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Crocus (Iridaceae) Species Relationships Based on the Anatomical Characters

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Abstract

Crocus belongs to the Iridaceae family which comprises 88-100 species. This genus is represented in Iran by 24 species. This study analyzed relationships among the eight taxa distributed in Iran using 11 leaf anatomical characters. UPGMA (Unweighted Paired Group using average) and WARD clustering were performed using the Euclidean distance after 1000 times bootstrapping. The principal component analysis (PCA) shows that the three main components represent 85% of the variation. The PCA of anatomical traits effectively delineated the species under investigation. Furthermore, the PCA Biplot demonstrated that anatomical features can successfully differentiate between species. Notably, the condition of C. haussknechtii was found to be closely related to the two species, *C. biflorus* and *C. michelsonii*, despite *C. haussknechtii* being classified within the Crocus section, while the other two species belong to the Nudicapus section.Our findings indicate that additional anatomical data and molecular data are necessary for reliable conclusions regarding the studied species.

Keywords: *Crocus*, Anatomy, UPGMA, PCA

Introduction

Iridaceae represents a large and diverse family that encompasses about 66 genera and 2244 species. The members of this family are mainly distributed in the southern hemisphere (Kamra et al. 2023). Among its members, *Crocus* L. comprises about 100 species extending from southwest Europe, central Europe to Turkey as well as parts of southwestern Asia extending to western China (Butnariu et al. 2022; Alavi-Kia et al. 2008; Petersen et al. 2008).

Dolatyari et al (2024) reported that alongside the cultivated *Crocus* species, there exist 23 additional species of this genus in Iran, of which 18 species are exclusive to Iran and the other five species are distributed only in Iran and neighboring countries. Iran servesas a significant center for diversification of this genus. Notable species whitin this genus found in include *Crocus sativus* L., *C. caspius* Fisch. & C. A. Mey. ex Hohen.; *C. speciosus* M. Bieb.; *C. almehensis* C. D. Brickell & B. Mathew; *C.*

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biflorus Mill.; *C. michelsonii* B. Fedtsch.; *C. gilanicus* B. Mathew.; *C. cancellatus* Herb.; *C. haussknechtii* (Boiss. & Reut. ex Maw) Boiss. Systematic investigations concerning this genus have been carried out by Pedro de Padua et al (2023), Munirah et al (2022), Uslu et al (2022), Pasche, (1994) and Pasche and kerndorff (1996 b ,1997). However, a comprehensive study of the anatomy of this genus has not been performed.

Anatomical characteristics have been considered as significant in the systematic studies of the genus *Crocus* (Raycheva et al. 2021; Ljubisavljević & Raca, 2020; Rudall and Mathew 1990). These features include the presence or absence of papillae, the configuration of the anticlinal walls in the epidermis cells, the thickness of the sclerenchyma layer surroundingvascular bundles, and the extent to which sclerenchyma extends beyond the lamina.

The leaves of most Crocus species exhibit a unique and distinctive cross-sectional shape, comprising a central square or rectangular 'keel' and two lateral 'arms', with their margins. The edges of these arms typicallyrecurv back toward the keel. The distinctive pale stripe that extend longitudinally along the center of the leaf results from the prescence of parenchymatous cells whitin the keel, which are devoid of chloroplasts and frequently disintegrate to create an air activity (Uslu et al. 2022).

In the transverse section of the *Crocus*, the palisade parenchyma is distinguished from the spongy parenchyma. This characteristic has also been noted in *Lilium candidum* (Torres-Pio et al. 2021; Ozen et al. 2012). To explore a relationships among species within the genus Crocus, a combination of morphological and anatomical characters, as well as biochemcal data, was employed (Siracusa et al. 2022; Rudall 1994).

Numerous phylogenetic investigation concerning the genus Crocus have been carried out, focusing primarily on molecular data. However, there is a notable absence of research that combines morphological and anatomical characteristics with molecular data (Anabat et al., 2022; Yılmaz, 2021b; Harpke et al. 2013; Seberg and Petersen 2009; Peterson et al. 2008). The Crocus taxonomy presents significant challenges, largely due to the incomplete genetic distinctions among the various species, which exhibit a wide range of habitats and morphologically heterogeneous traits (Dolatyari et al., 2024; Raycheva et al., 2023; Caiola et al. 2004).

Utilizing molecular data, anatomy, and pollen data is recommended to establish a dependable relationship of Crocus species

The aims of the present study were to provide anatomical features of the Crocus species found in Iran and to utilize these data to illustrate the relationships among the species.

Material and methods

Species of *Crocus* species were collected from various area of Iran (Table 1). A total of 40 plant specimens were analyzed. The plant samples were randomly selected and preserved in a solution of formalin-acetic acid-alcohol (FAA) with a composition of formalin, acetic acid, and ethanol (90%) in a ratio of 5:5:50% (Pegg et al., 2021; Jensen, 1962) for a duration of 48 to 72 hours.

The speciemens were then preserved at a temperature of 4 °C until they were sectioned. This was , followed by dehydration process utilizing a graded series of ethanol,culminating in embedding within 70% ethanol.

The transverse hand sections of the lamina and stem samples were prepared and subsequently washed with distilled water. They were then immersed in a 5% sodium hypochlorite solution for 20 min to facilitate clearing. Following this, the sections were rinsed with distilled water.

The samples were stained with methyl blue and Congo red before being mounted on slides using Canada balsam. Thin cut sections were examined under a microscope equipped with digital camera.

Data analysis

The anatomical data obtained, encompassing both quantitative and qualitative characters (Table 2), were standardized (Mean = 0, Variance = 1) and subsequently employed for multivariate analyses. To group the studied species and reveal their relationships, a combination of clustering and ordination methods were utilized (Gebrehiwot et al. 2020; Podani 2000).

UPGMA (Unweighted Paired Group using average) and WARD clustering were performed utilizing the Euclidean distance after 1000 times bootstrapping. In addition, Principal coordinate analysis (PCoA) was employed to categorize the studied species, while Principal Components Analysis (PCA) was performed to identify the most variable anatomical characters (Yazici et al., 2024; Podani 2000). For these analysesPAST (Paleontological Statistical software) version 3 was utilized (Hammer and Harper 2024; Hammer et al. 201

Results

The anatomical traits of the leaf are detailed in Fig. 1 and Table 2. The maximum cuticle thickness was recorded in C. almehensis at 11.55 µm, whilethe minimum thickness was found in C. hausskenechtii at4.12 µm. The greatest length of lower epidermis was observed in C. caspius measuring 20.42 µm, whereas C. hausskenechtii exhibited the shortest length at 11.34 µm. For spongy parenchyma, the highest measurement was in C. biflorus at 40.28 µm, with C. almehensis shwing the lowest at 20.72 µm. The longest vascular bundle was observed in S. speciosus at 124.85µm, while the C. hausskenechtii had the shortest at 70.45 µm. Additionally, C. almehensis had the highest number of vascular bundles at 14, in contrast to C. cancellatus which had the fewest at 6.67.

Species delimitation and species relationship

The identification of species boundaries through anatomical characters is a significant task. The WARD tree (Fig. 2) which is based on anatomical data, shows that C. biflorus and C. caspius are closely related, and both C. sativus and C. cancellatus species are also adjacent to each other. Additionally, C. almensis and C. speciosus are located in close to each other.

The PCA plot (Fig. 3) illustrates the clustering of the studied species based on anatomical data, which effectively differentiates between the various species. Furthermore, this figure displays the delimitation of the examined species (Fig. 3).

The principal component analysis (PCA) shows that the three main components represent 85% of the variation. Consequently, the trait of cuticle thickness (> 0.90) was one of the most variable traits in the first component. In the second component, the characteristics of the vascular bundle, including the length of the vascular bundle, the width of the vascular bundle, and the number of vascular bundles were identified as the most variable characters (exceeding 0.75). The third component highlighted the length and the width of the epidermis along with the width of the palisade parenchyma, as the most variable traits (surpassing 0.80). The PCA Biplot shows that anatomical characteristics play a significant role in species differentiation. Accordingly, character 1 (cuticle thickness) is the most influential factor in distinguishing C. hussknechtii and C. michelsonii from C. caspius. Additionally, character 8 (height of the parenchyma spongy) has contributed to the distinctiveness of C. caspius compared to the other species. Furthermore, character 9 (vascular bundle length) and character 11 (the number of vascular bundle) have led to the separation of C. speciosus and C. almehensis from one another (Fig.4).

Discussion

In this study, the interrelationships among eight Crocus species found in Iran were studied through eleven leaf anatomical traits. The specific characters utilized for each taxon in this investigation are presented in Table 2.

The notable finding is that C. haussknechtii is adjacent to C. biflorus and C. michelsonii, despite C. haussknechtii being classified within the Crocus section, while the other two species belong to the Nudicapus section, based on previous studies (Dolatyari et al. 2024; Petersen 2008; Rudall 1990).

A comparable situation has been observed in a study conducted by Anabat et al. (2022) and Coskun et al. (2010) regardingC. haussknechtii, which is the sister group of the Nudicapus section. Furthermore, research by Peterson et al. (2008) indicated that the three species C. biflorus, C. cancellatus and C. speciosus were classified within the same branch. In contrast, the current study has identified these species in three main branches, aligning with the findings of Yılmaz 2021, although it is worth mentioning that C. cancellatus and C. speciosus are positioned in close proximity to one anther (Golshani et al. 2020). The two species occupy almost identical regions of the tree, while C. biflorus is situated separately int a different area. Based on the anatomical data analysis conducted in this study, C. biflorus and C. caspius were categorized within the same branch, consistent with the classification stablished by Rudall and Maw (1990), as both species belong to the same section (Nudicapus) and series (Biflori) (Yılmaz 2021). However, Our findings significantly differ from from the sectional and serial classifications proposed by Petersen et al. (2008).

The anatomical characters of the leaf examined in this study successfully differentiateddistinguish the species, aligning with the findings done by Uslu et al. (2022) and Zedmir et al. (2010).

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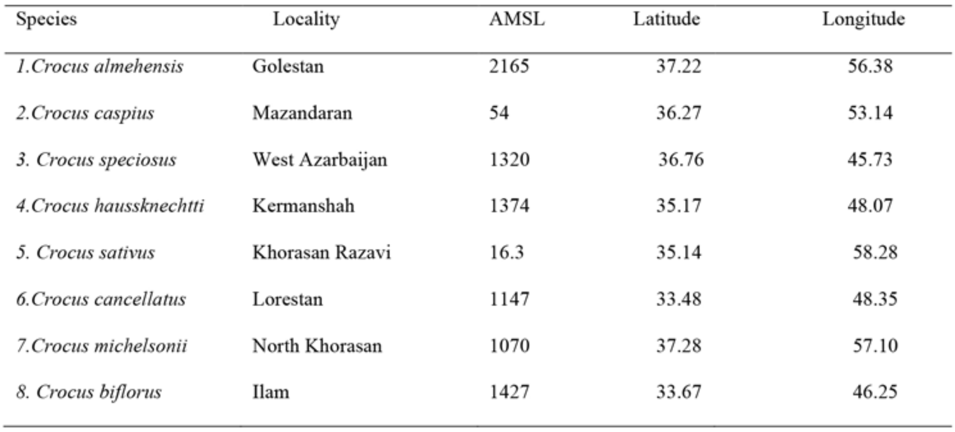
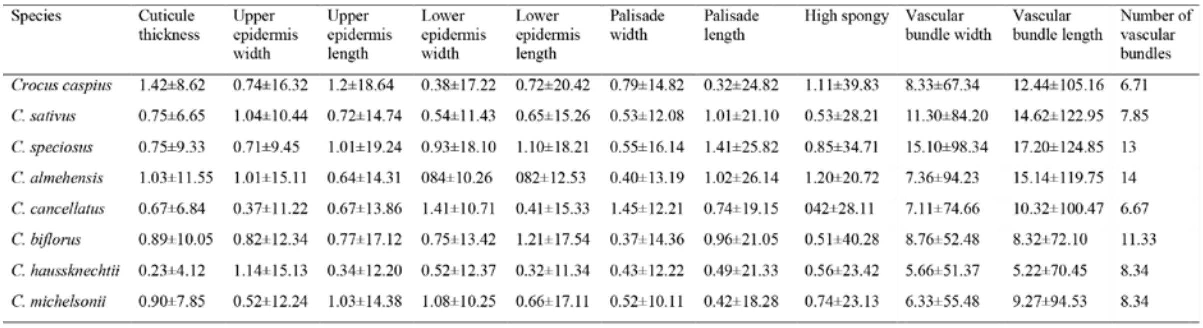
epidermis; Vb: vascular bundle; Pp: palisade parenchyma; Sp: spongy parenchyma; Ue: upper epidermis.

Table 1. Geographical locations and ecological characteristics of the studied *Crocus* species



Species	Locality	AMSL	Latitude	Longitude
1.Crocus almehensis	Golestan	2165	37.22	56.38
2.Crocus caspius	Mazandaran	54	36.27	53.14
3. Crocus speciosus	West Azarbaijan	1320	36.76	45.73
4.Crocus haussknechtti	Kermanshah	1374	35.17	48.07
5. Crocus sativus	Khorasan Razavi	16.3	35.14	58.28
6.Crocus cancellatus	Lorestan	1147	33.48	48.35
7.Crocus michelsonii	North Khorasan	1070	37.28	57.10
8. Crocus biflorus	Ilam	1427	33.67	46.25

Table2. Average of the anatomical characters measured on *Crocus* species (µm)



Species	Cuticule thickness	Upper epidermis width	Upper epidermis length	Lower epidermis width	Lower epidermis length	Palisade width	Palisade length	High spongy	Vascular bundle width	Vascular bundle length	Number of vascular bundles
Crocus caspius	1.42±8.62	0.74±16.32	1.2±18.64	0.38±17.22	0.72±20.42	0.79±14.82	0.32±24.82	1.11±39.83	8.33±67.34	12.44±105.16	6.71
C. sativus	0.75±6.65	1.04±10.44	0.72±14.74	0.54±11.43	0.65±15.26	0.53±12.08	1.01±21.10	0.53±28.21	11.30±84.20	14.62±122.95	7.85
C. speciosus	0.75±9.33	0.71±9.45	1.01±19.24	0.93±18.10	1.10±18.21	0.55±16.14	1.41±25.82	0.85±34.71	15.10±98.34	17.20±124.85	13
C. almehensis	1.03±11.55	1.01±15.11	0.64±14.31	084±10.26	082±12.53	0.40±13.19	1.02±26.14	1.20±20.72	7.36±94.23	15.14±119.75	14
C. cancellatus	0.67±6.84	0.37±11.22	0.67±13.86	1.41±10.71	0.41±15.33	1.45±12.21	0.74±19.15	042±28.11	7.11±74.66	10.32±100.47	6.67
C. biflorus	0.89±10.05	0.82±12.34	0.77±17.12	0.75±13.42	1.21±17.54	0.37±14.36	0.96±21.05	0.51±40.28	8.76±52.48	8.32±72.10	11.33
C. haussknechtii	0.23±4.12	1.14±15.13	0.34±12.20	0.52±12.37	0.32±11.34	0.43±12.22	0.49±21.33	0.56±23.42	5.66±51.37	5.22±70.45	8.34
C. michelsonii	0.90±7.85	0.52±12.24	1.03±14.38	1.08±10.25	0.66±17.11	0.52±10.11	0.42±18.28	0.74±23.13	6.33±55.48	9.27±94.53	8.34

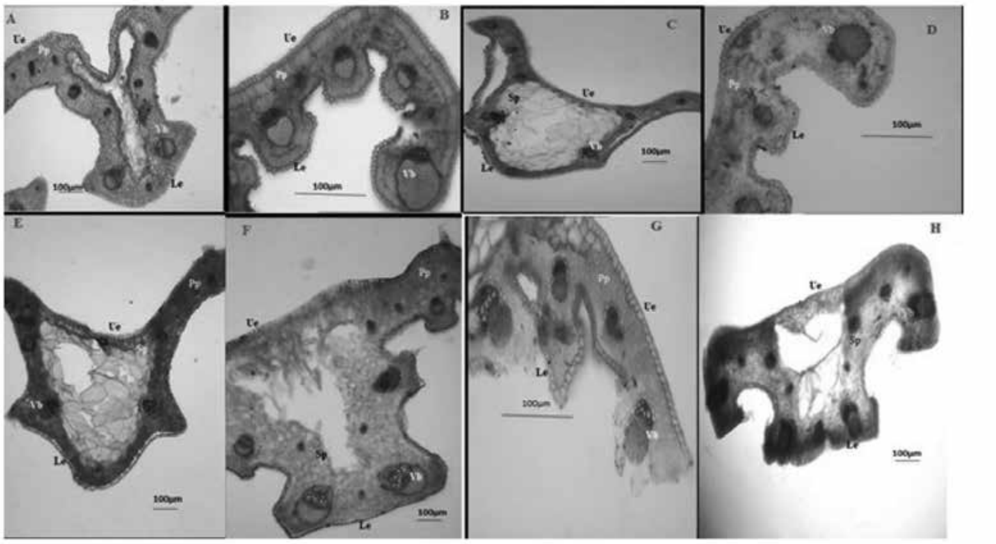


Figure 1. Appearance of leaf in *Crocus* species. A: *C. speciosus*; B: *C. almehensis*; C: *C. biflorus*; D: *C. cancellatus* ;E: *C. caspius*; F: *C. hausskenechtii*; G: *C. michelsonii*; H: *C. sativus* ; Le: lower epidermis; Vb: vascular bundle; Pp: palisade parenchyma; Sp: spongy parenchyma; Ue: upper epidermis.

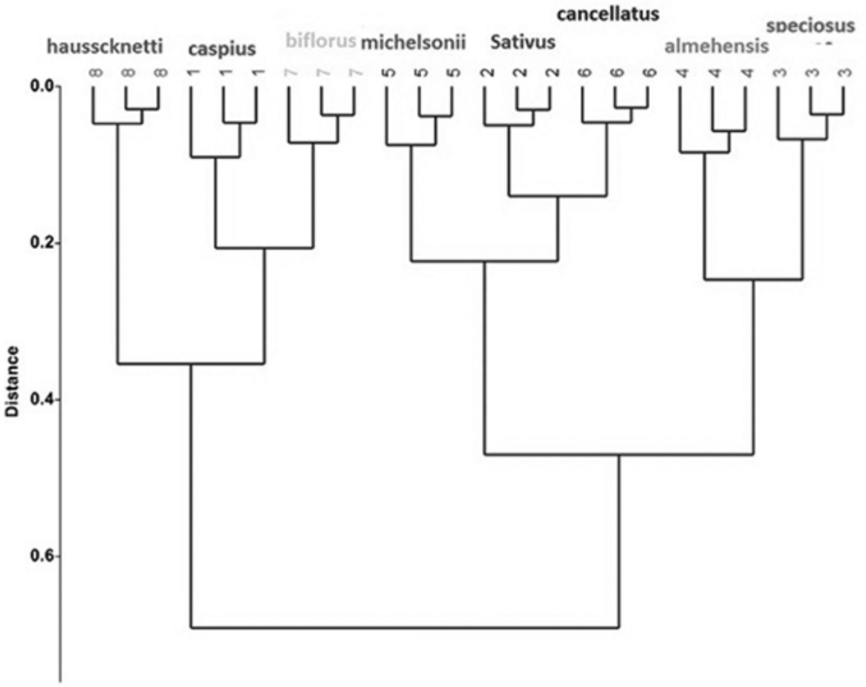


Figure 2. The WARD tree of the studied species based on anatomical data shows the proximity and distance between the species, as well as the delimitation of the species.

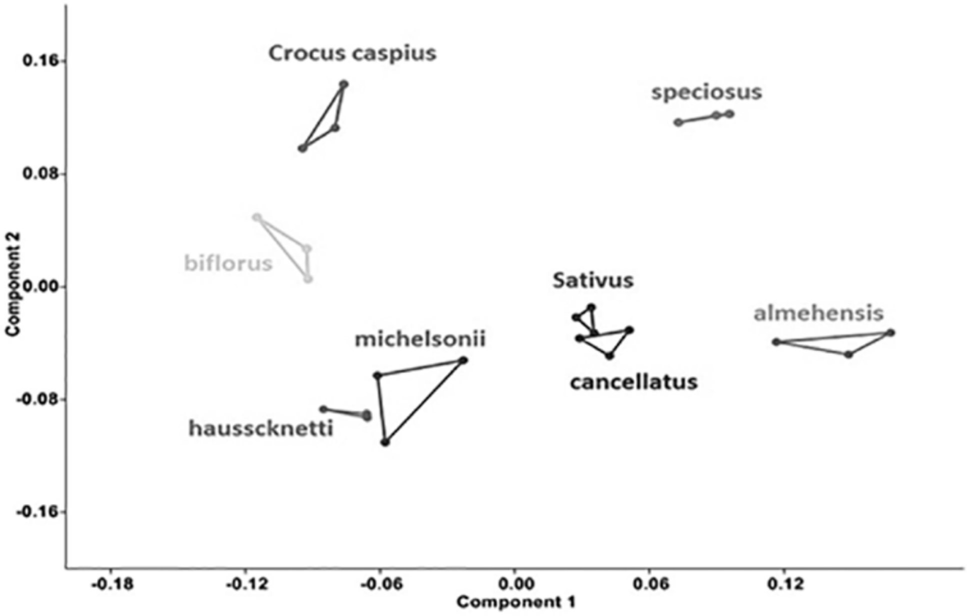


Figure3. The PCA plot of *Crocus* genus species based on anatomical data shows the grouping of the studied samples

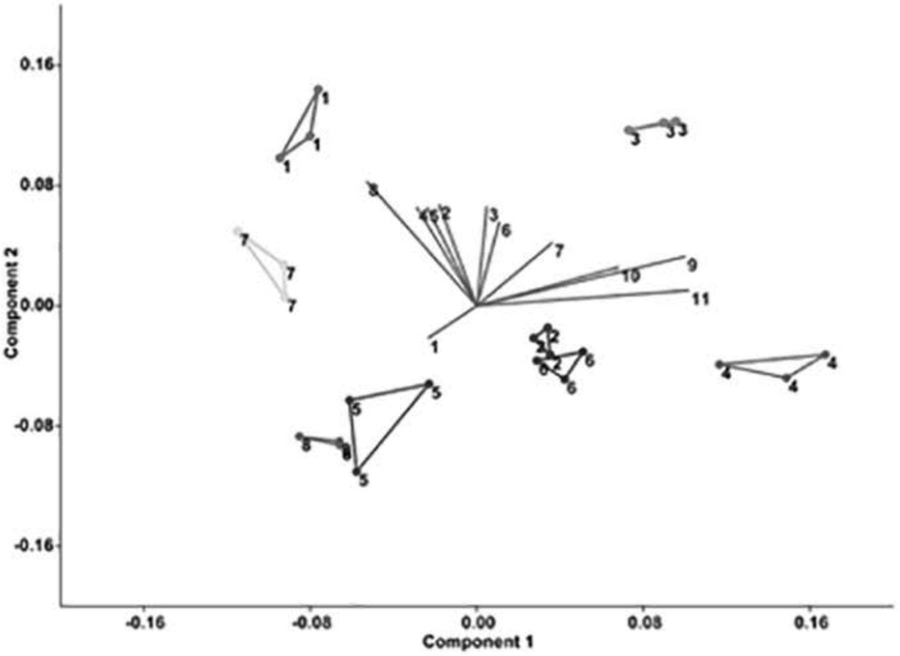


Figure 4. The PCA plot of *Crocus* genus species based on anatomical data shows the grouping of the studied samples

1.Crocus caspius, 2. C. sativus 3. C. Speciosus, 4. C. almehensis,5. C. michelsonii,6. C. cancellatus
7.C. biflorus,8. C. hausskenechtii.