Vol.9 No.4
Autumn 1998

DETERMINATION OF PHYLOGENETIC ALLIES IN SOME SPECIES OF *CROCUS* IN IRAN THROUGH THE STUDY OF SOLUBLE PROTEINS, PEROXIDASES AND POLYPHENOL OXIDASES

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Abstract

The taxonomic affinity of six species of *Crocus* (*C. cancellatus* subsp. *damascenus*, *C. almehensis*, *C. gilanicus*, *C. sativus*, *C. pallassi* subsp. *haussknechtii*, *C. speciosus* subsp. *speciosus*) from Iran were studied using different electrophoresis techniques. The similarity index of the species was computed by comparison of protein bands of corm and seed tissues, and the related dendrograms of affinity allies were drawn. The local populations of *C. cancellatus* constitute the first cluster of dendrogram and along with autumnal species form a common cluster. *C. almehensis*, which in contrast to other species is vernal, joined them at a farther distance.

Introduction

Crocus species are perennial plants adapted to overcome a dry dormant period in the form of an underground corm. Based on a variety of characters, there have been several classifications of the *Crocus*, and it must be noted that each system has both advantages and disadvantages [14]. The *Crocus* is a highly complex genus and its taxonomy depends on the fine details of its cataphylls, prophylls, bracts and bracteoles [14, 17].

Wendelbo [19] has reported that there are 80 species of *Crocus* and Mathew and Brighton have both mentioned 100 species of *Crocus* in their reports [13,14]. Wendelbo [19] and Mobayyen [15] have identified eight ornamental species and cultivated *Crocus* in Iran. This genus would fit reasonably well into each of the classifications of *Crocus* in its own period. However, nowadays we have a great deal of information making it possible to group the species in a more natural way, although a great deal of work is still needed if evolutionary lines are to be traced with any degree of confidence.

Numerous methods exist for protein analysis in order to estimate the toxonomic distance. In the study of proteins, similarities and diversities refer back to evolutionary changes. Sometimes, the application of gel electrophoresis techniques and the sole comparison of soluble protein components are not able to solve taxonomic complexities. When this is the case, the special staining of enzymes is more suitable and help to overcome this problem [5,11].

Unfortunately, chemotaxonomic studies are not carried out on the *Crocus* genus. Comparative studies of the proteins in particular help reveal evolutionary relations of

Keywords: *Crocus*; Taxonomic affinity; Enzyme

the species and the manner in which they are derived.

In the present paper, the relationship among some of the *Crocus* species of Iran is examined by comparative study of the seeds and corm tissue proteins. Also, the activity of the polyphenol oxidase and peroxidase enzymes are examined at the time of floral anthesis.

Materials and Methods

All the plants studied in the present investigation were collected during the period of floral anthesis then stored at -80°C for later analysis. Localities and organs of the material studied are listed in Table 1. These samples were homogenized in tris-glycine buffer (0.01 M, pH 7.2, containing a soluble polyvinylpyrrolidone) 1:2 w/v for corm and 1:7 w/v for seed tissue, in the cold [7]. The resulting homogenates were centrifuged at 13000 xg for 40 minutes at 0°C. The supernatant was then passed through eight layers of cheese-cloth. Protein content was determined by Lowry's Folin test [4,6] and equivalent quantities of different protein extracts were chosen for injection [10,16,18].

The profiles of the soluble proteins were studied by examining the extracts of seeds and fresh corms by disc-gel electrophoresis on 12.5, 10 and 7.5% polyacrylamide gels, according to the modified methods of Laemmli and Davis [10,18]. The gels were fixed (0.5-1 h) [13] and were stained immediately by coomassie brilliant blue R-250 solution [18] after which the excess dye was removed and the gels were placed in glass containers 7% (V/V) aceti acid for permanent storage.

For densitometrical analysis, a densitometer mode DR (CRT) Shimadzu was used. Relative mobility of th protein bands was computed according to the followin formula [10]:

$$Rm = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

Rm data of each profile with other profiles were compare and estimated; similarity index between species was als estimated [12]:

$$\text{Similarity index} = \frac{\text{number of similar pair bands}}{\text{number of different bands + number of similar pair bands}} \times 100$$

The results were then regulated in the form of symmetrical matrix on the basis of which the relationship between the examined plant specimens were summarize in a denodrogram.

Quantitative and qualitative activity of the polypheno oxidase and peroxidase in the Crocus corms were analyse by spectrophotometric and gel electrophoretic method [9].

Results

Characteristics of proteins in the corms and wil

Table 1. Introduction of species of *Crocus* being investigated

Species	Locality	Tissue		Abbreviations
		Corm	Seed	
C. almehensis C. Brickell and B. Mathew	Almeh	+	+	Ca
C. cancellatus subsp. *damascenus* (Herbert) B. Mathew	Arak	+	+	Cc1
C. cancellatus	Golpaygan	+	+	Cc2
C. cancellatus	Hamedan	-	+	Cc3
C. gilanicus B. Mathew	Syah-bisheh	+	-	Cg
C. pallasii Golob subsp. *haussknechtii* (Boiss and Reuter ex-Maw) B. Mathew	Hamedan	+	+	Ch
C. sativus L.	Natanz	+	-	Cs
C. speciosus (BIEB) subsp. *speciosus*	Golestan National Park	+	-	Csp

Table 2. Comparison of protein characteristics in the corm at the time of flowering

Species	Protein content per 100 g dry weight	Number of polypeptide bands	Range of M.W. (KDa)
C. almehensis	0.63	8	13-82
C. cancellatus 1	1.29	22	13-97
C. cancellatus 2	0.94	29	13-97
C. gilanicus	1.28	31	13-97
C. pallasii subsp. *haussknechtii*	0.96	28	13-82
C. sativus	0.89	30	13-82
C. speciosus	1.78	29	13-97

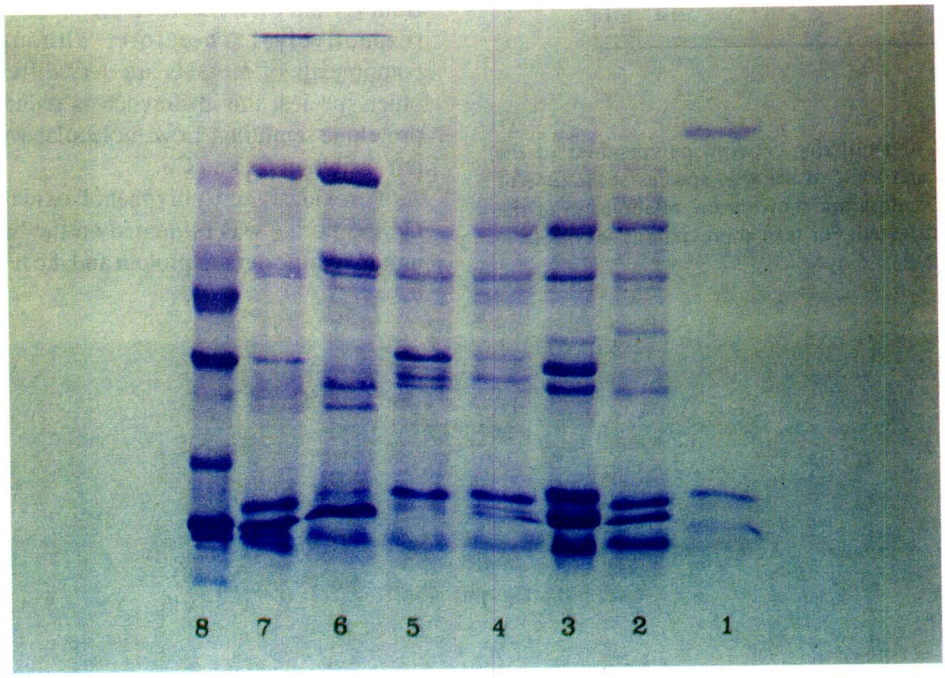
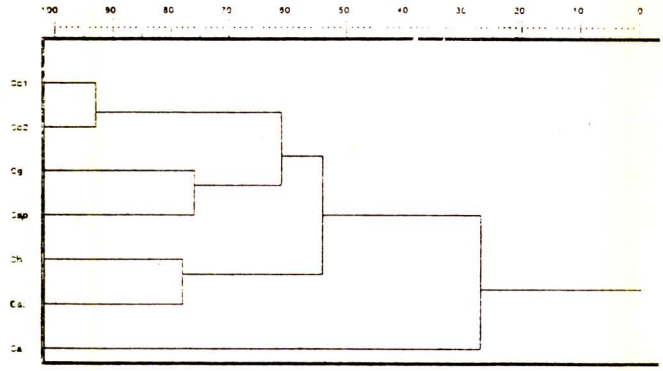


Figure 1. Profile of soluble proteins in corms of saffron and some of the wild species, during the period of floral anthesis. (SDS-PAGE 12.5%): Ca (1), Cg (2), Csp (3), Cc1 (4), Cc2 (5), Ch (6), Cs (7), standard proteins (8) [bovine albumin (66000), egg albumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20100), α-lactalbumin (14200)].

species of *Crocus sativus* L. from the viewpoint of the protein content and range of polypeptides molecular weight, are presented in Table 2. Comparison of the corm polypeptides during the special physiological period, floral anthesis, shows that two specimens of *C. cancellatus* with different localities have the greatest similarity (93.1%), while *C. almehensis* and *C. sativus* have the lowest (18.6%, Figs. 1,2).

Among the examined species, *C. almehensis* is the only species that blossoms during the spring, and its electrophoretic profile shows less similarity with other species (index of similarity below 30%). This species,



	Ca	Cc1	Cc2	Ch	Cg	Cs	Csp
Ca	100.0	25.0	25.6	20.7	25.8	19.6	27.6
Cc1		100.0	93.1	52.8	52.6	48.7	50.0
Cc2			100.0	54.1	57.9	50.0	61.1
Ch				100.0	47.5	78.8	46.2
Cg					100.0	45.4	76.5
Cs						100.0	41.5
Csp							100.0

Figure 2. Index of similarity between polypeptides of the corms of saffron and some of the wild species at the time of floral anthesis. Dendrogram showing the relationship of the *Crocus* taxa investigated. For taxa abbreviations see Table 1. (SDS-PAGE, 12.5%).

which flowers in the freezing season, revealed eight bands by SDS-PAGE method, none of which was placed into protein groups of 20-30 and 50-60 KDa, while other species revealed more bands in these groups (Table 2).

The affinity of different species of *Crocus* was also studied according to the Davis method in which protein bands become separated without dissociation into polypeptides (Fig. 3). Similarity indexes between corm proteins of specimens are arranged in Figure 4. Comparison of Figure 4 with Figure 2 (Laemmli method) shows a decrement in similarity percent of species with this method. As before, the greatest similarity was observed between Cc1 and Cc2 (66.7%) which is less than the similarity found using the Laemmli method (93.1%). By this method, the similarity between Cs and Ch reduces to 20%. *C. almehensis* has no protein bands similar to those of Csp, Cg and Cc, and, as in the Laemmli method, it is placed a long distance from other species, its distance from Cs and Ch is, however, reduced (16.7% and 11.1% respectively). Therefore, although polypeptide components of proteins are very different from Ca and other species, this difference is reduced as a result of protein assembling. Low molecular weight proteins are not visible in Cs and Ch.

Peroxidase and polyphenol oxidase activity in the *Crocus* corms was estimated on the basis of absorption unit in minute per mg protein and the results are shown in

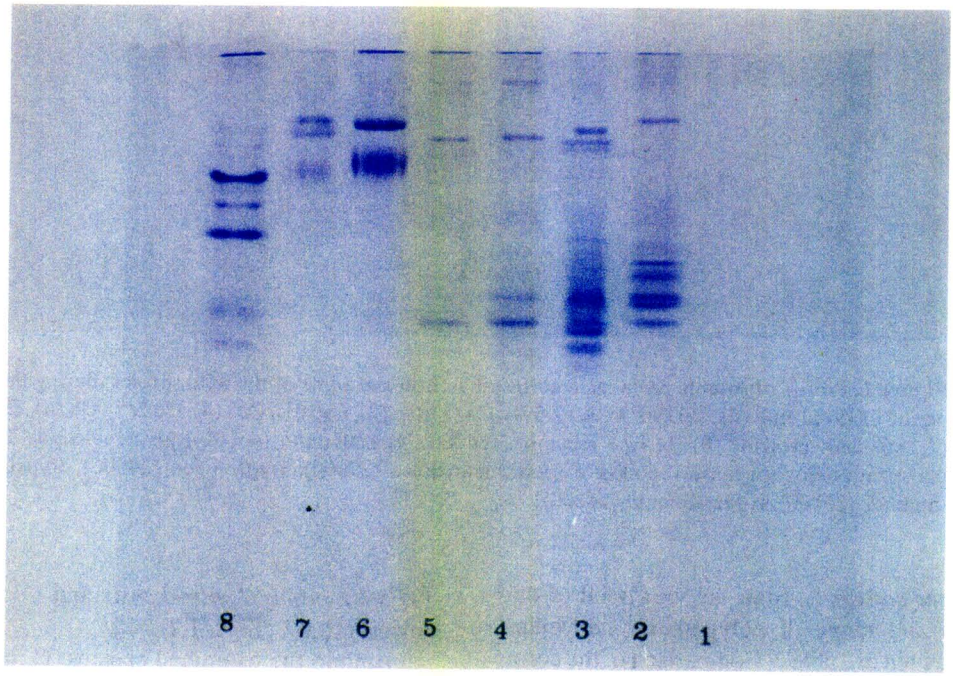
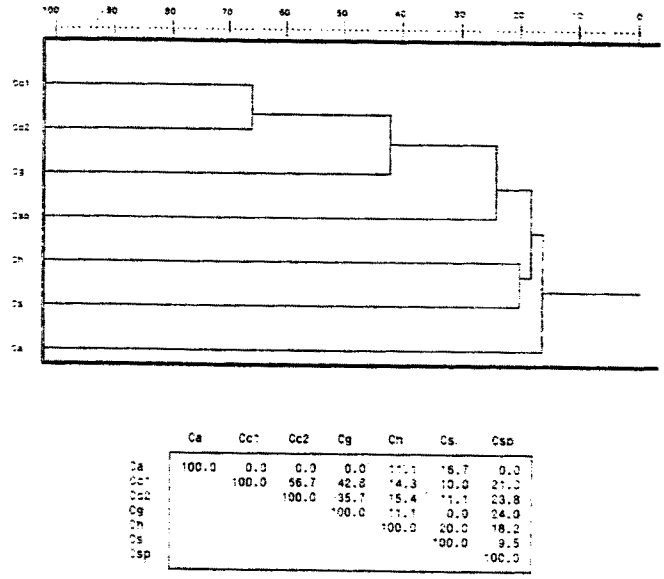


Figure 3. The electrophoretic profile of soluble proteins in corms of the *Crocus* taxa investigated during the flowering period (PAGE, 12.5%): Ca (1), Cg (2), Csp (3), Cc1 (4), Cc2 (5), Ch (6), Cs (7), standard proteins (8).



	Ca	Cc1	Cc2	Cg	Ch	Cs	Csp
Ca	100.0	0.0	0.0	0.0	11.1	16.7	0.0
Cc1		100.0	56.7	42.8	14.3	10.0	21.0
Cc2			100.0	35.7	15.4	11.1	23.8
Cg				100.0	11.1	0.0	24.0
Ch					100.0	20.0	18.2
Cs						100.0	9.5
Csp							100.0

Figure 4. Index of similarity between soluble proteins of the corms of *Crocus* taxa during the period of floral anthesis. Dendrogram showing the relationship of the *Crocus* taxa investigated. (PAGE, 12.5%).

Table 3. The comparison of peroxidase activity in the corm tissue reveals that the activity of the enzyme in corms of *C. almehensis*, which flowers during the spring, is considerably higher than others and the corms of *C. gilanicus* have the lowest activity. Determination of polyphenol oxidase activity in the corms, during the period of floral anthesis, shows that *Crocus sativus* L. has the highest and *C. speciosus* has the least enzyme activity. However, regarding the number of enzymatic bands, Ca and Cc have the maximum bands (6-7 bands) and Cs, Ch and Cg, each with three bands, have minimum polyphenol oxidase isozymes (Table 3). Corms of the saffron *Crocus* (*Crocus sativus* L.), which were harvested from saffron fields at Gonabad and Natanz, were similar in the number and situation of enzymatic bands (Fig. 5).

The estimation of relative mobility of polyphenol oxidase bands by PAGE shows many differences between autumnal specimens and vernal species (*C. almehensis*) and confirms the results of a study of proteins using the Laemmli and Davis methods (Fig. 6). Two specimens of *C. cancellatus* differ only in the presence of one enzymatic band (Rm = 0.598 in Cc1). *C. sativus* L. and *C. pallasii* subsp. *haussknechtii* have three bands with polyphenol oxidase activity (Rm = 0.75, 0.7, 0.917) that are entirely alike (Fig. 5).

Whereas corms may be influenced by environmental, geographic, and seasonal conditions, seed protein has a more stable physiological state, consequently, a comparative study of protein units of seeds was carried out. Results obtained from seed protein analysis of four species using the SDS-PAGE method show that the greatest similarity is observed between Cc1 and Cc2 (63.3%) and the least between Cc1 and Ca (I.S.= 36.5%) (Figs. 7,8). Comparison of the results obtained from tests on corms and seeds using SDS-PAGE reveals that *C. almehensis*, which in later experiments is placed far from other taxa, shows greater similarity with usage of seed tissue (Figs. 2,8).

Analysis of the undenatured seed proteins in some

Table 3. Comparison of peroxidase and polyphenol oxidase activity in corms of the Crocus taxa at the time of floral anthesis

Species	Protein content per 100 g dry weight	Peroxidase activity*	Polyphenol oxidase activity*	Number of polyphenol oxidase bands
C. almehensis	0.63	4.078	0.048	6
C. cancellatus 1	1.29	0.966	0.058	7
C. cancellatus 2	0.94	0.405	0.095	6
C. gilanicus	1.28	0.080	0.054	3
C. pallasii	0.96	0.322	0.024	3
C. speciosus	1.78	0.379	0.010	5
C. sativus	0.89	0.610	0.267	3

*Absorption units in min. per mg proteins

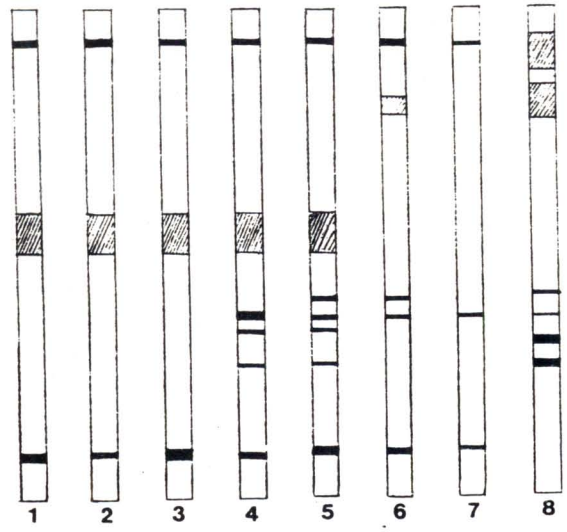


Figure 5. Diagrammatic representation of the polyphenol oxidase activity in corms of *Crocus* taxa investigated at the time of floral anthesis (PAGE, 7.5%): Cs (Gonabad) (1), Cs (Natanz) (2), Ch (3), Cc2 (4), Cc1 (5), Csp (6), Cg (7), Ca (8).

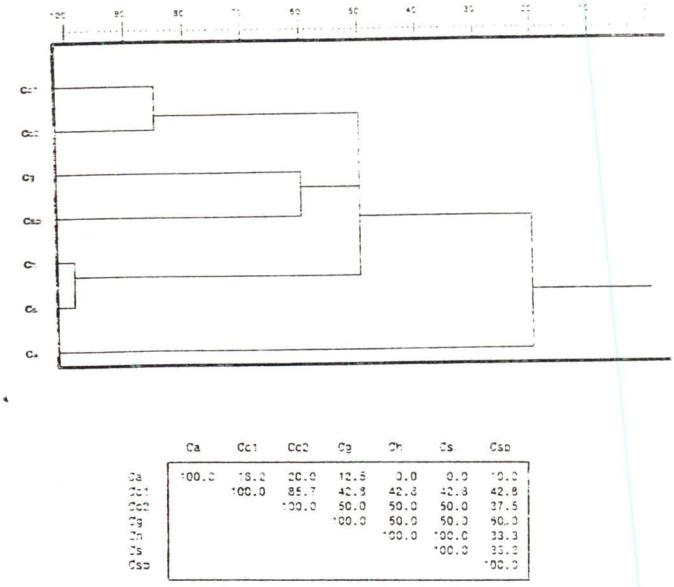


Figure 6. Index of similarity between some of the *Crocus* species from the viewpoint of polyphenol oxidase activity. Dendrogram showing relationship of the *Crocus* taxa with attention activity of this enzyme.

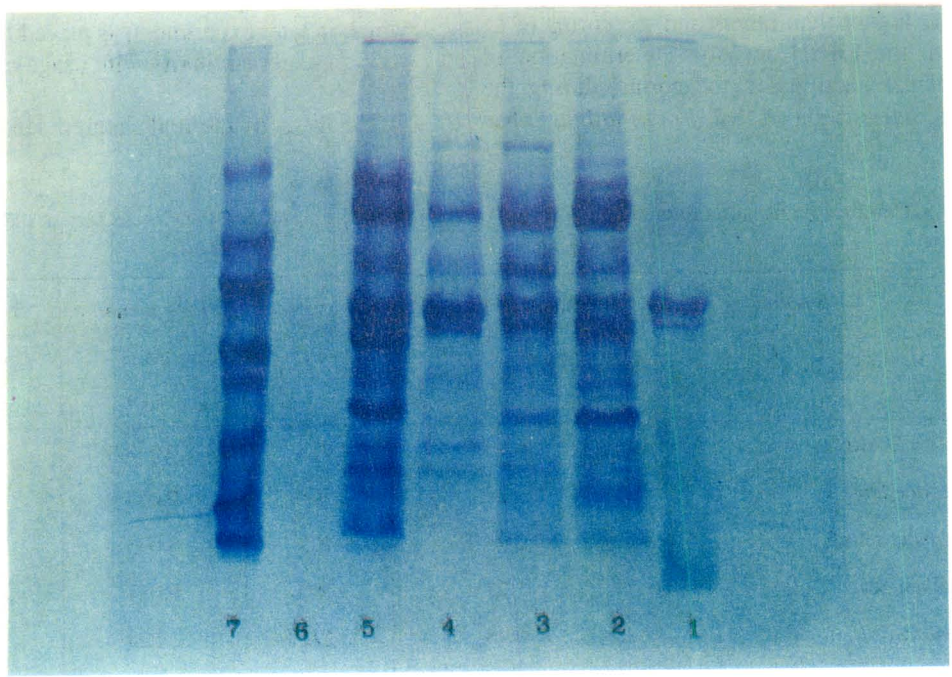
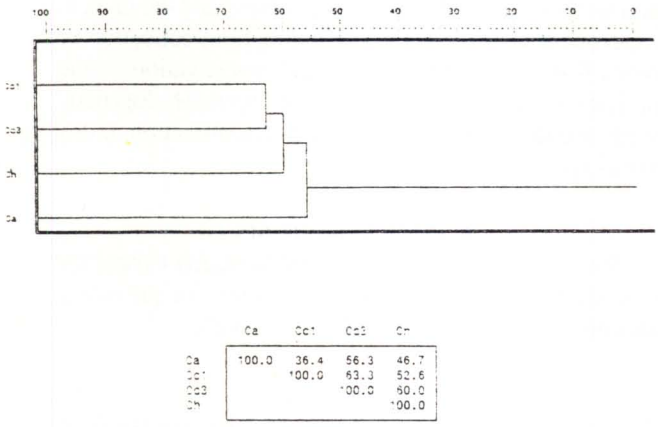


Figure 7. Electrophoretic profile of soluble polypeptides in seed tissue of *Crocus* species (SDS-PAGE, 12.5%) Cc (1), Cc1 (2), Cc (3), Ca (4), Ch (5), *Crocus michelsonii* (6), standard proteins (7). Samples of numbers 1 and 6 appeared weakly, for this reason, they were not taken into account.



	Ca	Cc1	Cc3	Ch
Ca	100.0	36.4	56.3	46.7
Cc1		100.0	63.3	52.6
Cc3			100.0	60.0
Ch				100.0

Figure 8. Index of similarity between seed polypeptides in some *Crocus* species (SDS-PAGE). Dendrogram showing taxonomic affinity of species.

species of *Crocus* by PAGE shows numerous protein bands within the region of low molecular weight proteins (Fig. 9). In general, these results confirm the results obtained using the SDS-PAGE method.

As indicated in Figure 10, the greatest similarity is observed among seeds of *C. cancellatus*. This similarity is 83.3 and 72.7% between Arak specimens and Hamedan and Golpaygan specimens, respectively. The least similarity exists between *C. almehensis* and *C. cancellatus* of Arak and Hamedan (12.5%).

In the electrophoresis of the *Crocus* corms using the Davis method, the similarity index of Ca with four specimens (Cc1, Cc2, Cg and Csp) was zero, while utilization of seed tissues presented a more logical similarity index (Figs. 4,10).

Discussion

The genus *Crocus* has an entirely old world distribution, the majority of the recognized species occurring in the Balkans and Turkey. The limits of the whole genus are therefore within the range 10°W to 80°E and 30°N to 50°N [14]. Nine species of them are known in Iran and it is necessary to mention that since these species are placed in different sections, the exact determination of phylogenetical relations is difficult.

Investigation of the relationship between studied species using the comparison of electrophoretic profile of corm and seed tissues, in particular the SDS-PAGE method, showed that two specimens of *C. cancellatus* subsp. *damascenus* (collected from Arak and Golpaygan) had maximum similarity and made the first cluster of dendrogam. A study of seed polypeptides and polyphenol

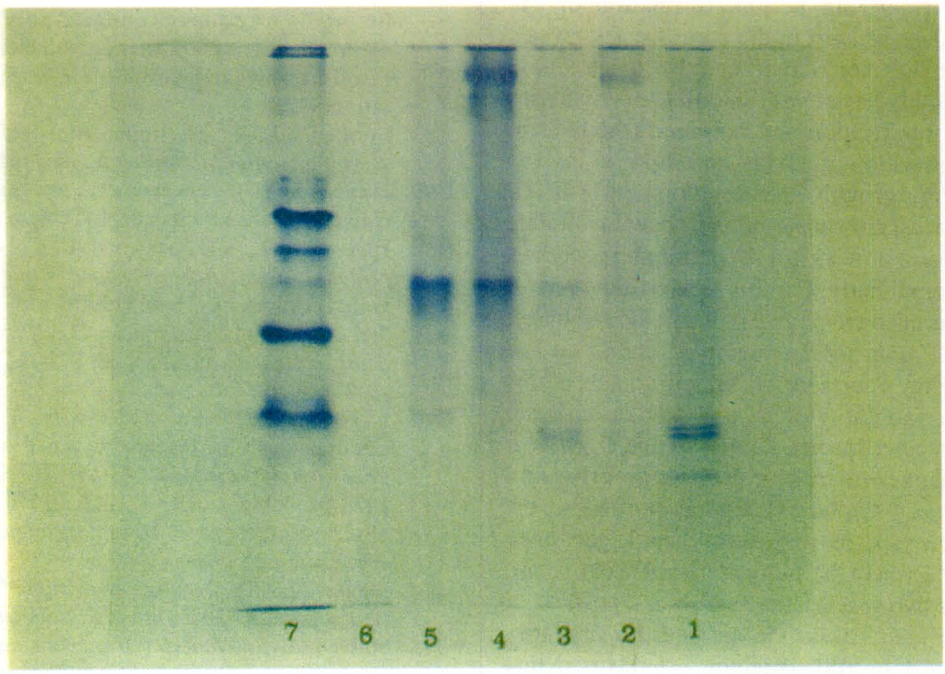
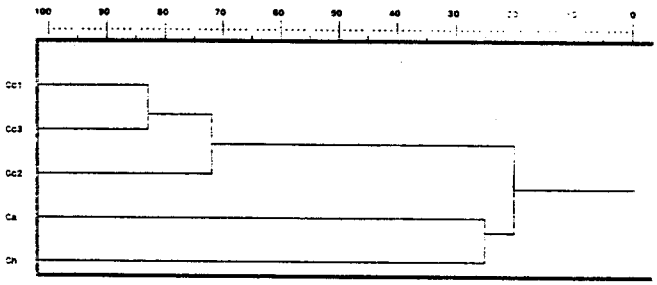


Figure 9. Electrophoretic profile of soluble protein in the seeds of some *Crocus* taxa investigated (PAGE, 10%): Cc3 (1), Cc1 (2), Cc2 (3), Ch (4), Ca (5), *C. michelsonii* (6), standard proteins (7).



	Ca	Cc1	Cc2	Cc3	Ch
Ca	100.0	12.5	5.4	12.5	25.0
Cc1		100.0	72.7	83.3	20.0
Cc2			100.0	72.7	20.0
Cc3				100.0	20.0
Ch					100.0

Figure 10. Comparison of index of similarity between soluble proteins of seed tissue in the *Crocus* (PAGE). Dendrogram showing their relationship.

oxidase activity in corms confirmed this result.

It seems therefore that these specimens are a local population and co-species [1,8]. At the time of floral anthesis, Cg and Csp, from the variety of polypeptides in the corm, resemble one another greatly. These taxa, along with Cc, make a common cluster, while the spring *Crocus*, *C. almehensis*, using both methods is placed very far from the others.

During the flowering period, estimation of the polyphenol oxidase activity in the corm tissues reveals that *C. almehensis* has more activity and also its peroxidase activity is remarkably excessive (Table 3). The peroxidases have a role in lignification and hormone metabolism, specially in degradation of auxins, and show an inverse relationship with growth and development [4]. *C. almehensis* has a fast growth period and, under favourable climatic conditions, flowers in a short time and generates new corms concomitantly. For this reason, although all the examined corms were physiologically the same age, the corms of *C. almehensis* resembled corms on the threshold of ageing. Therefore, the protein content of this species and the variability of its polypeptide units are clearly less than other species. *C. sativus* and *C. pallasii* subsp. *haussknechtii* are morphologically and karyologically very similar [3]. Protein profiles of the corms of these two species on polyacrylamide gels show great similarity. Even in the study of polyphenol oxidase activity of these two species, the number and position of the bands were identical on the gel and the similarity percent was equal to 100. Brighton [3] reported that triploid vigour of the saffron *Crocus* may have originated from either the species *C. cartwrightinus* or *C. tomasii*.

Since, in the Crocus genus, the protein units of corms and seeds have not yet been investigated and the present experiment is a first step in this direction, collection of seeds from all over the world and investigation of the proteins is essential for an exact and universal phylogenetic study and interspecific relationship determination of this genus species.

Acknowledgements

We are grateful to the National Research Center for Genetic Engineering and Biotechnology for providing facilities during the course of this research.

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