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Genetic relationships among species of Iranian crocus (*Crocus* spp.)

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ABSTRACT

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Fifteen microsatellite markers were employed to evaluate genetic relationships among different Iranian species of *Crocus* spp. (*C. sativus*, *C. haussknechtii*, *C. cancellatus, C. speciosus,* and *C. caspius*) from ten geographical regions (Ghaen, Gonabad, Estahban, Ferdows, Veresk, Reno, Ali abad, Kali abad, Neka, and Eslam abad). Of these, 12 primers revealed polymorphism among *Crocus* spp. Average polymorphism information content (PIC) and genetic diversity valueswere 0.33 and 0.41, respectively. Cluster analysis using the neighbor-joining (NJ) algorithm based on Nei's genetic distance separated all the cultivated clonesfrom wild ones and differentiated all species. Utilizing principal coordinate analysis (PCoA) and analysis of molecular variance (AMOVA), significant heterogeneity was observed within *Crocus* species. Based on the genetic distance (0.0311) andfixation index (F_{ST}=0.33, P>0.05) between Estahban (29° 12' N and 54° 03' E) and Ferdows (34° 01' N and 58° 16' E), it seems that accessions from Estahbanmay have originated from Ferdows genotypes. Indeed, results revealed that *C. haussknchii* is the closest wild species to cultivated ones.

Keywords: *Crocus* spp., genetic diversity, simple sequence repeats

INTRODUCTION

Cultivation of saffron (*Crocus sativus* L.) dates back to 2500-1500 BC and probably originated in Iran and Greece, from where it later spread to India, China, the Mediterranean Basin, and Eastern Europe (Tammaro, 1987; Negbi, 1999). Saffron is classified in the family Iridaceae (*Iris*) in the genus *Crocus*, which consists of about 80 species (Mathew, 1982). All alliesof the *Crocus* genus are diploid, except for *Crocus sativus* L., which is triploid in genetic make up ($2n = 3x = 24$) and is propagated vegetatively by means of bulbs or corms (Mathew, 1977; Brighton, 1977; Fernandez, 2004).

Saffron is a crop of prime economic importance, with exceptional therapeutic properties (Rios *et al*., 1996). It is mostly used as an aromatic spice and colorant in many foods worldwide, but its medicinal benefits have also been recognized from the time of ancient civilizations to the present day (Rios *et al*., 1996; Ferrence and Bendersky, 2004). In recent years, the therapeutic value of saffron in cancer and cerebrovascular and cardiovascular diseases has been well documented (Nair *et al*., 1991; Abdullaev, 2007; Escribano *et al*., 1996; Rios *et al*., 1996).

Morphological comparisons of *C. sativus* have revealed some differences in intensity of flower color, pollen size, number of style branches and stamens, and viability (Caiola *et al*., 2001; Piccioli, 1932). Although there are phenotypic variations within cultivated saffron, until now no genetic diversity has been observed (Caiola and Zaier, 2004; Alavi-Kia *et al*., 2008). This limited genetic background in cultivated saffron is attributed to its asexual propagation, and by successive selection during breeding (Alavi-Kia *et al*., 2008). However, other factors that may contribute to the low levels of molecular polymorphism are the marker techniques applied and the amount of diversity among the natural population used (Singh *et al*., 1998).

Nowadays, molecular markers have been utilized for to differentiate, identify, and evaluate genetic variation of many cultivars (Karim *et al*., 2010; El-Tarras *et al*., 2007; Gul *et al*., 2007). Microsatellites or simple sequence repeat (SSR) markers have many advantages over other markers used in genetic studies, for they are highly polymorphic, locus specific, and readily transferable, and have been applied successfully to detect genetic diversity and construct molecular maps in several plant species (Cuc *et al*., 2008; Bowcock, 1994) such as rice (Kumar and Bhagwat, 2012). The use of SSR markers for breeding and other applied research in

plants requires developing a large number of SSRs for the species of interest. Although Nemati *et al*. (2012) developed 12 microsatellite markers that are currently available for *C. sativus* L., there is urgent need to discover and develop more polymorphic SSR markers for efficient management of the germplasm of this valuable plant.

Iran is the world's main producer of saffron, accounting for 93.7% of global production, with approximately 50,000 ha under cultivation, which annually produce more than 230 tonnes of dry stigmata (Ghorbani, 2007). In addition to *C. sativus* L., Iran is also home to eight wild species of *Crocus* including *C. haussknechtii*, *C. cancellatus*, *C. caspius*, *C. speciosus*, *C. almehensis*, *C. gilanicus*, *C. michelsonii*, and *C. biflorus* (Mathew, 1982).

In the present study, we used microsatellite markers isolated by Rubio-Moraga *et al*. (2009) to evaluate the efficiency of these SSR markers in detecting genetic polymorphisms in Iranian saffron. Another aim of this study was to investigatethe transferability of these SSR markers to other *Crocus* species.

MATERIAL AND METHODS

Plant materials

Thirty-eight saffron genotypes (*C. sativus* L.) and 28 allies (including *C. haussknechtii, C. caspius, C. speciosus*, and *C. cancellatus*) were collected from 10 different regions of Iran (Table 1).

DNA isolation and SSR analysis

Total genomic DNA was extracted from fresh leaves of 66 genotypes from 5 species of *Crocus* using the procedure of Doyle and Doyle (1987) with minor modifications. The concentration and quality of the extracted DNA was measured using electrophoresis and 0.7% TAE agarose gel. The DNA was diluted to 20 ng/µl and stored at -20°C.

First, by choosing some samples, the optimum annealing temperatures were identified using a gradient PCR program. Final PCR amplifications were then carried out in a Veriti thermal cycler (Applied Biosystems, California, USA) in a total volume of 15 µl, which included 20 ng DNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.06 pmolof each primer and 0.5 U *Taq*DNA polymerase (Fermentas, Sankt Leon-Rot, Germany). The PCR program includedinitial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30s, annealingat 47-55°C and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. PCR products were visualized by 4% metaphor agarose gel (Lonza, Rockland, USA). A 50-bp DNA ladder (Fermentas, Sankt Leon-Rot, Germany) was used to identify the alleles as a molecular weight standard for band scoring that was loaded in each gel.

Data analysis

SSR amplified fragments were manually scored across all genotypes for more accuracy. Number of alleles per locus, expected heterozygosity (H_e), observed heterozygosity (H_o), polymorphism information content (PIC), and inbreeding coefficient or fixation index (F) (Nei, 1987) were calculated using Power Marker ver. 3.25 (Liu and Muse, 2005). An NJ dendrogram was constructed using Power Marker software (Liu and Muse, 2005) and the minimum evolution method.

Analysis of molecular variance (AMOVA) was performed using Arlequin 3.1 software (Schneider *et al*., 2000) to partition the total molecular variance among and within all populations, as well as to test the significance of partitioned variance components using a permutation procedure. The polymorphic SSR loci were examined for detecting diversity measures in the 38 cultivated and 28 wild saffron accessions. Genetic distances were estimated according to Nei (1987), and principal coordinate analysis (PCoA) (Gower, 1966) and neighbor-joining (NJ) analysis were performed.

RESULTS AND DISCUSSION

SSR polymorphism

Fifteen SSR primers designed by Rubio-Moraga *et al*. (2009) were employed to differentiate the studied populations. Of those, 12 primers (Table 2) could generate sharp and polymorphic bands ranging in size from 200 to 350 bp. In total, 21 alleles were produced among different *Crocus* species using these markers. Data analyzed using Power Marker ver. 3.25 are summarized in Table 2.

Polymorphism information content values were calculated as the marker index to show the informativeness of utilized markers with the means 0.33 by the range of variation from 0.22 to 0.41. The most informative marker was related to CSMIC14 and CSMIC62. According to Xie *et al*. (2010), the developed markers were located at the medium PIC level (0.25<PIC<0.5) utilizing the assessment of various *Crocus* spp.

The obtained results are in agreement with previous research (Bounphanousay *et al*., 2008) in which the PIC of utilized microsatellites were in the range of 0.4. Genetic diversity values range from 0.25 to 0.49 with an average of 0.41. By examining the deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using the pairwise method at the 5% probability level between all pairs of loci, no significant linkage disequilibrium was revealed, except CSMIC26, CSMIC46, and CSMIC47 loci.

Table 1. List of *Crocus* species collected from different regions of Iran that were used in this study.

No.	Code	Species	Ploidy level	Geographical region
1	SAGO72	*C. sativus*	3X	Gonabad, Khorasan, Iran
2	SAGO74	*C. sativus*	3X	Gonabad, Khorasan, Iran
3	SAGO73	*C. sativus*	3X	Gonabad, Khorasan, Iran
4	SAGO71	*C. sativus*	3X	Gonabad, Khorasan, Iran
5	SAGO76	*C. sativus*	3X	Gonabad, Khorasan, Iran
6	SAGO66	*C. sativus*	3X	Gonabad, Khorasan, Iran
7	HASVL4	*C. haussknechtii*	2X	Veresk, Mazandaran,Iran
8	SAGO69	*C. sativus*	3X	Gonabad, Khorasan, Iran
9	SAGO75	*C. sativus*	3X	Gonabad, Khorasan, Iran
10	SAGH23	*C. sativus*	3X	Ghaen, Khorasan, Iran
11	SAGH29	*C. sativus*	3X	Ghaen, Khorasan, Iran
12	SAGH30	*C. sativus*	3X	Ghaen, Khorasan, Iran
13	SAGH1	*C. sativus*	3X	Ghaen, Khorasan, Iran
14	SAGH12	*C. sativus*	3X	Ghaen, Khorasan, Iran
15	SAES9	*C. sativus*	3X	Estahban, Fars, Iran
16	ES10SA	*C. sativus*	3X	Estahban, Fars, Iran
17	ES11SA	*C. sativus*	3X	Estahban, Fars, Iran
18	ES1SA	*C. sativus*	3X	Estahban, Fars, Iran
19	SAES3	*C. sativus*	3X	Estahban, Fars, Iran
20	SAES15	*C. sativus*	3X	Estahban, Fars, Iran
21	SAES14	*C. sativus*	3X	Estahban, Fars, Iran
22	ES8SA	*C. sativus*	3X	Estahban, Fars, Iran
23	SAES2	*C. sativus*	3X	Estahban, Fars, Iran
24	SAES6	*C. sativus*	3X	Estahban, Fars, Iran
25	SAES12	*C. sativus*	3X	Estahban, Fars, Iran
26	SAES4	*C. sativus*	3X	Estahban, Fars, Iran
27	SAES7	*C. sativus*	3X	Estahban, Fars, Iran
28	SAES5	*C. sativus*	3X	Estahban, Fars, Iran
29	SAF13	*C. sativus*	3X	Ferdows, Khorasan, Iran
30	SAF3	*C. sativus*	3X	Ferdows, Khorasan, Iran
31	SAF7	*C. sativus*	3X	Ferdows, Khorasan, Iran
32	SAF10	*C. sativus*	3X	Ferdows, Khorasan, Iran
33	SAF14	*C. sativus*	3X	Ferdows, Khorasan, Iran
34	SAF9	*C. sativus*	3X	Ferdows, Khorasan, Iran
35	SAF1	*C. sativus*	3X	Ferdows, Khorasan, Iran
36	SAF6	*C. sativus*	3X	Ferdows, Khorasan, Iran
37	SAF12	*C. sativus*	3X	Ferdows, Khorasan, Iran
38	SAF8	*C. sativus*	3X	Ferdows, Khorasan, Iran
39	SAF4	*C. sativus*	3X	Ferdows, Khorasan, Iran
40	SPSAD2	*C. specious*	2X	Ali abad, Gorgan, Iran
41	SPSAD1	*C. specious*	2X	Ali abad, Gorgan, Iran
42	SPSAD3	*C. specious*	2X	Ali abad, Gorgan, Iran
43	SPSAD5	*C. specious*	2X	Ali abad, Gorgan, Iran
44	HASVL5	*C. haussknechtii*	2X	Veresk, Mazandaran, Iran
45	HASVL1	*C. haussknechtii*	2X	Veresk, Mazandaran, Iran
46	HASVL2	*C. haussknechtii*	2X	Veresk, Mazandaran, Iran
47	HASK2	*C. haussknechtii*	2X	Kali abad,Golestan, Iran
48	HASK3	*C. haussknechtii*	2X	Kali abad,Golestan, Iran
49	HASK1	*C. haussknechtii*	2X	Kali abad, Golestan, Iran
50	HASK5	*C. haussknechtii*	2X	Kali abad, Golestan, Iran
51	HASVL3	*C. haussknechtii*	2X	Kali abad, Golestan, Iran
52	CACN5	*C. caspius*	2X	Neka, Mazandaran, Iran
53	CACN3	*C. caspius*	2X	Neka, Mazandaran, Iran
54	CACN6	*C. caspius*	2X	Neka, Mazandaran, Iran
55	CACN1	*C. caspius*	2X	Neka, Mazandaran, Iran
56	CACN2	*C. caspius*	2X	Neka, Mazandaran, Iran
57	CNCR33	*C. cancellatus*	2X	Reno, Mazandaran, Iran
58	CNCR5	*C. cancellatus*	2X	Reno, Mazandaran, Iran
59	CNCR2	*C. cancellatus*	2X	Reno, Mazandaran, Iran
60	CNCR4	*C. cancellatus*	2X	Reno, Mazandaran, Iran
61	HAHR1	*C. haussknechtii*	2X	Reno, Mazandaran, Iran
62	HAHR2	*C. haussknechtii*	2X	Reno, Mazandaran, Iran
63	HAHR3	*C. haussknechtii*	2X	Reno, Mazandaran, Iran
64	CAHI4	*C. caspius*	2X	Eslam abad, Mazandaran, Iran
65	CAHI5	*C. caspius*	2X	Eslam abad, Mazandaran, Iran
66	CAHI2	*C. caspius*	2X	Eslam abad, Mazandaran, Iran
67	CAHI1	*C. caspius*	2X	Eslamabad,Mazandaran, Iran

Genetic relationships among Crocus genotypes

Principal coordinate analysis (PCoA) based on a genetic similarity matrix was used to demonstrate the genetic relationships among accessions. The first three eigenvectors explained 55.2% of the total molecular variation, which accounted for 32.88%,

Table 2. Parameters obtained using 12 polymorphic SSR markers.

Marker	Alleles No.	Chi square P-value	H_e	H_o	F	PIC
CSMIC14	3	0.00	0.4600	0.24	0.48	0.41
CSMIC23	2	0.14*	0.2500	0.30	0.23	0.22
CSMIC25	2	0.00	0.4500	0.71	0.34	0.35
CSMIC36	2	0.00	0.4959	0.66	-0.33	0.37
CSMIC44	2	0.00	0.4100	0.59	0.45	0.32
CSMIC46	2	0.19*	0.4775	0.69	-0.43	0.36
CSMIC47	2	0.14*	0.2500	0.30	0.65	0.22
CSMIC50	2	0.00	0.5000	0.59	-0.15	0.37
CSMIC53	2	0.00	0.3900	0.54	0.22	0.31
CSMIC55	2	0.00	0.3750	0.01	0.95	0.30
CSMIC59	2	0.00	0.5000	0.75	-0.53	0.37
CSMIC62	2	0.00	0.4559	0.27	0.33	0.40
Mean	2.16		0.4100	0.47		0.33

13.07%, and 9.25%, respectively, of the observed variation (Fig. 1). PCoA revealed a pattern in which the accessions were attributed to nine groups.

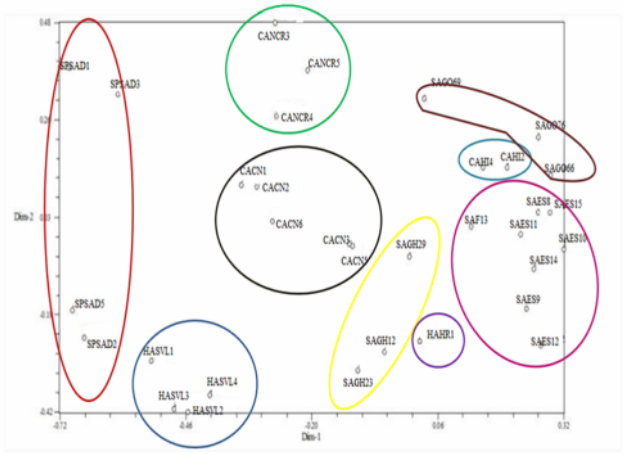

Fig. 1. Relationships among *Crocus* genotypes visualized by principal coordinate analysis (PCoA).

Assessment of genetic variation

Analysis of molecular variance (AMOVA) ($P< 0.05$) for 1000 permutations for all population comparisons was conducted to partition the genetic variation among and within groups. In the present study, the results of AMOVA demonstrated that the greatest variation can be attributed to within populations (75.68%) (Table 3). In this study, we found that the lower level of genetic variation among populations within species (8.69%) in comparison with that of within populations may be caused by the small population size in wild species. Different genetic variations among and within species and populations are summarized in Table 3.

A dendrogram constructed based on the neighbor-joining algorithm using Power Marker revealed a pattern in which the genotypes were assigned to two main clusters (groups A and B) (Fig. 2). In this grouping, one group included all the cultivated genotypes, and the other included the wild ones. Our results are in agreement with those obtained by Beiki *et al*. (2010), who differentiated cultivated genotypes from wild ones using 14 polymorphic RAPD primers.

Group B consisted of *C. haussknechtii* from

Table 3. Analysis of molecular variance (AMOVA) in 66 saffron genotypes based on 12 polymorphic SSR markers.

S. O. V.	df	SS	MS	% of variance	F-statistic
Among species	4	19.040	0.15638	15.63	FCT=0.15
Among populations Within species	6	11.333	0.08698	8.69	FSC=0.10
Within population	121	91.620	0.75719	75.68	FST=0.24
Total	131	121.992	1.00054		

Reno, Veresk, and Eslamabad, *C. cancellatus* from Reno, *C. speciosus*from Ali abad and Kali abad, and *C. caspius* from Neka. Each species was completely differentiated from the others. *Crocus sativus* accessions from different geographical regions were included in group A. Unlike previous researchers (Rubio-Moraga *et al*., 2009; Alavi-Kia *et al*., 2008) who have reported there is no genetic variation within *C. sativus*, microsatellites indicated the genetic diversity among *C. sativus* belonged to different regions of Iran. Based on genetic similarity, the cultivated genotypes may have originated from the common parents. Spontaneous mutation and artificial selection may have led to existing cultivars (Afzal *et al*., 2004).

Utilizing principal coordinate analysis, all species were separated into nine groups, except *C. sativus*from Ferdows and *C. sativus* from Estahban. In this grouping, *C. sativus* was assigned to three groups. Considering the constructed dendrogram for different species from various geographical regions (Fig.2), results of the PCoA (Fig.1) and of the AMOVA (Table 3), the existence of genetic diversity among *C. sativus* was observed. Results showed that there are significant differences among *C. sativus* from Gonabad, Ghaen, and Ferdows due

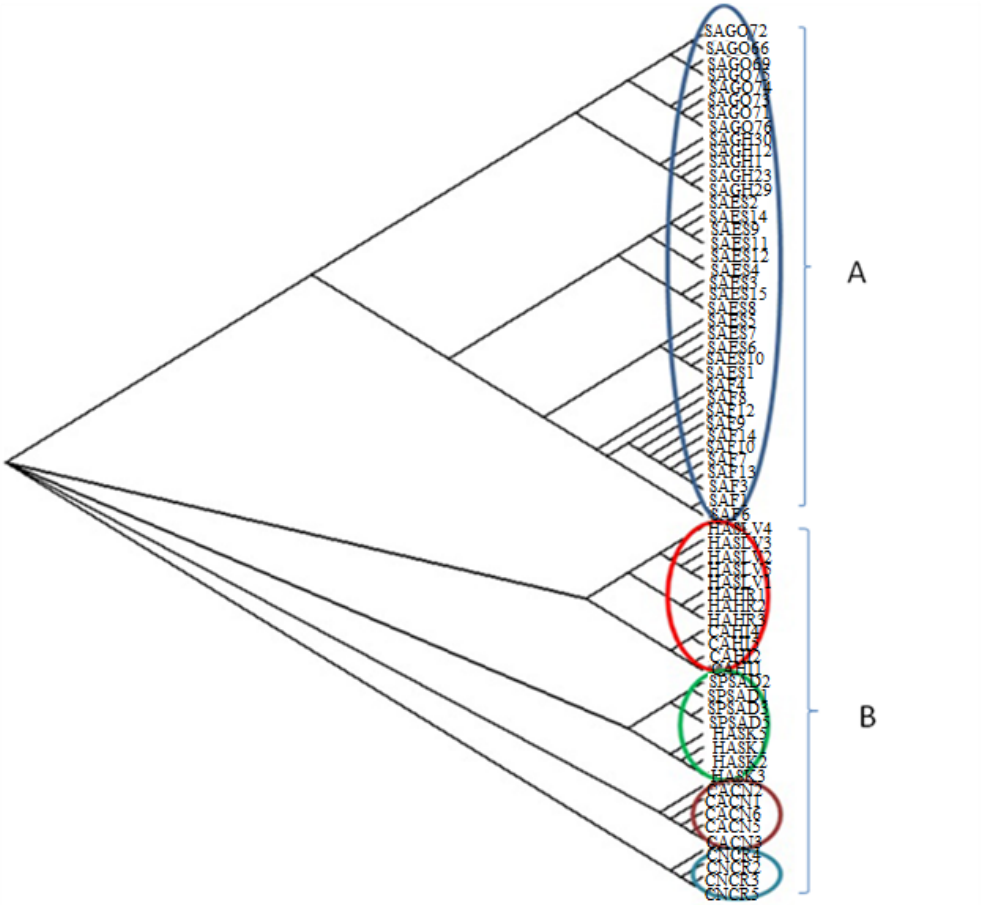

Fig. 2. Dendrogram constructed using neighbor-joining analysis based on Nei's genetic distance index for various *Crocus* spp. Note: Group A includes *Crocus sativus* (cultivated genotypes) and group B includes *C. haussknechtii* from different geographic regions, plus *C. cancellatus, C. speciosus* from different geographic regions, and *C. caspius* (wild species).

to their location in separate clusters. Despite the geographical distance between Ferdows and Estahban, genotypes from these locations were grouped in same cluster. This was further confirmed by the genetic distance (0.0311) between those genotypes, which was the smallest one. Regarding the fixation index (F_{ST}=0.33, P>0.05) between these two groups, it seems that the accessions from Estahban may have originated from Ferdows genotypes (Table 4).

Table 4. Distance between cultivated *Crocus* based on the pairwise method in lower diagonal F_{ST} P values in upper diagonal (P>0.05).

	C. sativus Gonabad	*C. sativus* Ghaen	*C. sativus* Ferdows	*C. sativus* Estahban
***C. sativus* Gonabad**	**0**	**0.0000+-0.0000**	**0.00000+-0.0000**	**0.00195+-0.0014**
***C. sativus* Ghaen**	**0.34**	**0**	**0.00000+-0.0000**	**0.00000+-0.0000**
***C. sativus* Ferdows**	**0.06**	**0.30**	**0**	**0.33203+-0.0156**
***C. sativus* Estahban**	**0.053**	**0.24**	**-0.02**	**0**

This finding—differentiation among cultivated *Crocus*—is not in accordance with previous resultsreported by Rubio-Moraga *et al*. (2009). Using 30 RAPD, 48 ISSR, and 15 microsatellite primers, all *C. sativus* accessions revealed the same pattern bands. These previous results led to classifying saffron as a monomorphic species (Alavi-Kia *et al*., 2008; Rubio-Moraga *et al*., 2009), which is not in accordance with our results. Like other researchers (Beiki *et al*. 2010), we also found genetic diversity among various cultivated species of Iran, however, robustness of microsatellite was obvious as compared with RAPD which did not show the relationship between Estahban and Ferdows genotypes. All Estahban genotypes were taken from Khorasan and were planted in Estahban where they turned intolocal population.

Generally, compared with other important plants, the variability of saffron started to decrease rapidly due to artificial selection by humans over a long period of time and due to the asexual propagation of the species.

Based on the results of this study, *C. haussknchii*is the closest wild species to cultivated ones. It has been reported that *C. cartwrightianus* and *C. thomassi* are more similar to *C. sativus* both morphologically and cytogenetically. In addition, dominant molecular markers (AFLP, RAPD) were used to confirm this close relationship (Moretzsohn *et al*., 2004, Zubor *et al*., 2004); however, another study indicated that *C. almehensis* and *C. mickelsonii* are more closely related to saffron (Alavi-Kia *et al.* 2008). Today, despite sufficient morphological and cytogenetical characters, problems are encountered when attempting to categorize *Crocus* species. Identifying the relationship among different *Crocus* species poses a real challenge due to the lack of suitable collection and adequate molecular characterization.

In this study, the smallest and the greatest genetic distance were shown to exist between *C. haussknchii* and *C. sativus* (0.09), and between *C. haussknchii* and *C. specious* (0.66), respectively. Saffron was introduced as an alloploid plant (Beiki *et al*., 2010), and it seems *C. haussknchii* may be one of its wild ancestors. Bagheri and Vesal (2002) introduced *C. haussknechtii* as the closest wild relative of saffron (*C. sativus*) based on morphological characteristics.We suggest that more powerful molecular markers should be applied to gain a better understanding of saffron categorization.

CONCLUSIONS

In this study, microsatellites revealed high genetic variation among and within saffron species, especially among *Crocus sativus*. Despite having different ecotypes, each species was differentiated, revealing that *C. haussknchii* may be one of saffron's ancestors. Further research of the genetic diversity and population structure of Iranian saffron using SSR markers is needed to identify the origin and wild relatives of this valuable species. Also, it would be useful to breeders to determine how genetic diversity, as analyzed with molecular markers, relates to phenotypic variability and, more importantly, how it reflects the variability of important traits.

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