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Phylogenetic Assessment of Some Species of *Crocus* Genus Using DNA Barcoding

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ABSTRACT

DNA barcoding is a simple method for the identification of any species using a short genetic sequence from a standard genome section. The present study aimed at examining the nuclear and chloroplast diversity as well as the phylogenetic relationships of eight species of saffron including four spring-flowering and five autumn-flowering species from different parts of Iran, using the nuclear barcode and chloroplast genes to specify their evolutionary pathway and to examine their genetic affinity. First, PCR was performed using the primers designed based on the chloroplast barcodes of *mat*K, *trn*L, and *rbc*L, and nuclear barcode of ITS. Then, the PCR products were purified and sequenced. The results of the phylogenetic tree indicated that the examined saffron species of Iran were separated from each other based on the sequences of ITS, *mat*K, and *trn*L barcodes. In the *rbc*L barcode, the relationships of a number of species remained unresolved, and this genetic region could not appropriately discriminate the species. The ITS barcode was introduced as the best barcode due to its discriminatory power, a high number of SNPs, and its comprehensiveness in most species. Moreover, the *mat*K and *trn*L barcodes were identified as complementary barcodes. The use of the four genetic regions in comparison with their individual use has caused these species to be well separated. Overall, the obtained phylogram showed that the cultivated species were more similar to *C. pallasii* subsp. *haussknechtii* species.

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Introduction

Nowadays, marker-based and DNA-based methods are used to identify plants. In comparison with the classic methods based just on morphology (Mathew, 1982), DNA-based methods are cheap, fast, and highly accurate in species identification as well as biodiversity assessment (Hebert *et al*., 2003). The term DNA barcode sequence refers to a very short standard DNA sequence of a gene or a well-known intergenic region that can be used to identify which species the animal, plant, or mushroom belongs to. DNA barcoding can be considered as a novel and inspiring method in taxonomic research due to its high potential for the detection and identification of species (Valentini *et al*., 2009). There is a remarkable variation among plant species regarding the proposed sequences, as a result of which different regions of the chloroplast and nuclear DNA have been introduced as a standard sequence in plants (Chase and Fay, 2009). In 2009, the Consortium for the Barcoding of Life used the chloroplast genes to suggest the *rbc*L + *mat*K marker combination as a standard plant barcode, which has a desirable sequencing quality and high discriminatory power for plant species (Mahadani and Ghosh, 2013). The *rbc*L, *mat*K, and ITS barcodes have been examined by many researchers in the identification and documentation of plant diversity (Chen *et al.,* 2010). ITS region is one of the most widely used nuclear DNA sequences and is often suitable at lower taxonomic levels to reconstruct the phylogeny and identify the closely-related

species and sexes (Chase *et al*., 2005; Kress *et al.,* 2005; Yousefzadeh *et al*., 2012;). The *trn*L-F region is one of the important regions in the chloroplast genome, which has been used in many studies to examine the phylogenetic relationships among plant species (Yousefzadeh *et al*., 2014; 2019). Many studies on cyanobacteria, algae, and plants have been derived from the evaluation of their evolutionary history using the intron *trn*L (Borsch *et al*., 2003). Recent research on saffron samples obtained from different countries as well as Iran have confirmed their differences at the DNA level (Grilli Caiola *et al*., 2004; Siracusa *et al*., 2013). *Crocus sativus* L., commonly known as saffron*,* is a member of the family Iris (Iridaceae*)* and one of the most important genera of Iridaceae with more than 90 species (Mathew, 1982). Since ancient times, this plant has been cultivated in some parts of the world including Iran, India, Greece, Morocco, Spain, and Italy. Some historical evidence suggests that the native habitat of saffron is Iran, and nine various species of *Crocus* are native to Iran (Mathew, 1982), of which three species are spring-flowering and the other six species are autumn-flowering plants (Kafi, 2002).

The stigma of *Crocus sativus* contains glycoside-derived esters, which are responsible for its properties of aroma (safranal), flavor (picrocrocin), and color (crocin) (Siracusa *et al*., 2013). The *C. pallasii* subsp. *haussknechtii* species, with the vernacular name of "pišuk", is one of the most widely used volunteer plants in the western provinces of Iran including Kermanshah, Ilam, Lorestan, and Hamadan and can be harvested in the spring. Vavilov stated that the Middle East including Asia Minor, Turkistan, and Iran is the original source of saffron. Recent research has indicated that Greece is the first place where the domestication of saffron began, and the *C. Cartwrightianus* species has been introduced as the closest relative of saffron (Frello and Heslop-Harrison, 2000). Furthermore, *C. pallasii* species have been considered as the ancestors of saffron in various references (Brandizzi and Grilli Caiola, 1998; Zubor *et al*., 2004). Before starting and designing a saffron breeding program, it is essential to gain thorough information about its ancestors. As Iran has a very rich germplasm of plant species and is one of the most significant regions for plant distribution, especially the genus *Crocus*, and given that the true ancestor of saffron is one of the mysteries that are still not precisely resolved, the present study aimed at identifying the evolutionary pathways of the cultivated saffron and specifying its genetic affinity by examining its chloroplast diversity and phylogenetic relationships and comparing the obtained results with those of the wild species using the DNA barcoding method.

Materials and Methods

Sample collection

Samples of wild species of saffron were collected from the selected regions mentioned in the flora of Iran in winter and spring (Mazhari, 2000) Leaf samples were frozen in the nitrogen liquid and stored in a freezer at -80°C before performing DNA extraction (Table 1).

Table 1. Samples collection sites.

Species	province	locality	Altitude (m)	longitude (m)	latitude (m)
C. almehensis	Golestan	Alme	2165	56.38	37.22
C. pallasii subsp. *hausskenechtii*	Kordestan	Ghorve	1900	49.24	33.55
C. caspius	Gilan	Talesh	1200	48.49	37.22
C. speciosus	Alborz	Karaj	1261	50.56	35.46
C. michelsonii	North Khorasan	North of Ghuchan	1947	58.2	37.22
C. cancellatus	Markazi	Shazand	1700	48.55	33.25
C. biflorous	Kordestan	Sanandaj	1894	46.48	35.46

DNA extraction

Leaf samples were extracted using the GeneAll kit (South Korea) according to the manufacturer's instructions. Then, the quantity and quality of genomic DNA were evaluated using agarose gel electrophoresis and NanoDrop. The GeneAll kit, the ComboGp-50p model, was used for DNA purification.

PCR and gel electrophoresis

In the present study, the sequence of the ITS region of the nuclear genome and the sequences of the chloroplast *mat*K, *rbc*L, and *trn*L were used to evaluate the phylogenetic of the examined taxa (Table 2). The polymerase chain reaction (PCR) was performed using the Bio-Rad thermocycler (at a 30 µl volume including 15 µM Master Mix, 1.5 µL Primer, and genomic 200-50 ng). The thermal cycles consisted of an initial stage of priming at 95 ° C for 5 minutes, 35 cycles at 95°C for 45 seconds, 65-68°C (depending on the different primers) for 40 seconds, and 72°C for 2 minutes. At the end of the reaction, a final expansion step was performed at 72°C for 10 minutes. The products of the polymerase chain reaction were loaded onto a 1% agarose gel and electrophoresed at 85 volts. The results of the PCR amplification of these regions were sent to Macro Corporation of Korea to perform sequencing.

Table 2. Characteristics of primers used

Gene name	Primer name	Primer sequence	Amplicon size (bp)	Reference
ITS	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	500-700	White *et al.*, 1990
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′		White *et al.*,1990
*mat*K	*mat*K-F	5′-CGATCTATTCATTCAATATTTC-3′	900-1000	Cuenoud *et al.*, 2002
	*mat*K-R	5′-TCTAGCACACGAAAGTCGAAGT-3′		Cuenoud *et al.*, 2002
*rbc*L	*rbc*L-F	5′-ATGTCACCACAAACAGAGACTAAGC-3′	700-800	Levin *et al.*, 2003
	*rbc*L-R	5′-GTAAAATCAAGTCCACCRCG-3′		Kress and Erichson, 2009
*trn*L	*trn*L-F	5′-GGAAATCGGTAGACGCTACG-3′	900-1000	Gismondi et al. 2013
	*trn*L-R	5′-ATTTGAACTGGTGACACGAG-3′		Gismondi et al. 2013

Data analysis

The entire sequences of PCR products were edited by BioEdit v7.2.6, and a combination of all the analyzed barcodes (ITS + *mat*K + *rbc*L + *trn*L) was aligned using the ClustalW2$_{ver2.1}$ software. The final phylogenetic analysis was performed by applying the maximum parsimony and the maximum likelihood methods to analyze the phylogenetic relationships based on the data aligned using the Mrbayes $_{v3.2}$ software. In order to evaluate the validity of branches, the bootstrap method (Felsentein, 1985) was used with a thousand replications. Specification of the percentage of the nucleotide sequence identity with a National Center for Biotechnology Information Database (NCBI) was performed using the Local Alignment Search Tool (Blast).

Results

Examination of the molecular data

Data obtained from ITS

The analysis of data related to the sequence of nrDNA ITS using the Bayesian method constructed one phylogenetic tree with two main clades. In the first clade, *C. michelsonii* with the same species in the NCBI makes a sister group strongly supported (100%) and is placed as the sister to large sub-clade of series *Crociris* (*C. bannaticus* its sole member), *Verni*, *Versicolores,* and *Crocus.* The *C. pallasii* subsp. *haussknechtii* and cultivated *C. sativus* species, with a stability of 100, occurred in one sister group with a large evolutionary distance from other species. Furthermore, the mentioned result was confirmed by the size of the branch length created in the phylogenetic tree. The rest of the species were also found in the other branches, in which *C. caspius* with the same species in the NCBI were found in one sister group with the stability of 100. Moreover, *C. almehensis* and *C. biflorus* species from series *Biflori* were isolated from the sister species of *C. cancellatus* and *C. gargaricus* from series *Reticulati* with the stability of 88 (Fig. 1). The last clade is consisting of the remaining species not mentioned yet that within *C. speciosus* is in sister group with *C. pulchellus* in series *Speciosi*. This clade is again in sister group with *C. almehensis* from series *Biflori* and *C. cancellatus* from series *Reticulati*. The unresolved position of these three series has been reported before (Peterson *et al*., 2008).

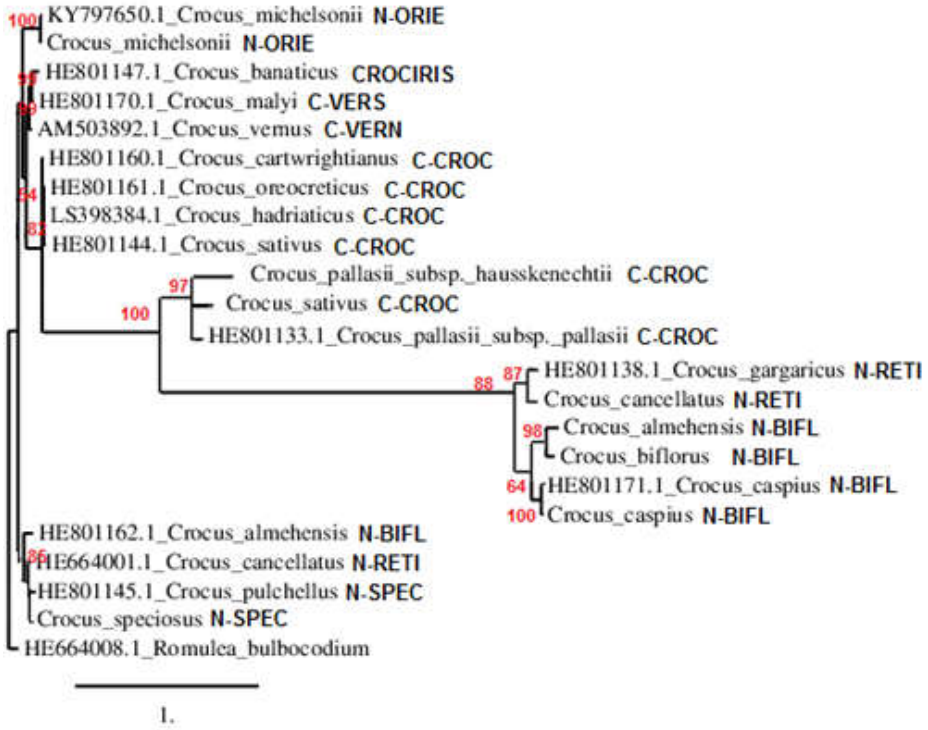

Fig. 1. The phylogenic tree derived from the analysis of ITS region data by the Bayesian method, the number above each branch shows the bootstrap value

matK gene sequence analysis

The phylogenetic tree obtained from this analysis had a large trichotomy consist species belonged to section *Crocus* series *Crocus* followed by three series of *Reticulati*, *Biflori* and *Speciosi* together and the last clade consists of the only member of section *Crociris* (*C. bannaticus*) which is in sister group with the *C. malyi* and *C. vernus* with the stability of 100. In the sub-branch of this clade, *C. michelsoni* embedded in the sister group with *C. caspius*. The position of these species in the sub-branches was identical to the position provided by the ITS data analysis. The *C. pallasii* subsp.-*haussknechtii* species had the highest similarity with the *C. sativus* from NCBI and formed a sister group. (Fig. 2). Zarini (2013) examined the genotypes of the cultivated and wild saffron species using the protein band patterns and found that *C. michelsonii* species had the minimum similarity with the cultivated genotypes. In the mentioned study, none of the three wild species including *C. speciosus*, *C. cancellatus*, and *C. michelsonii* indicated any significant similarity with the crop species, which confirmed the results obtained from analyzing the *mat*K gene.

*rbc*L gene sequence analysis

The results of this tree analysis also divided the species into two main groups (Fig. 3). Within the below clade, the *C. Pallasii* subsp.-*haussknechtii* and the *C. sativus* are both placed in a large, strongly supported (99%), but very unresolved clade also including the rest of the species. *trn*L. The analysis of the data obtained from the *trn*L gene sequence indicated a similar topology to that of the species and ancestors examined in other studies (Ahadi *et al.*, 2015). In the obtained tree, the taxa within the group formed a mass trichotomy. Within this large calde, species of section *Nusiscapus* series *Biflori* (*C. almehensis* and *C. biflorus*) are embedded with weakly supported (55). The other clade consists of a large group of all section *Crocus*, *Nudiscapus* and *Crociris* (*C. bannaticus*) (Fig. 4).

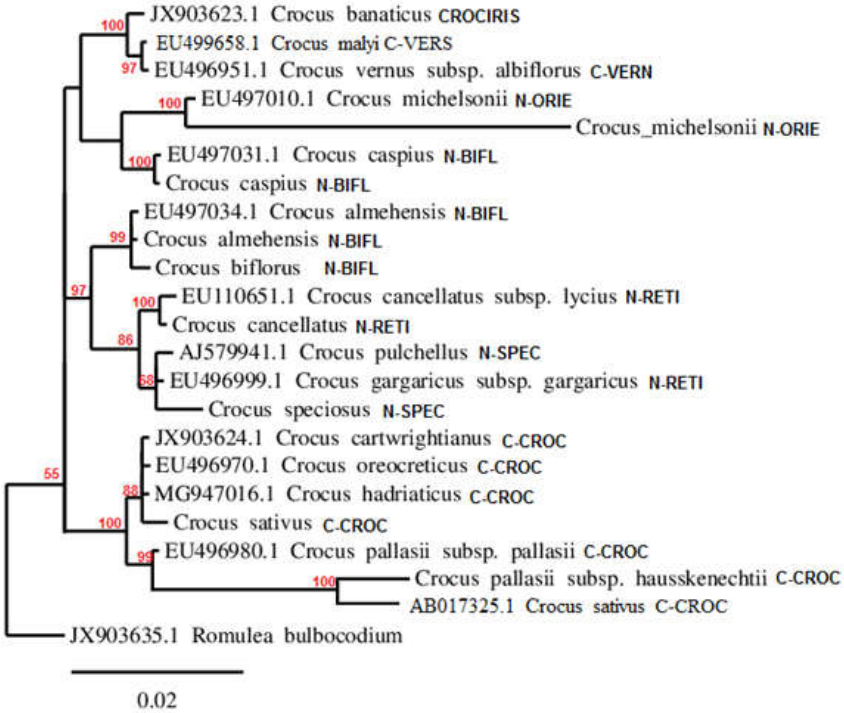

Fig. 2. The phylogenic tree derived from the analysis of *mat*K region data by the Bayesian method, the number above each branch shows the bootstrap value.

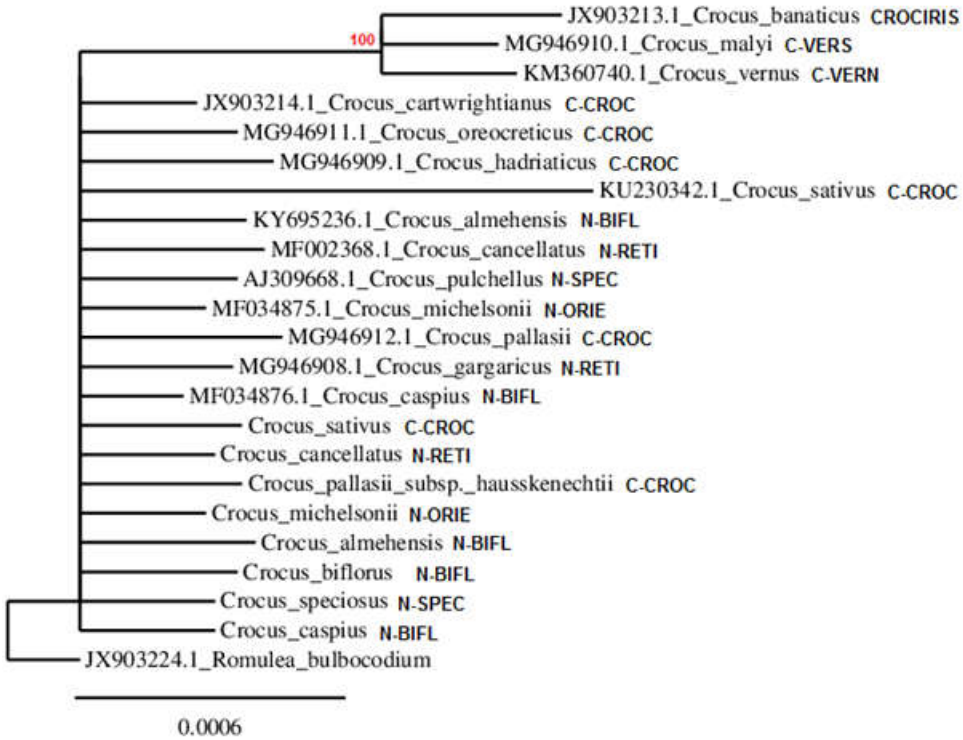

Fig. 3. The phylogenic tree derived from the analysis of *rbc*L region data by the Bayesian method, the number above each branch shows the bootstrap value.

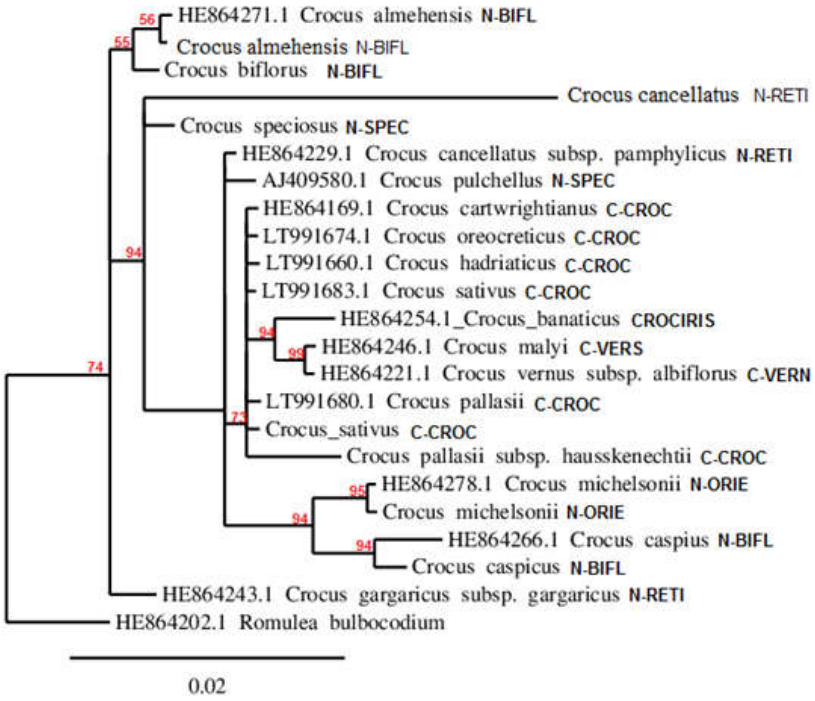

Fig. 4. The phylogenic tree derived from the analysis of *trn*L region data by the Bayesian method, the number above each branch shows the bootstrap value.

Combination analysis of *mat*K and *rbc*L

The phylogenetic analyses of the combined chloroplast loci (*mat*K and *rbc*L) resulted in polytomy which is divided into four branches (Fig. 5). The first clade contains section *Crocus*. In this group, *C. pallasii* subsp. *haussknechtii* had indicated the maximum similarity to *C. pallasii* subsp. *pallasii* and occurred in a separate sister sub-group. The cultivated saffron is embedded in the sister group of the *Crocus* series. The next clade is *C. michelsoni* (series *Orientales*) which is in sister to *C. caspius* (series *Biflori*). In the third group, *C. bannaticus* is embedded in sister with *C. vernus* with a weakly supported (67). In the last branch, the species of three series of sections *Nudiscapus* (*Reticulati*, *Biflori,* and *Speciosi*) are placed together. The explanation of such a classification is elucidated later in the combination of four barcodes.

Combination analysis of ITS, *mat*K, and *trn*L

Analysis of the data from the combination of ITS, *mat*K, and *trn*L barcodes (Fig. 6) divided the studied species into two clades. In one of these branches, *C. michelsonii* occurred in the sister group with its similar species in the NCBI, and the remaining species occurred in the other larger branch which is divided into two clades: one strongly supported (100) including *C. almehensis* which is in sister to *C. cancellatus*, and these two species form the sister group to species of series *Speciosi* (*C. speciosus* and *C. pulchellus*). The second group consists of the remaining species. All of the species along with the group of *C. cartwrightianus*, *C. oreocreticus*, *C. hardriaticus*, and *C. sativus* obtained from NCBI occurred in another sister group. Moreover, in another group, *C. pallasii* subsp. *haussknechtii* is sister to C. pallasii subsp. Pallasii and cultivated saffron. These three species are in the sister group of species belong to series *Biflori* and *Reticulati*. Apart from some differences present the genus include in these series, many of the species of these two series are so similar in morphological characteristics, such as corm tunics (Wolter, 1990). Combining these three barcodes did not improve the resolution of the phylogenetic very much in comparison to using ITS only.

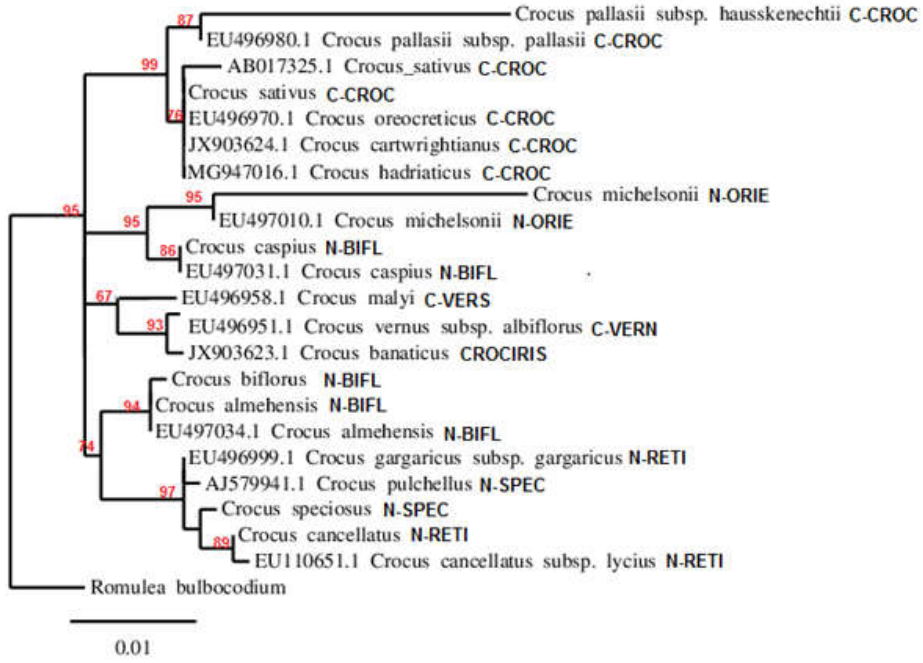

Fig. 5. The phylogenic tree derived from the analysis of *mat*K+*rbc*L region data by the Bayesian method, the number above each branch shows the bootstrap value.

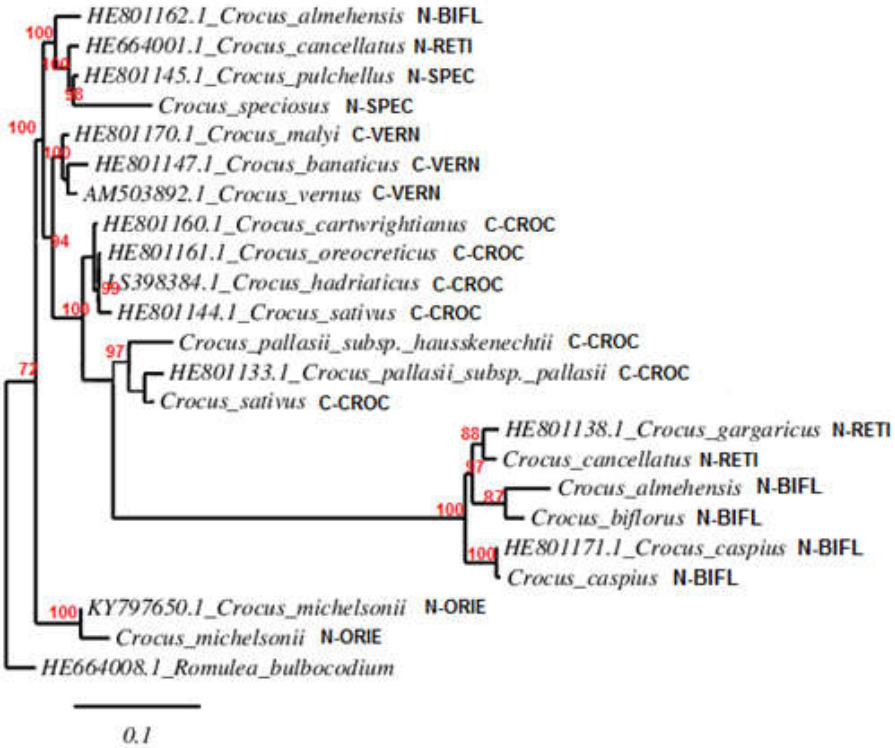

Fig. 6. The phylogenic tree derived from the analysis of ITS+*mat*K+*trn*L region data by the Bayesian method, the number above each branch shows the bootstrap value.

Combination analysis of ITS, *mat*K, *rbc*L, and *trn*L

The whole studied species obtained from the combination of the nuclear and chloroplast markers form a polytomy structure including four clades (Fig. 7). In comparison to Mathew's (1982) taxonomic treatment of *Crocus*, our phylogenetic analysis identified congruence and conflicts on different taxonomic levels. The analysis of the combined data resulted in a tree that grouped *C. bannaticus*, the only member of subgenus *Crociris*, embedded within a clade including species of section *Crocus* (*C. malyi*) and is placed as the sister group with species of series *Versicolores* (weakly supported 64%). The second strongly clade forms a monophyletic group in series *Orientales* including *C. michelsonii* as a sister group with the same species in NCBI. Series *Orientales* are winter-flowering and morphologically well-defined primarily by corm tunics with a carpet of fine fibers. The third moderately supported includes three series of *Biflori* and *Reticulati* and *Speciosi* together. Another large strongly supported clade (96%) includes species of series *Crocus* and *Nudiscapus* as a dichotomy. Our analysis revealed that *C. pallasii* subsp. *haussknechtii* is more closely to the *C. sativus* and *C. pallasii* subsp. *pallasii* and are separated from the clade consisting of species of *C. caspius*, *C. almehensis, C. biflorus* (series *Biflori*) and also *C. cancellatus* and *C. gargaricus* (series *Reticulati*). The other sub-clade consists of series *Crocus.* Section *Crocus* is not monophyletic in the present data analysis (Fig. 7).

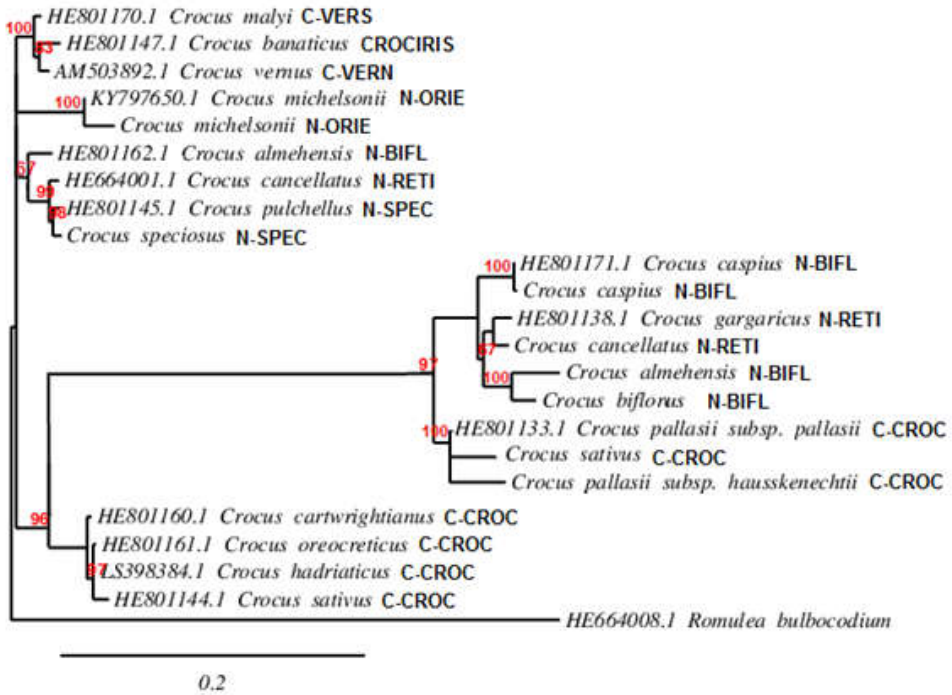

Fig. 7. The phylogenic tree derived from the analysis of ITS+*mat*K+*rbc*L+*trn*L region data by the Bayesian method, the number above each branch shows the bootstrap value.

Discussion

The results of this study show that among the studied genetic regions, the nuclear ITS gene region performed better than the other genes and separated all the examined species from one another. Furthermore, the *mat*K gene region was one of the good genetic regions for separating and distinguishing the studied species. The low diversity of DNA of chloroplast in *Crocus* species was previously examined in a study conducted by Seberg *et al*., (2009). Even in the present study, the use of six genetic regions could not be useful for separating the species. In a study by Haung *et al*., (2015) In the previous study, the distinguish between cultivated saffron

and some Chinese herbs was assessed resulting that ITS could make a distinction at the intra-species level using the *rbc*L and *trn*H-*psb*A barcodes at the inter-species level. In another study, it was found that the use of the ITS and *mat*K markers was effective in the identification of *Crocus* species (Villa *et al*., 2016). In addition, it was shown that the markers of ITS, *mat*K, *trn*L, and *rbc*L performed better to create interspecies divergence (Li *et al*., 2011). Based on our data, although the alignment of chloroplast *trn*L and ITS regions have similar lengths, the uniparentally inherited chloroplast *trn*L region provided relatively few polymorphic sites (140) in comparison to the ITS region (270). Hence, the phylogenetic relationships among species were not well resolved when the chloroplast region was analyzed alone. Phylogenetic relationships among species were hence not well resolved when analyzing the chloroplast region alone. This was in agreement with the previous result indicating five chloroplast regions in *Crocus* with a low mutation rate (Petersen *et al.*, 2008; Seberg and Petersen, 2009). The Consortium for the Barcoding of Life (CBOL) (2009) introduced *mat*K + *rbc*L genes as the best common barcode option for plants. This topology again is nicely supported by the chloroplast analysis of Petersen *et al*. (2008) that resulted in such a classification and grouping in different series of *Crocus*. Although, the combination of *mat*K and *rbc*L did not improve the resolution of the phylogenetic tree apparently. The posterior probabilities along the backbone of the tree increased the combined analysis in comparison to using ITS only.

In all studied sequences, *C. pallasii* subsp. *hausskenechtii* indicated the maximum similarity to the cultivated species. The study conducted by Sanei *et al*., (2007) which examined *C. pallasii* according to karyotype data and the studies performed by Alsayied *et al*. (2015) and Harpke *et al*. (2013) using nuclear, ribosomal and plastidy single-copy gene sequences confirmed the findings of the present study. Similarly, the result of the present research was in line with the report of Namayandeh *et al.* (2012) on wild and cultivated saffron species using the microsatellite markers. Beiki *et al*. (2010) examined the variability and genetic relationships of 30 different *Crocus* genotypes using RAPD marker. The results revealed that all cultivated saffron genotypes indicated the maximum similarity with *C. cancellatus* which was in contrast with the findings of the present study. Furthermore, *C. michelsonii* and *C. almehensis* revealed the maximum similarity to the cultivated species in the study conducted by Alavi-Kia *et al*. (2008).

The position of *C. banaticus* within section *Crocus* resembles also to the results of Petersen *et al*., (2008) and Harpke *et al*., (2015). *C. banaticus* has to be construed as a morphological peculiar taxon with autapomorphies characteristic such as small leaf diameter, introse anthers, inner perianth-segments being much shorter than the outside and several style splitting, lilac branches, slender and elongate ovoid capsule (Dahlgen *et al*., 1985; Kerndorff *et al*., 2015). As stated in Mathew's study (1982) *C. bannaticus* had needle-like crystals like those of series *Verni* which verify the placement of them in the sister group. The relationship between *Biflori*, *Reticulati* and *Speciosi* is unresolved (Peterson *et al*., 2008). In Mathew's estimation the separation of these sections is still justified and was believed to discern some of the most challenging taxonomic problems within the genus, while Harpke *et al*. (2014) stated for these species that oxalate-crystals have a clear structure with only needle-like and sand-like (Peterson *et al*., 2008).

The close relationship between *C. sativus* and *C. pallasii* is supported by morphological similarity (Mathew, 1982). *C. sativus* is a sterile triploid only known from cultivation. It is usually thought to be hybrid with *C. cartwrightianus* (Ahrazem *et al*., 2010; Larsen *et al*., 2015; Harpke *et al*., 2018) which is in contrast with our results. Given that until now it has been presented that *C. cartwrightianus* is the most probable and also the sole ancestor of the cultivated saffron (Nemati *et al*., 2019), but efforts should be devoted to finding the probable parents of saffron. However, despite the results of the mentioned studies, it is conjectured that *C. pallasii* is one of the potential parents of saffron (Gismondi, 2013; Erol, 2014), which is confirmed by the findings of the present study.

In the point of flowering time, the species vary from late autumn through to early and there be autumn-, winter-, and early spring-flowering populations of *Crocus* (Mathew, 1982). *C.*

cancellatus occurs in the same area as the species of series *Biflori, Reticulati,* and *Crocus*, but *C. almehensis* become of being spring-flowering, endemic to Iran, in contrast to the autumn-flowering species of this clade, which are is widely distributed, make an unlikely explanation for the unexpected relationship. The disparate flowering time of the species of this clade and the other species under discussion is probably not of great significance (Peterson *et al*., 2008; Nemati *et al*., 2018). The suggested relationship between species of series *Biflori* and *Reticulati* are considerably more complicated. For the reasons stated above, this is not surprising in view of the morphological similarity between them. Morphologically *C. caspius* is not a typical member of series *Biflori* so its position in the sister group with *Reticulati* species is not too unexpected (Peterson *et al*., 2008). This possibility can be taken into consideration that the species occurring in one group have had a different evolutionary pathway due to their growth in different regions and the impact of different environmental conditions on them, which ultimately led to diversity in these species (Mirzaei *et al*., 2001).

The species of section *Crocus* are placed in a clade together with *C. bannaticus* and a group of species regularly included in the series *Reticulati* of section *Nudiscapus*, accordingly, this position of section *Nudiscapus* makes them non-monophyletic (Peterson *et al*., 2008).

Our phylogenetic analysis of *Crocus* does not follow the principal classification of Mathew (1982) into subgenera and sections; nevertheless, the grouping into series is weakly supported. In the present study, four barcodes which were recommended by various researchers were used to investigate their power to separate the wild and domesticated species of *Crocus*. The relationship between three series of *Biflori* and *Reticulati* and *Speciosi* was unresolved according to our phylogenetic data and need revision as well as the subgenus conception of Mathew's classification. These findings are in accordance with chloroplast data reported by Peterson (2008).

Since *C. sativus* lacks genetic diversity or at least has a small variety, improvement of the qualitative and quantitative traits would be difficult for its breeders and botanists. All cultivated saffron species are sterile and generate the same gene pool that makes it difficult for breeding strategy and crop development in the future. Therefore, genetic enhancement of saffron requires the knowledge of the biology of source species or wild relatives. Wild species often contain useful gene attributes such as drought, heat, and cold tolerance, which can be used in breeding programs for cultivated species, so that hybrid breeding strategies with the pertinent species are effective in yield and quality improvement of saffron (Agayev, 2007; shokrpour *et al*., 2019). Though the phylogeny has clarified some relationships between super-specific taxa, more data are clearly needed for fully resolving the phylogenetic tree. Our results confirm the importance of multi-locus studies for the inference of phylogenetic relationships in a series of *Crocus*. An ideal barcode should be able to be replicated using a pair of universal primers in all species of a genus or even a family. The intergenic regions of ITS, *mat*K, and *trn*L could separate all the studied species of saffron (Kress *et al*., 2005, 2007; Lahaye *et al*., 2008). Furthermore, the tree derived from the *rbc*L gene sequence was unable to discriminate different saffron species, which indicated the ineffectiveness of this gene in this study (Seberg and Petersen, (2009). Since the interpretation of the evolutionary pathway based on the presented tree was not reliable; the triple combination of ITS, *mat*K, and *trn*L barcodes was examined along with the four-component combination. The intergenic and nuclear regions of the ITS had an identification power of 100%, a high PCR amplification capability and could separate all the examined species. Consequently, according to the presented results, this genetic region can be recommended as a barcode in the studied plants. Moreover, it was revealed that the combination of four markers of ITS, *mat*K, *trn*L, and *rbc*L achieved better to create interspecies divergence. Among the studied species of saffron, *C. pallasii* subsp. *hausskenechtii* showed the most similarity to the cultivated species.

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Conflicts of interest

The authors have declared that no competing interests exist.

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