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Species delimitation and relationship in *Crocus* L. (Iridaceae)

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Abstract – The genus *Crocus* L. (Iridaceae) is monophyletic and contains about 100 species throughout the world. *Crocus* species have horticultural, medicinal and pharmacological importance. Saffron is the dried styles of *C. sativus* and is one of the world's most expensive spices by weight. Controversy exits about the taxonomy of the genus and the species relationship. Exploring genetic diversity and inter-specific cross-ability are important tasks for conservation of wild taxa and for breeding of cultivated *C. sativus*. The present study was performed to study genetic variability and population structure in five *Crocus* L. species including *Crocus almehensis* Brickell & Mathew, *C. caspius* Fischer & Meyer, *C. speciosus* Marschall von Biberstein, *C. haussknechtii* Boissier, and *C. sativus* L. by inter simple sequence repeat (ISSR) molecular markers. We also used published internal transcribed spacer (ITS) sequences to study species relationship and compare the results with ISSR data. The results revealed a high degree of genetic variability both within and among the studied species. Neighbor joining (NJ) tree and network analysis revealed that ISSR markers are useful in *Crocus* species delimitation. Population fragmentation occurred in *C. caspius* and *C. sativus*. Both ISSR and sequenced based analyses separated *C. sativus* from the other studied species. Close genetic affinity of *C. sativus* and *C. pallisii* and inter-specific gene flow was supported by both data sets.

Key words: *Crocus*, gene flow, ISSR, ITS

Introduction

The genus *Crocus* L. (Iridaceae) is monophyletic and consists of about 100 recognized species (Petersen et al. 2008). These taxa occur from Western Europe and northwestern Africa to Western China with the center of species diversity in Asia Minor and on the Balkan Peninsula (Harpke et al. 2003). The genus is well characterized and morphologically distinct but karyologically very heterogeneous. The genus is of ecological, horticultural, culinary and pharmacological importance (Sik et al. 2008). Saffron is the dried styles of *C. sativus* and is one of the world's most expensive spices by weight. Moreover, the styles of *C. sativus* and some other *Crocus* species contain carotenoids that inhibit cancer cell proliferation (Chryssanthi et al. 2007).

Crocus L. is a genus of perennial geophytes. The floral and foliar organs are completely formed before the summer drought when they enter dormancy and wait for moisture from autumn rains and melting snow. The flowers are cross-pollinated by e.g. bees, bumble-bees or moths (Jensen and Jacobsen 2003).

Nine wild species of *Crocus* L. have been described from Persia and some adjacent areas (Idem and Mathew 1975, Wendelbo 1977, Matine 1978). *Crocus* taxonomy is controversial and has been based primarily on morphology, as well as chromosome number. The genus *Crocus* is divided into two subgenera: subgenus *Crociris* containing *C. banaticus* and subgenus *Crocus* comprising the remaining species. The subgenus *Crocus* is further divided into two sections: section *Crocus* and section *Nudiscapus*. However, the lack of clear distinctive characters, the wide range of habitats and the heterogeneity of the morphological traits and cytological data make the taxonomy of *Crocus* difficult (Norbak 2002). The *Crocus* species grow in various geographical regions and therefore face different environmental and ecological conditions. Adaptation of plant taxa to such a dispersed distribu-

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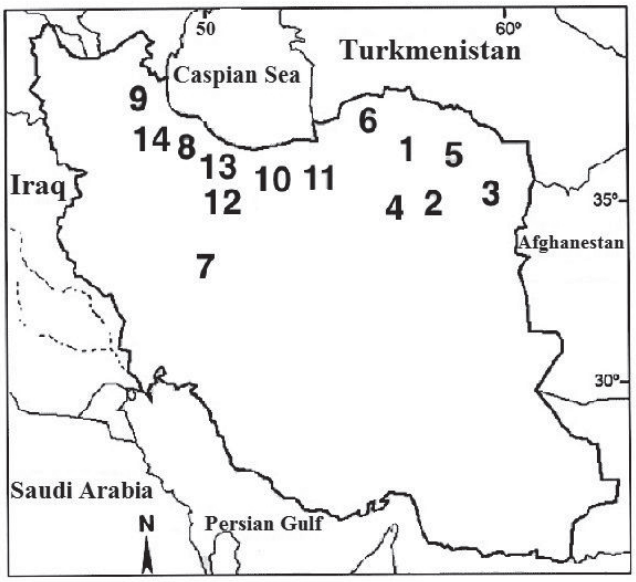

Fig. 1. Distribution map of the studied *Crocus* populations. Populations are marked with numbers from 1-14 according to the Tab. 1.

tion usually is accompanied by extensive genetic variability that can be present either in the form of allelic variability or allelic uniqueness of some populations (Petit et al. 1998).

There have been several attempts to study *Crocus* taxa by molecular markers revealing species genetic variability (see for example, Caiola et al. 2004, Alavi-kia et al. 2008, Sik et al. 2008, Beiki et al. 2013). However, none was aimed at studying population genetic structure and gene flow among the species and populations. Exploring the genetic structure and genetic variability of the wild relatives of cultivated plants is important for breeding purpose. Tracing the useful traits among wild plants can broaden the gene pool available for human use. These traits and genes can be incorporated in the crop plant through hybridization. The genus *Crocus* contains out-crossing species and most of its species are cross-compatible even with *C. sativus*. Thus, studying the genetic diversity and population fragmentation in the genus is an important task for future breeding of this important crop plant (Larsen 2011). The objectives of the present study are: i) to explore genetic variability within and among the species, ii) to identify population fragmentation and gene flow among these species, and iii) to reveal the species relationship.

Neutral molecular markers have been used extensively for species delimitation and genetic diversity analyses (see for example, Sheidai et al. 2013, 2014). These molecular markers are extensively used in population genetics analyses that can shed light on different levels of genetic variation and the partitioning of variability within/among populations. It can also identify inbreeding as well as selfing versus outcrossing, effective population size and population bottleneck. These analyses may be of help in planning effective management strategies for endangered and/or invasive species (Chen 2000).

At present no investigation into population genetic structure, gene flow or genetic fragmentation of *Crocus* species in Iran has been reported. We have carried out population genetic analysis of 14 populations of five *Crocus* species for the first time in the country. We used Inter-simple sequence repeat (ISSR) markers to study genetic diversity since this marker is reproducible, cheap and easy to work with (Sheidai et al. 2013, 2014). Moreover, recent studies have shown that arbitrary amplified dominant markers like ISSRs can solve phylogenetic relationships of closely related recently radiated taxa at low taxonomic levels (Poczai 2011). We used ITS sequences of the NCBI to build up a phylogenetic tree of *Crocus* species growing in Iran and compare the result with the ISSR tree obtained.

Materials and methods

Plant material

Ninety plant specimens were collected from 14 populations of five *Crocus* L. species. The species studied are: 1- *Crocus almehensis* Brickell & Mathew, 2- *C. caspius* Fischer & Meyer, 3- *C. speciosus* Marschall von Biberstein, 4- *C. haussknechtii* Boissier, and 5- *C. sativus*L. (= *C. officinalis* Pers.). Details of the studied populations are provided in Table 1. Plants of each species are marked with numbers: *C. sativus* = 1–26, *C. almehensis* = 27–33, *C. haussknechtii* = 34–41, *C. caspicus* = 42–83, *C. speciosus* = 84–90.

DNA extraction and ISSR assay

Fresh leaves were collected randomly in each of the studied populations and dried in silica gel powder. Genomic DNA was extracted using CTAB protocol with activated charcoal (Krizman et al. 2006). The quality of extracted DNA was examined by running it on 0.8% agarose gel. Ten ISSR primers; (AGC)5GT, (CA)7GT, (AGC)5GG, UBC810, (CA)7AT, (GA)9C, UBC807, UBC811, (GA)9A and (GT)7CA commercialized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25 μL volume containing 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 0.2 μM of a single primer, 20 ng genomic DNA and 1 U of Taq DNA polymerase (Bioron, Germany). The amplifications' reactions were performed in Technethermocycler (Germany) with the following program: 5 min initial denaturation step at 94 °C, 30 s at 94 °C; 1 min at 50 °C and 1 min at 72 °C. The reaction was completed by a final extension step of 7 min at 72 °C. The amplification products were visualized by running them on 2% agarose gel, followed by the ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

ISSR bands obtained were coded as binary characters (presence = 1, absence = 0). The following genetic diversity parameters were determined in each population: percentage of allelic polymorphism, allele diversity (Weising 2005), Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Freeland et al. 2011).

Tab. 1. *Crocus* species and populations, their locality and voucher number.

Species	Province	Locality/Population (No. sample)	Altitude (m)	Longitude	Latitude	Voucher No.
C. sativus	Khorasan-Razavi	Jovein/Pop1 (1-7)	1100	36.42	57.25	2014600
C. sativus	Khorasan-Razavi	Kashmar/Pop2 (8-11)	1603	35.14	58.28	2014601
C. sativus	Khorasan-Razavi	Torbate-Heydariyyeh/Pop3 (12-15)	1450	35.1	59	2014602
C. sativus	Khorasan-Razavi	Bardaskan/Pop4 (16-19)	1370	33.25	59.42	2014603
C. sativus	Khorasan-Razavi	Tabas village/Pop5 (20-26)	1500	36.24	51.42	2014604
C. almehenis	Golestan	Almeh mountain/Pop6 (27-33)	2165	37.22	56.38	2014605
C. haussknechtti	Markazi	Shazand/Pop7 (34-41)	1900	33.55	49.24	2014606
C. caspius	Gilan	Rudbar-Reshtehrud/Pop8 (42-48)	320	37	49.27	2014607
C. caspius	Gilan	Rezvanshahr/Pop9 (49-55)	1200	37.32	48.49	2014608
C. caspius	Mazandaran	Sari, Agriculture university/Pop10 (56-62)	30	36.34	53.11	2014609
C. caspius	Mazandaran	Sari, Jamkhanehvillage/Pop11 (63-69)	64	36.35	53.14	20146010
C. caspius	Mazandaran	Ghaemshahr/Pop12 (70-76)	55	36.27	52.51	20146011
C. caspius	Rostamabad	Gilan/Pop13 (77-83)	2500	36.53	49.14	20146012
C. speciosus	Gilan	Rostam-abad/Pop14 (84-90)	2500	36.53	49.14	20146013

ITS sequences

ITS sequence data were obtained from NCBI for representative *Crocus* species growing in Iran. The species and their accession numbers are: *Crocus biflorus* subsp. *alborcoronatus* (LT222365), *C. almehensis* (HE801162), *C. cancellatus* subsp. *pamphylicus* (LM993449), *C. speciosus* subsp. *ilgazensis* (HE801120), *C. caspius* (LT222445), *C. gilanicus* (HE801172), *C. sativus* (DQ094185) and *C. pallasii* (HE664002).

Data analysis

Genetic diversity and population structure

ISSR bands obtained were scored as binary characters. Genetic diversity parameters were determined in each population. These parameters were Nei's gene diversity (H), Shannon information index (I), number of effective alleles, and percentage of polymorphism (Weising 2005, Freeland et al. 2011). Nei's genetic distance was determined among the studied populations and used for clustering (Weising 2005, Freeland et al. 2011).

For grouping of the plant specimens, Neighbor Joining (NJ) clustering and NeighborNet methods of networking were performed after bootstrapping 100 times (Freeland et al. 2011, Huson and Bryant 2006).

The Mantel test was performed to check the correlation between geographical distance and the genetic distance of the studied species (Podani 2000). PAST ver. 2.17 (Hamer et al. 2012), DARwin ver. 5 (2012) and SplitsTree4 V4.13.1 (2013) programs were used for these analyses.

Significant genetic difference among the studied populations and provinces were determined by AMOVA (analysis of molecular variance) test (with 1000 permutations) by using GenAlex 6.4 (Peakall and Smouse 2006), and Nei's Gst analysis of GenoDive ver.2 (2013) (Meirmans and Van Tienderen 2004). The population genetic differentiation was studied by Gst_est (standardized measure of genetic differentiation) (Hedrick 2005), and D_est (Jost measure of differentiation (Jost 2008). In order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program, Hickory (ver. 1.0) (Holsinger et al. 2003) was used to estimate parameters related to genetic structure (theta B value).

The genetic structure of populations was studied in two different approaches. Firstly with the use of the Bayesian based model STRUCTURE analysis (Pritchard et al. 2000), and secondly by the maximum likelihood-based method of K-Means clustering.

For STRUCTURE analysis, data were scored as dominant markers (Falush et al. 2007). The Evanno test was performed on the STRUCTURE result to find the proper number of K by using delta K value (Evanno et al. 2005). We performed K-means clustering as done in GenoDive ver. 2. (2013). Two summary statistics, pseudo-F, and Bayesian information criterion (BIC), provide the best fit for K-means clustering (Meirmans 2012).

Gene flow

Gene flow was determined by two different approaches. First was the calculation of Nm, an estimate of gene flow from Gst by PopGen ver. 1.32 (1997) as: Nm = 0.5 (1 – Gst)/ coefficient of gene differentiation (Gst). This approach considers equal amounts of gene flow among all populations (which may not be correct for all situations), Secondly, a population assignment test based on maximum likelihood (ML) as performed in GenoDive ver. 2. (2013). The latter approach does not consider equal amounts of gene flow among the studied populations.

Recently, Frichot et al. (2013) introduced the statistical model called latent factor mixed models (LFMM), which

tests correlations between environmental and genetic variations while estimating the effects of hidden factors that represent background residual levels of population structure. We used this method to check if ISSR markers showed correlation with the environmental features of the studied populations. The analysis was done with the LFMM program Version: 1.2 (2013).

Results

Genetic diversity

Genetic diversity parameters were first determined for the studied populations and then for the five studied species. Data with regard to genetic diversity in 14 studied populations are presented in Table 2. The highest values for an effective number of alleles occurred in population Pop13 (1.37), followed by Pop9 (1.32). The highest value of genetic diversity due to population (Hs) occurred in population Pop13 (0.25), followed by Pop10 (0.23).

The genetic diversity parameters in the species studied are presented in Table 3. The highest value of gene diversity (He) occurred in *C. saspicus* (0.23), while *C. haussknechtii* had the lowest value (0.10). The highest value of genetic polymorphism occurred in *C. caspicus* (92.45), while the lowest one occurred in *C. speciosus* (32.08).

Tab. 2. Genetic diversity parameters in 14 studied *Crocus* populations. Ae = effective number of alleles, Hs = genetic diversity due to population, and Ht = total genetic diversity.

Population	Sample No.	Ae	Hs	Hs/Ht
Pop1	10	1.196	0.128	0.05
Pop2	4	1.098	0.069	0.03
Pop3	4	1.223	0.164	0.07
Pop4	4	1.151	0.119	0.05
Pop5	4	1.158	0.123	0.05
Pop6	7	1.256	0.174	0.07
Pop7	8	1.201	0.142	0.06
Pop8	7	1.271	0.18	0.08
Pop9	7	1.326	0.226	0.1
Pop10	7	1.352	0.234	0.1
Pop11	7	1.185	0.128	0.05
Pop12	7	1.223	0.151	0.06
Pop13	7	1.374	0.253	0.11
Pop14	7	1.234	0.153	0.06

Tab. 3. Genetic diversity parameters in the studied *Crocus* species. N= number of populations, Na = number of alleles, Ne = number of effective alleles, I = Shanon information index, He = gene diversity, Uhe = unbiassed gene diversity, %P = percentage of polymorphism.

Species	N	Na	Ne	I	He	UHe	%P
C. sativus	26	1.245	1.277	0.268	0.171	0.175	62.26
C. almehensis	7	0.849	1.189	0.186	0.120	0.129	39.62
C. haussknechtii	8	0.830	1.171	0.165	0.105	0.113	37.74
C. caspicus	42	1.849	1.355	0.373	0.233	0.235	92.45
C. speciosus	7	0.679	1.209	0.177	0.120	0.129	32.08

Population genetic structure

AMOVA test produced significant genetic difference (PhiPT = 0.51, P = 0.010) among the studied populations. Moreover, Gst = 0.49 (P = 0.001), the Hedrick standardized fixation index (G'st = 0.59, P = 0.001) and the Jost differentiation index (D-est = 0.20, P = 0.001) revealed the genetic differentiation of the studied populations. Pair-wise Fst values determined among 14 studied populations revealed that all populations differed significantly due to their genetic differences.

Among species, AMOVA analysis produced significant difference among the studied species (PhiPT = 0.31, P = 0.010). It also revealed that genetic variability is higher within species (69%) than among species (31%). The Hickory test also produced a high Theta B value (0.4), supporting AMOVA.

Since neighbor joining (NJ) tree and Neighbor-net diagram of ISSR molecular markers produced similar results, only NJ is presented and discussed (Fig. 2). Plant specimens of each species were placed in separate clusters. This reveals that ISSR molecular markers can delimit the studied species. The NJ tree and Neighbor-net diagram also revealed within-species genetic variability (among populations genetic difference), as some of the populations in one species were located far from the other members of the same species. For example, populations of *S. caspius* were distributed in two major clusters. The NJ tree of ISSR data revealed close genetic affinity between *C. speciosus*, *C. almehensis* and *C. caspicus*.

K-Means clustering produced k = 2 according to pseudo-F value (13.94), which is in agreement with the number of major clusters produced by the NJ tree, while it produced k = 10 according to the BIC value (577.12). This is in agreement with the number of sub-clusters in the NJ tree. Moreover, the Evanno test produced the best k = 9 based on delta k. Therefore obtaining K = 9–10 indicates the population genetic fragmentation in *Crocus* species studied.

STRUCTURE plot (Fig. 3) based on k = 9, separated the five populations of *C. sativus* studied into two genetic groups. Populations Pop1 and Pop2, which formed the first genetic group, differed in their allele content from populations Pop3–5 of the second genetic group. All these populations are located in Khorasan-Razavi Province. Therefore, *C. sativus* showed population fragmentation in this province.

C. caspius (Pop8-13) also showed population fragmentation. These populations are located in the Gilan and Mazandaran provinces. Populations Pop8 and Pop9 of Gilan Province showed genetic similarity, but differed in their allele composition from the other populations. The populations Pop10-13 of Mazandaran Province showed great allele diversity and differed significantly from each other.

The Mantel test performed after 5000 permutations produced significant correlation (r = 0.41, P < 0.01) between ge-

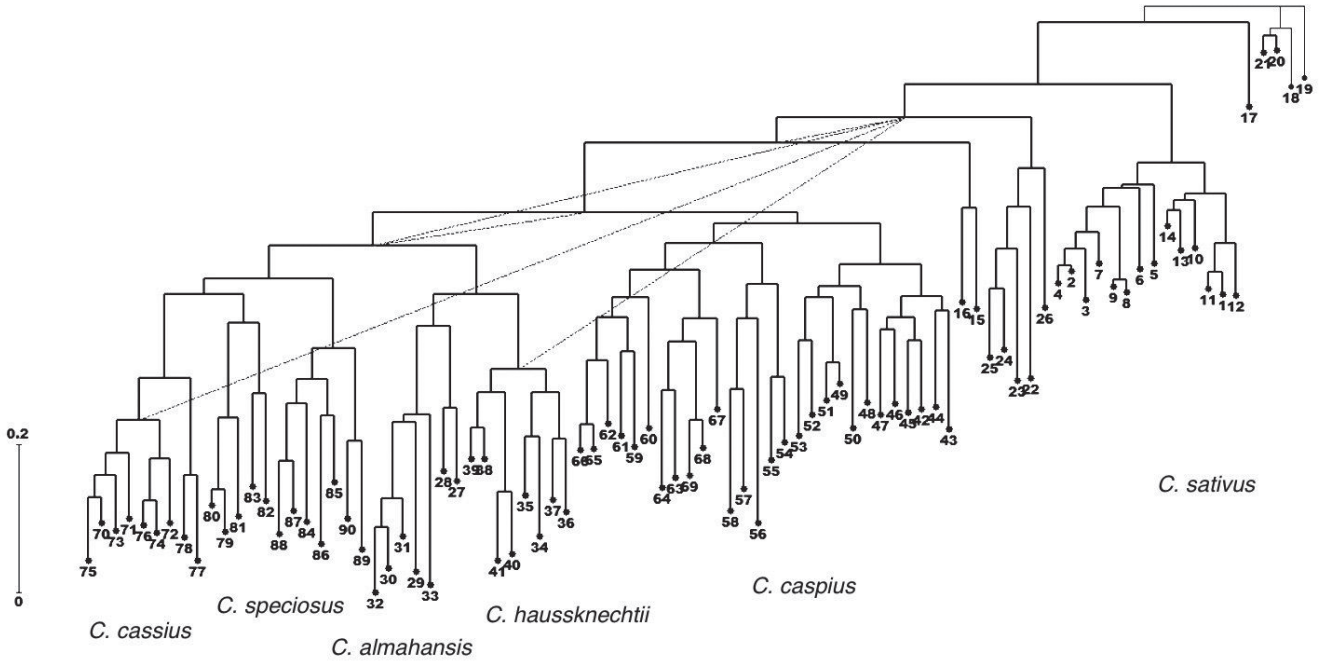

Fig. 2. Reticulogram of the *Crocus* species studied based on a neighbor joining tree of inter simple sequence repeat (ISSR) data. Populations are marked with numbers from 1–90 according to the Tab. 1.

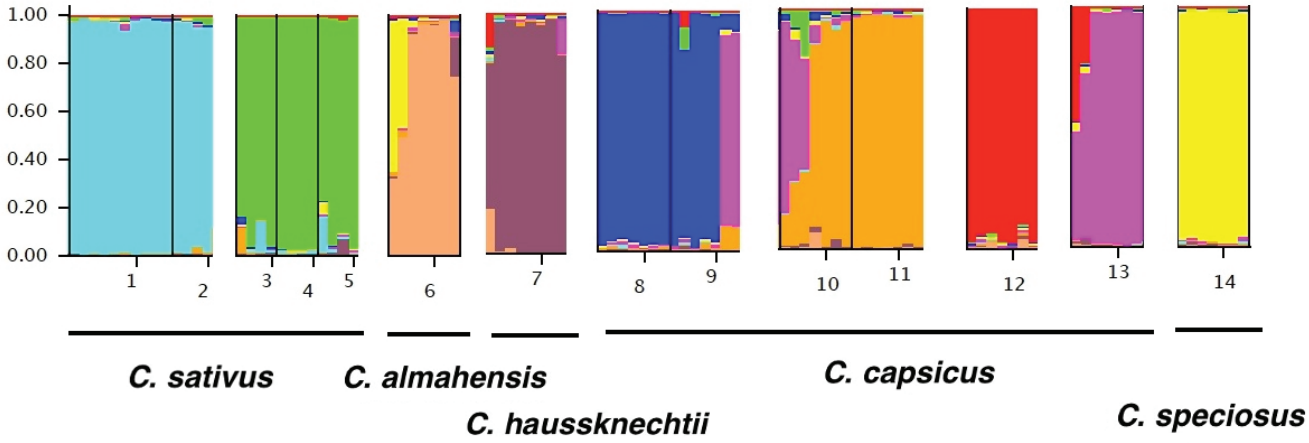

Fig. 3. STRUCTURE plot of *Crocus* species and populations based on inter simple sequence repeat (ISSR) data. Populations are marked with numbers from 1–14 according to the Tab. 1.

netic distance and geographical distance of the studied populations. Therefore, IBD (Isolation by distance) occurred in *Crocus* species.

Gene flow

The mean Nm = 1.03 was obtained for ISSR loci studied. This is a high value and indicates a high level of gene flow among the studied populations. Moreover, the STRUCTURE plot revealed some degree of genetic admixture in the studied species. For example, population Pop6 (*C. almehensis*) had some alleles from population Pop14 (*C. speciosus*). Similarly, populations Pop12 and Pop13 as well as Pop10 and Pop13 of *C. caspicus* had some shared alleles (similarly colored segments). The reticulograms of the species based on the NJ tree (Fig. 2) also showed some degree of gene flow among the studied species. It particularly revealed gene flow among *C. sativus* and the other *Crocus* species.

LFMM analysis revealed that some of the ISSR loci had -log10 (p-value)>1.50 (P<0.05). These loci are adaptive loci in the studied populations, such as ISSR loci 1, 2, 10, 19, 24, 25, and 29. Some of these loci are among highly shared loci (Nm >1), while some had lower Nm values. Therefore, both shared alleles and those that were confined to some geographical populations had adaptive value.

Species relationship based on ITS sequences

The model test analysis revealed that K2 + G (the Kimura two parameter model + Gamma) is the best model to fit ITS data in the studied species. The species relationship determined by ML, NJ, and maximum parsimony produced simi-

lar results. Therefore, the ML tree is presented and discussed here (Fig. 4). The ML tree produced two major clades. The first major clade consists of 5 species, *C. almahensis*, *C. biflorus*, *C. speciosus*, *C. cancellatus* and *C. caspius*. *C. almahensis* and *C. biflorus* showed a higher degree of genetic affinity and were placed close to each other. Similarly, *C. speciosus* and *C. cancellatus* were placed close to each other, while *C. caspius* joined the other species at a great distance. The second major cluster contained *C. gilanicus, C. pallasii* and *C. sativus*. The phylogenetic tree obtained on ITS was in agreement with the ISSR tree presented above. Both molecular analyses revealed close genetic affinity among *C. almahensis*, *C. speciosus*, and *C. caspius*. Similarly, both analyses revealed that *C. sativus* is genetically different from these three species and joins them at some distance. The reticulogram (Fig. 5.) obtained based on ITS was in agreement with ISSR reticulograms and revealed the occurrence of gene flow and/or ancestral shared alleles among the studied species.

Discussion

Exploring the wild relatives of cultivated plants is important in order to have better picture on the genetic variability of related plant taxa, thereby broadening the available gene pool for human needs. Usually, wild relatives of important crop plants may contain useful genes that can be introduced to the crop plant that has been under selection pressure for long time. Extensive selection and uniform breeding practice can lead to genetic erosion and lower genetic variability of crop plants (Poczai 2011). The present investigation revealed the presence of a high degree of genetic variability both within and among *Crocus* species. Due to the high crossability potential among *Crocus* species, there are possibilities of transferring useful genes in these taxa.

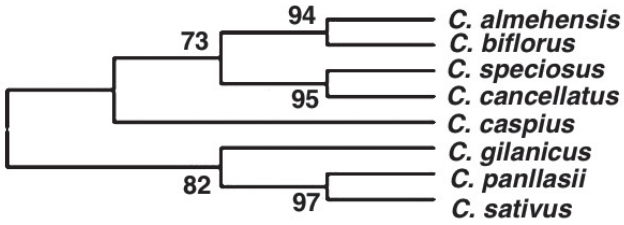

Fig. 4. Maximum likelihood phylogeny tree of *Crocus* species based on ITS sequences.

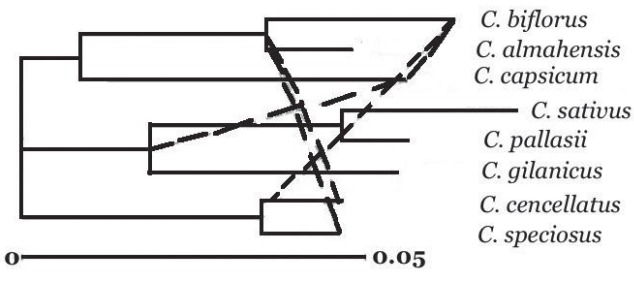

Fig. 5. Reticulogram of the *Crocus* species based on ITS sequences.

To recognize different gene pools in plant species, it is necessary to study the possible population genetic fragmentation due to habitat differences. The present study showed that some *Crocus* species do show genetic fragmentation of populations. The population fragmentation is connected with their geographical location. This was particularly true for the cultivated species *C. sativus* and for *C. caspius*. Genetic differences of geographical populations in *C. sativus* are particularly interesting as these plants can be selected for desirable agronomic traits and be used for further breeding purpose.

Larsen (2011) studied hybridization compatibility among species of subgenus *Crocus* series *Crocus sativus* by making 88 crosses. The results indicated that essentially all species of series *Crocus* can be hybridized. This occurs due to cross-pollination and self-incompatibility suppressing self-fertilization in these species (Chichiricco 1990, 1996). It is suggested that the considerable genetic resources found in different *Crocus* species could be used to genetically improve *C. sativus*. In fact, the hybrids obtained in the genus *Crocus* show an increased frequency of unreduced gametes and it could be possible to use these hybrids in the production of future triploids (Øragaard et al. 1995.

Habitat fragmentation along with extensive use of plants by humans and deforestation result in reduced rate of gene flow among populations (Hou and Lou 2011). STRUCTURE analysis and K-Means clustering revealed population fragmentation in the studied *Crocus* species. However, these populations are not completely disconnected as the population assignment test, reticulation analysis and Nm estimation showed some degree of gene flow among them. Therefore, these populations obtain some degree of genetic variability. This situation is similar to a meta-population. A meta-population is an assemblage of local populations that usually are small and are linked by loose relationships, i.e., there is some gene flow among them. In such cases, gene flow among local populations could mitigate losses of genetic variation caused by genetic drift in local populations and thus save them from extinction via so-called "genetic rescue" (Richards 2000).

Genetic diversity is an important aspect of species genetic continuity. It could be used for study of plant adaptation potential that copes with environmental changes during a plant's life history (Çalişkan 2012, Sheidai et al. 2013, 2014). A high degree of within-population genetic diversity was observed in the studied populations (AMOVA test revealed that 69% of total genetic variability is present within a population and 31% was present among populations). Such a result could be related to the out-crossing nature of *Crocus* taxa (Larsen 2011).

The Mantel test revealed a pattern of isolation by distance across the distribution range of the studied *Crocus* populations. Therefore, gene flow is most likely to occur between neighboring populations and as a result, more closely located populations tend to be more genetically similar to one another (Hutchison and Templeton 1999). Larsen (2011) reported between-population genetic differentiation in *Crocus hadri-*

aticus of Mainland Greece and Peloponnese. He considered isolation by distance as the main reason for this genetic differentiation. He stated that individuals tended to cross with individuals from neighboring locations rather than with individuals from more distant sites. This results in adaption to local conditions. LFMM analysis of ISSR data in our study revealed that some of the genetic loci have an adaptive nature. Therefore, the combination of genetic divergence, limited gene flow and local adaptation has played a role in the diversification of *Crocus*. In conclusion the present study may provide some useful information about population genetic structure and genetic variability of *Crocus* species that may be used in the conservation of these important species and also for hybridization with cultivated *Crocus sativus*.

C. sativus has 2n = 3x = 24 chromosomes and morphologically belongs to Series *Crocus*. It is believed that the ancestors of the species should be searched between one or two diploid species of series *Crocus* with 2n = 16. Six species fulfill these criteria: *Crocus cartwrightianus*, *C. thomasii*, *C. hadriaticus*, *C. oreocreticus*, *C. matheweii*, and *C. pallasii*.

The ancestor of *C. sativus* is still in question. For example, based on morphological, cytological and molecular analyses Larsen (2011) considered *C. cartwrightianus* the most probable ancestor for *C. sativus*. Zubor et al. (2004) found that *C. sativus* shares a high number of morphological similarities with *C. cartwrightianus* and *C. thomasii*, while Frizzi et al. (2007) considered *C. cartwrightianus* the only ancestor of *C. sativus*. However, according to Caiola et al. (2004), *C. cartwrightianus*, *C. hadriaticus*, *C. asumaniae* and *C. pallasii* share most similar genetic fragments with *C. sativus*. Recently, Alsayied et al. (2015), using IRAP (inter-retroelement amplified polymorphism) molecular markers, investigated the genetic diversity and relationships of *Crocus sativus* and its relatives and suggested that the most likely ancestors of *Crocus sativus* are *C. cartwrightianus* and *C. pallasii* subsp. *pallasii* (or close relatives). This is in agreement with our result as *C. pallasi* (synonym *C. haussknechtii*), was closely related to *C. sativus* in both ISSR and ITS phylogenetic trees obtained.

We can conclude that ISSR markers seem to be useful in *Crocus* species delimitation and also in the study of species relationships. *Crocus sativus* and *C. caspicus* showed population fragmentation, while some degree of gene flow occurred among the studied species.

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