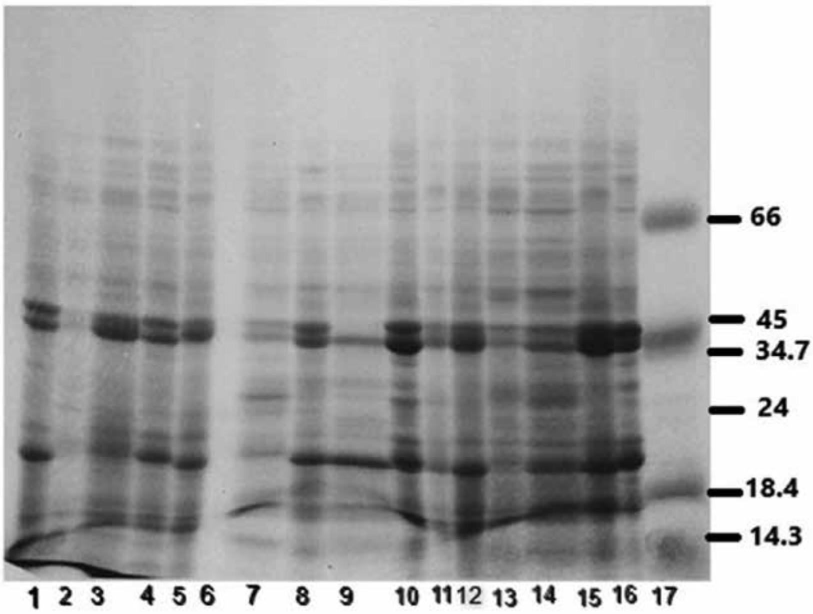

Fig. 2. SDS-PAGE polyacryl amid gel electrophoresis of seed proteins extracted of *Alcea* species studied: sequences of taxa from left to right: 1: *A. tarica*; 2: *A. aucheri*; 3: *A. sulphurea*; 4: *A. iranshahrii*; 5: *A. mazandaranica*; 6: *A. mozaffarianii*; 7: *A. koelzii* (Kohgiluyeh accession); 8: *A. koelzii* (Arak accession); 9, 10: *A. arbelensis*; 11: *A. rechingeri*; 12: *A. kurdica* var. *laxiflora*; 13: *A. schirazana*; 14, 15, 16: *A. kurdica*; 17: Marker

Table 3. Jaccard similarity coefficients for protein profiles of 24 species and four varieties of the genus *Alcea* from 50 samples representing the relatedness of similarity between the whole couples of *Alcea* species

Cod	an	Su-d	Su	lo	gl.d	ani	st	ca	t	gl	ta	w	sa	fl	fl.a	gh	w-li	go	po	an	tr	ku	sc	la	re	ko	ko-e	mo	ma	ir	au	
an	1.000																															
Su-d	0.632	1.000																														
Su	0.452	0.438	1.000																													
lo	0.424	0.371	0.824	1.000																												
gl.d	0.429	0.348	0.406	0.352	1.000																											
ani	0.500	0.429	0.543	0.600	0.500	1.000																										
st	0.542	0.520	0.677	0.687	0.321	0.353	1.000																									
ca	0.609	0.520	0.625	0.636	0.480	0.586	0.571	1.000																								
t	0.489	0.412	0.624	0.882	0.424	0.600	0.636	0.687	1.000																							
gl	0.344	0.419	0.611	0.714	0.344	0.576	0.471	0.515	0.667	1.000																						
ta	0.375	0.406	0.639	0.743	0.375	0.559	0.545	0.564	0.743	0.839	1.000																					
W	0.400	0.357	0.629	0.686	0.400	0.500	0.531	0.531	0.735	0.719	0.867	1.000																				
sa	0.364	0.394	0.667	0.722	0.364	0.543	0.529	0.529	0.722	0.812	0.903	0.839	1.000																			
fl	0.333	0.364	0.639	0.649	0.333	0.514	0.500	0.500	0.694	0.781	0.871	0.906	0.967	1.000																		
fl.a	0.406	0.394	0.714	0.771	0.406	0.543	0.576	0.576	0.824	0.706	0.788	0.781	0.818	0.788	1.000																	
gh	0.333	0.364	0.639	0.694	0.333	0.514	0.500	0.500	0.694	0.781	0.871	0.906	0.967	0.933	0.844	1.000																
w-li	0.344	0.375	0.611	0.667	0.344	0.529	0.429	0.471	0.667	0.750	0.727	0.867	0.812	0.781	0.812	0.839	1.000															
go	0.323	0.355	0.556	0.657	0.281	0.515	0.412	0.455	0.611	0.800	0.774	0.655	0.806	0.774	0.750	0.833	0.929	1.000														
po	0.367	0.400	0.556	0.657	0.323	0.515	0.455	0.500	0.657	0.742	0.833	0.710	0.806	0.774	0.806	0.833	0.862	0.926	1.000													
an	0.267	0.393	0.472	0.571	0.357	0.464	0.406	0.452	0.571	0.645	0.733	0.613	0.710	0.677	0.710	0.733	0.750	0.815	0.885	1.000												
tr	0.441	0.429	0.730	0.833	0.441	0.611	0.647	0.647	0.886	0.722	0.800	0.743	0.778	0.750	0.778	0.750	0.676	0.622	0.667	0.583	1.000											
ku	0.424	0.412	0.676	0.730	0.382	0.600	0.588	0.588	0.730	0.667	0.694	0.639	0.771	0.743	0.722	0.743	0.676	0.611	0.611	0.571	0.833	1.000										
Sc	0.500	0.433	0.583	0.639	0.448	0.645	0.531	0.485	0.639	0.618	0.647	0.588	0.983	0.556	0.583	0.556	0.528	0.514	0.559	0.471	0.743	0.735	1.000									
la	0.370	0.357	0.444	0.600	0.423	0.533	0.419	0.375	0.500	0.471	0.500	0.441	0.486	0.500	0.486	0.457	0.429	0.412	0.455	0.412	0.600	0.687	0.815	1.000								
re	0.406	0.394	0.667	0.722	0.452	0.687	0.529	0.486	0.676	0.657	0.639	0.541	0.622	0.595	0.622	0.595	0.611	0.600	0.600	0.559	0.730	0.722	0.761	0.825	1.000							
ar	0.438	0.382	0.649	0.750	0.438	0.719	0.472	0.472	0.703	0.686	0.667	0.611	0.649	0.622	0.649	0.622	0.639	0.629	0.629	0.543	0.757	0.750	0.871	0.710	0.906	1.000						
ko	0.444	0.379	0.500	0.600	0.500	0.778	0.394	0.484	0.656	0.529	0.514	0.500	0.543	0.514	0.543	0.514	0.486	0.471	0.471	0.489	0.611	0.679	0.700	0.704	0.867	0.774	1.000					
ko.e	0.222	0.211	0.233	0.219	0.222	0.192	0.261	0.208	0.219	0.207	0.161	0.172	0.156	0.161	0.156	0.125	0.094	0.100	0.100	0.111	0.206	0.219	0.259	0.261	0.233	0.226	0.292	1.000				
mo	0.414	0.356	0.566	0.611	0.519	0.667	0.455	0.548	0.657	0.500	0.571	0.606	0.556	0.528	0.600	0.628	0.459	0.405	0.444	0.441	0.714	0.758	0.767	0.714	0.647	0.727	0.786	0.269	1.000			
ma	0.375	0.406	0.595	0.649	0.517	0.656	0.457	0.545	0.694	0.583	0.657	0.674	0.639	0.611	0.639	0.611	0.541	0.486	0.528	0.529	0.800	0.794	0.750	0.700	0.686	0.765	0.767	0.241	0.897	1.000		
ir	0.375	0.214	0.412	0.515	0.500	0.448	0.379	0.379	0.515	0.438	0.516	0.503	0.500	0.489	0.500	0.469	0.438	0.419	0.467	0.414	0.529	0.515	0.607	0.600	0.500	0.581	0.615	0.250	0.571	0.567	1.000	
au	0.408	0.390	0.660	0.725	0.459	0.680	0.531	0.489	0.675	0.667	0.645	0.543	0.620	0.590	0.622	0.590	0.618	0.570	0.580	0.560	0.740	0.720	0.780	0.622	0.250	0.518	0.567	0.544	0.822	0.450	0.890	1.000

Abbreviations: an: *A. angulata; Su-d: A. sulphurea* (Damavand accession); Su: *A. sulphurea* (Firuzkuh accession); lo: *A. glabrata Alef. var. microcarpa;* gl. d: *A. tarica;* ani: *A. gorganica* (Khorasan accession); St: *A. striata;* Ca: *A. calverti* var. *albiflora;* t: *A. tiliacea* ; gl: *A. glabrata*; ta: *A. tabrisiana; W: A. wilhelminae;* Sa: *A. sachsachanica;* Fl.: *A. flavovirens*; Fl.a: *A. flavovirens* var. *albiflora*; gh: *A, ghahremanii*; W-li*: A. wilhelminae* var. *leariloba;* go: *A. gorganica;* po: *A. popovii; an: A. gorganica* (Khorasan accession); tr: *A. transcaucasica*; ku: *A. kurdica*; Sc: *A. shirazana*; la: *A. kurdica* var. *laxiflora*; re: *A. rechingeri*; ko: *A. koelzii*; ko-e: *A. koelzii* (Kohgiluyeh accession); mo: *A. mozaffarianii*; ir: *A. iranshahrii*; au: *A. aucheri*

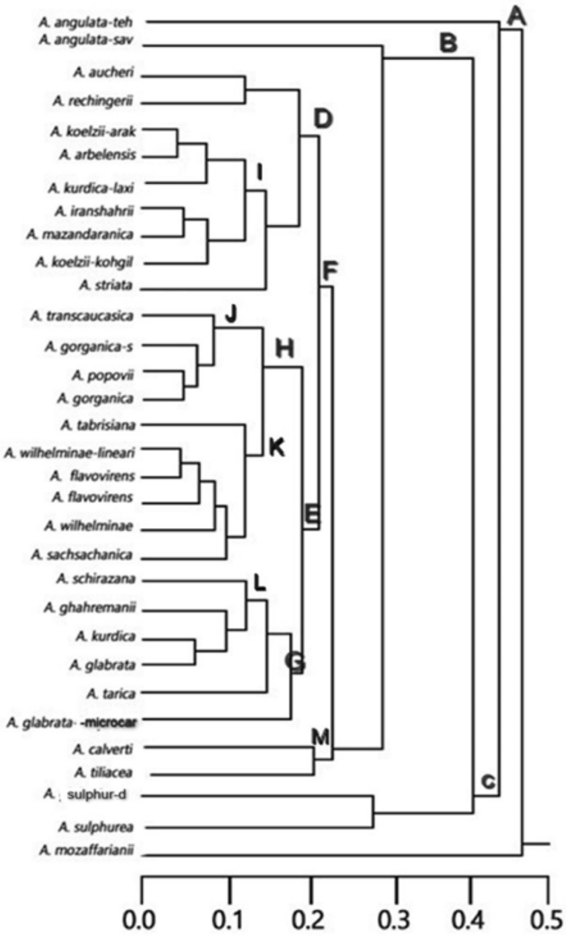

Fig. 3. Cluster analyses of SDS-PAGE patterns observed in Alcea accessions (Jaccard association coefficient index)

we conducted using some nuclear and chloroplast genes of *Alcea* species in the Irano-Turanian region, the phylogenetic trees did not closely match the species groupings based on morphological traits. The position of the species in the phylogenetic tree showed greater similarity to the dendrogram obtained from the present study (Escobar et al., 2012).

Furthermore, the placement of *A. rechingeri*, *A. koelzii*, and *A. arbelensis* in the same cluster confirms the morphological results (Pakravan, 2008). These species are all part of the same species group, characterized by mericarps that possess broad wings and lack folds, with their distribution located in western Iran. Additionally, *A. kurdica* var. *laxiflora* is also placed in this cluster, confirming the closeness of *A. laxiflora* to *A. kurdica,* leading to its classification at the varietal level of *A. kurdica* (Pakravan, 2001) On the other hand, a population of *A. koelzii* with red flowers, collected from the Kohgiluyeh and Boyer-Ahmad province, is located in this cluster. This taxon is separated from *A. koelzii* (with white flowers) at the 0.1 level in the dendrogram (Fig. 3), which suggests the variation of this taxon. Thus, it can be proposed as a variety of A. koelzii. However, a definitive statement about the position of this taxon requires further research in other biosystematics fields.

In the second cluster (branch H), the placement of *A. gorganica* and *A. popovi* alongside the subcluster of species group *A. flavovirens* align with the phylogenetic tree obtained from molecular phylogeny. Furthermore, *A. gorganica* is located next to *A popovi*, which differ only in flower color and wrinkling mericarp. Their placement in adjacent branches confirms their close relationships. Moreover, a sample identified as *A. sycophylla* in the Flora Iranica (Riedel, 1976), which is investigated morphologically (by the author), is placed in a branch next to *A. gorganica* and *A. popovi*. Since this sample shows no difference from *A. gorganica*, the presence of *A. sycophylla* Iljin & Nikitin in Iran, based on the samples reported by Riedel, is not confirmed.

In the subcluster K, the placement of *A. wilhelminae*, *A. flavovirens*, and *A. sachsacanica* Iljin together in the same branches fully confirms the morphological results as well as the phylogenetic tree obtained from molecular phylogeny. All of these species all classified within the *A. flavovirens* species group and exhibit several morphological similarities. These characteristics include a sparse, star-shaped hairy covering, palmate leaves with relatively deep lobes, and mericarps that possess well-developed wings and radially arranged wrinkles.

The positioning of *A. schirazana* within a common cluster alonside *A. kurdica,* is consistent with the morphological results. However, the placement of *A. schirazana* in a cluster with *A. glabrata*, *A. ghahremanii*, and *A. tarica* does not correspond with the morphological data. This discrapancy arises because all these species exhibit sparse hairs and mericarps that are either nearly wingless or possess degenerated wings. On the other hand, the placement of *A. tarica*, which has been introduced in recent years for the Flora of Iran (Pakravan, 2008), in a distinct cluster next to this one, further supports the distinction of this species.

Furthermore, the placement of *A. calverti* (Boiss.) Boiss. and *A. tiliacea* (Bornm.) Zohary, in a separate cluster (Fig. 3 subcluster M), does not align with the morphological results.

The placement of *A. sulphurea* far from *A. rhyticarpa* and *A. angulata*, which share many morphological similarities (having dense woolly hairs, shallowly cut leaves, and wingless mericarps), is not confirmed. *A. sulphurea* was placed in the *A. aucheri* species group by Zohary (1963a). *A. flavovirens* var. *alba*, with its white flowers, hairy ovaries, and veined sepals, is distinct from *A. flavovirens.* Therefore, the placement of *A. flavovirens* var. *alba* in a cluster distant from *A. flavovirens* indicates that this taxon could be elevated to the species level (Fig. 3), as its genetic distance is greater than that of a variety. However, this would require further investigations into gene sequencing.

A. mozaffarianii Ghahreman, Pakravan & Assadi was introduced in recent years for the Flora of Iran (Ghahreman et al. 2000). The placement of this species in a branch separated from other species confirms the distinction of this species as an independent and distinct unit.

From the results of the seed protein analysis in *Alcea* species, it can be concluded that the use of seed proteins is very useful for separating closely related taxa (such as varieties *A. kurdica*, *A. flavovirens*, and *A. glabrata*). Still, it has limited use in resolving interspecific relationships, which can be attributed to the phenotypic plasticity of morphological traits in the genus *Alcea*. As Escobar et al. (2012) concluded from their phylogenetic study, high species diversity in *Alcea* is due to rapid and recent radiation and low molecular divergence observed within the genus *Alcea.* Our work provides the first seed protein study in *Alcea* species.

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