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Study of phylogenic relationships of some wild and crop species of Iranian *Crocus* by ITS nuclear loci

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Abstract: ITS (Internal Transcription Spacer of nuclear ribosomal DNA) regions method was applied to identify and study the phylogenetic relationships existing between 41 individuals of Crocus, including 8 species collected in Iran (4 crop and 8 wild individuals) and 16 species (2 crop and 27 wild individuals) already registered in GenBank (NCBI). PCR amplifications were performed using the primer designed on the nucleotide sequence of nuclear region (ITS). A total of 12 sequences were obtained and registered in NCBI database. In particular, 4 of these sequences were not already present in the scientific library. Nucleotide polymorphic sites were counted for ITS (N = 97). The results showed that, the Cytosine base is the most abundant nucleotide. Relatively high conservation was observed in the ITS regions of the populations (0.761). Each sample could be distinguished from the others in the phylogenic trees developed based on the data obtained by ITS barcode genes. In general, ITS sequence indicating high resolution at the species level is very suitable for phylogenetic studies in the Crocus family. Phylogenetic analysis demonstrated the genetic relationship between crop saffron and wild Crocus species. According to the results of this study, the wild species of *C. cartwrightianus* and *C. oreocreticus* were considered as the closest relatives of the saffron. The results of the study also indicated that, the different ecotypes of *C. sativus* may have evolved through independent events probably due to the geographic and environmental pressures.

Introduction

Crocus is a monocot belonging to Iridaceae family (Mathew, 1982), *Crocus* series *Crocus,* which is distributed from Italy in the West to the Caucasus in the East with the center of diversity on the Balkan Peninsula and Asia Minor (Larsen *et al*., 2015). Only one species of this genus is source of saffron (*C. sativus*). It is triploid and its stigma is picked up manually and is desiccated for production of saffron (the world's most expensive spice) (Grilli Caiola *et al*., 2004). There are different opinions about the origin of saffron. Vavilov introduced the Middle East (Asia, Turkestan, and Iran) as the original distribution area of saffron (Vavilov, 1951). Some others consider the origin of this species in a wide region, expanding from Iran to Greece (Tammaro, 1987). Some evidence suggests that, the *C. sativus* is an autotriploid that has evolved in Attica by combining two different genotypes of *C. cartwrightianus* (Nemati *et al*., 2019). In addition to crop species, such as *C. sativus* L., Iran has also 8 wild species of *Crocus* including *C. haussknechtii, C. cancellatus, C. caspius, C. speciosus, C. almehensis, C. gilanicus, C. michelsonii, and C. biflorus* (Mathew, 1982).

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Other species of *Crocus* are popular as garden plants and collection species (Petersen *et al*., 2008). Considering the commercial desire with respect to this genus, surprisingly few studies have been conducted to clarify its phylogeny. The application of marker methods has not been suitable to uncover relationships among distantly related taxa within the series (RAPD: Grilli Caiola *et al*., 2004; AFLP/SSR: Erol *et al*., 2014; Larsen *et al*., 2015; IRAP: Alsayied *et al*., 2015).

In case of molecular biology, DNA barcode technique is a highly accurate scientific tool for taxonomic identification at species level, which is based on the high rate of mutations occurring in target sequences during the evolution (Gismondi *et al*., 2012). Advances in sequencing technology and bioinformatics computations have transformed DNA into one of the main and new sources of information for studying genetic evolution relationships, discovering unknown species, and identifying biodiversity (Asadi *et al*., 2015). This method first proposed by Hebert *et al.* (2003) shows a simple relationship between all stages of life at levels beyond species. The Consortium for the Barcode of Life is trying to develop this method (Casiraghi *et al*., 2010). The standard nucleotide sequence analysis (ITS, internal transcription spacer of nuclear ribosomal DNA) has been successfully used to identify and classify several plant species (Kress and Erickson, 2008; Seberg and Petersen, 2009; Gismondi *et al*., 2012).

Single Nucleotide Polymorphisms (SNPs) are other important markers for studying the genetics. In particular, they relate sequence changes to the phenotypic changes (Kim and misra, 2007; Xu *et al*., 2009). Harpke *et al*. (2013) analyzed phylogenetic relationships and evolution of the number of chromosomes in 115 samples of *Crocus* using 110 species, via trnL-F chloroplast sequence, ITS nuclear sequence, and a part of pCOSAt103 single-nucleus gene. Gismondi *et al*. (2013) studied Italian and Spanish saffron species using ITS, rbcL, matK, and trnH-psbA barcodes, and they demonstrated an independent event in generation of *Crocus sativus* species in Spain and Italy. Erol *et al*. (2015) postulated the existence of a new species of this genus in Turkey, the *Crocus musagecitii*, using morphological traits of molecular data (ITS, ITS1 + ITS2 + 5.8SrDNA and ETS). Kerndorff *et al*. (2017) supported the existence of a new species of this genus in Turkey and Iran, the *Crocus adamii GAy*, using morphological traits and ITS nuclear sequence. Finally, Nemati *et al*. (2018) analyzed sequences of two chloroplast (trnL-trnF, matK-trnK) and three nuclear (TOPO6, ribosomal DNA ETS, and ITS) marker regions to infer phylogenetic relationships among all species belonging to series *Crocus*.

Phylogenetics considers the macroevolutionary processes occurring between species and attempts to explain the line of ancestry leading to the current distribution of species in time and space (Lemey *et al*. 2009). Accordingly, the present study was aimed to achieve the genetic diversity status and structure among *Crocus* populations, as well as the possibility of differentiating, categorizing and determining the relationships between species and determining the location of Iranian *Crocus*, in the phylogenetic tree of the *Crocus* genus using the ITS nuclear loci.

Material and Methods

The plant material was collected from 7 wild *Crocus* species including 8 individuals *C. michelsonii, C. speciosus* deylaman, *C. speciosus* roudbar, *C. caspius, C. almehensis, C. sp*. Eslamabad, *C sp* harsin, and *C. sp* sonqor, as well as crop species including four individuals *C. sativus* fars, *C. sativus* khorasan razavi, *C. sativus* nehbandan, and *C. sativus* zabol, in different regions of Iran. Detailed information is presented in Tab. 1. For each species, 10 samples were considered and analyzed. These plant species cover some wild and crop species in Iran.

DNA extraction

DNA was extracted from *Crocus* leaves based on the method proposed by Dellaporta (without kits) (Dellaporta *et al*., 1983). In summary, the plant material (100 milligrams), was powdered under liquid nitrogen using a pestle and mortar and was suspended in Dellaporta buffer (containing Tris-HCl, EDTA, NaCl, β-Mercaptoethanol, and water). Then, 40 µL of SDS 10% was added to them. At the next stage, 100 µL of cold potassium acetate was added. Then, 500 µL of chloropharm-isoamyl alcohol mixture (24:1) was added to the sample, which resulted in the formation of three phases. The higher phase, containing DNA and being highly transparent, was transferred into a new sterile microtube containing 2 µL of RNase A. Then, 700 µL of cold isopropanol was added and placed at -20°C. The samples were centrifuged, and supernatant was wasted, while DNA pellet was washed with 400 µL of alcohol 70%. The final DNA was dried in a sterile hood, and was dissolved in 100 µL of deionized water, and then was stored at -20°C for further use.

TABLE 1

Passport information of *Crocus* species used in this study

Species	Flowering time	Province	County	Altitude	Latitude	Longitude	Number of samples
C. caspius	Autumn	Gilan	Rasht	161	37.44	49.96	10
C. speciosus	Autumn	Gilan	Roudbar	1340	36.50	49.43	10
C. speciosus	Autumn	Gilan	Deylaman	1573	36.52	49.05	10
C. sp	Autumn	Kermanshah	Harsin	1542	34.11	47.27	10
C. sp	Autumn	Kermanshah	Sonqor	1805	34.39	47.41	10
C. sp	Autumn	Kermanshah	Eslamabade	1350	34.07	43.36	10
C. michelsonii	Spring	Khorasan North	Bojnurd	1376	37.27	57.18	10
C. almehensis	Spring	Hamedan	Hamedan	1850	34.75	48.53	10
C. sativus	Autumn	Khorasan Razavi	Torbat	1354	35.20	59.22	10
C. sativus	Autumn	Fars	Estahban	1720	29.07	54.02	10
C. sativus	Autumn	South Khorasan	Nehbandan	1100	31.48	60.01	10
C. sativus	Autumn	Sistan and Baluchestan	Zabol	475	31.04	61.53	10

TABLE 2

Primer pair names (F: forward; R: reverse), their sequences, target region and relative Tm (°C) used in PCRs

Primer pair name	Sequence[a] (5'-3')	Amplified region	Tm (°C)
ITS F	TCCTCCGCTTATTGATATGC	ITS	57.5
ITS R	CCTTATCATTTAGAGGAAGGAG	ITS	

[a]References: Kress and Erickson (2008), Seberg and Petersen (2009), Gismondi *et al.* (2012, 2013)

TABLE 3

GenBank accession numbers for DNA sequences used in this paper

KY797650	KY886372	HG518184	LS398384	LT222445	DQ224364
KY797651	KY860628	LS398408	LS398383	LN864720	DQ224363
KY797648	KY828970	HE801118	LS398387	LM993456	KT357294
KY797649	KY860627	LS398371	LS398386	HE664001	KT357293
KY614361	KY860629	HG518198	LS398391	HE663998	LM993445
KY886374	AB699586	LS398398	LS398415	HE801129	HE664018
KY886373	DQ094185	LS398397	LS398414	HE801128	AY351379

PCR amplification and gel electrophoresis

Purified DNA was amplified using PCR method in a 50-µL reaction mixture containing 2 µL of DNA template, 2 µL of each primer (Tab. 2) with a concentration of 10 pM and 25 µL of Master Mix 2X (Ampliqon, Germany) (including 100 mM Tris-HCl pH of 8.5, 32 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.2 units of Ampliqon Taq DNA polymerase). Deionized distilled water was used for the final volume. DNA was replicated using BioRad ICycler thermocycler under the following condition: (a) initial denaturation at temperature of 95°C for 15 min, (b) 38 cycles of denaturation at 95°C for 1 min, annealing of primers at sufficient temperature for each primer pair (as described in Tab. 2) for 45 s, extension at 72°C for 1 min; (c) final extension at 72°C for 15 min. The reaction was stored at 4°C. The PCR products were separated on 1% agarose gel, using buffer TAE 1X (40 mM Tris; 1 mM EDTA, 20 mM acetic acid; pH = 8.5) by adding Fluoro Vue Nucleic Asid Gel Stain 2 µM 10000X (Smobio), and were visualized under UV light (Gel Doc 2000 BIO-RAD). All PCR reactions were performed at least in triplicate.

Data analyses

All amplicons were sequenced by Macrogene (South Korea). The sequence quality was evaluated using SeqMan DNASTAR software 12.2 (Schwei, 2015). The sequences were visualized by BioEdit program v7.0.5.3 (Hall, 1999). ITS sequences of each sample were compared using ClustalX multiple sequence alignment program v2 (Larkin *et al.*, 2007). To certify the amplifications, the identity of each amplified sequence was compared to that of 16 species (2 crop and 27 wild individuals) already registered in GenBank (NCBI) using search tool (Tab. 3). All detectable variable sites were included in the analysis. Phylogenetic analysis was performed using MEGA7 software (Kumar *et al.*, 2016). In MEGA7 software, all necessary parameters were set as reported in the study by Gu *et al.* (2013). The genetic tree was drawn applying Tamura-Nei model, maximum likelihood method, where 1000-bootstrap validation system and genetic indices were evaluated using DNA SP software v5.10.01 (Librado and Rozas, 2009), NETWORK v4600 (Bandelt *et al.*, 1999) and HIV databases (https://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html). An orchid case (Orchis mascula, ID: AY351379), extrapolated by NCBI database was also added to this analysis as out-group species.

Results

ITS sequences analyses

The success rate of ITS region amplification was equal to 94% in the studied *Crocus* plant samples, where 93% of them were successfully sequenced. The length of the amplicons was verified on agarose gel (Fig. 1). All sequences were in alignment (by BLASTn) with GenBank database, and all of them were attributed to other ITS sequences of *Crocus* species already submitted to the NCBI. The results of this study corresponded to 12 ITS sequences present on the NCBI site. After searching the databases, it was found that, 4 ITS sequences found in this study had not already been registered in the database and were successfully deposited in the database accordingly (KY797650, KY886374, KY886373, and KY886372).

In the phylogenic tree (MEGA7 software), *Crocus* genus species were strongly separated (Fig. 2) according to ITS barcode data. The number of variable sites for the specific ITS sequences obtained in this study was measured as 97 (SNPs).

The analysis results of the nucleotide sequences of ITS region in the *Crocus* family showed that, the highest and lowest abundance rates belonged to the Cytosine base (31.14%) and adenine base (18.0%), respectively (Tab. 4). Estimation of nucleotide substitution in the *Crocus* family showed that, high levels of pyrimidine substitution were obtained such that this level was obtained as 31.09 and 19.81% for thymine-cytosine and cytosine-thymine conversion, respectively (Tab. 5).

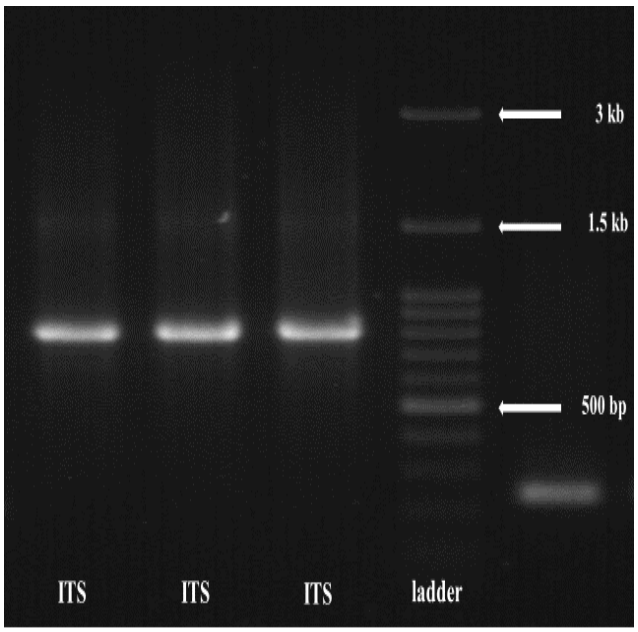

FIGURE 1. DNA extracted from saffron, amplified by PCR and visualized, by UV light, after separation on 1% agarose gel. ITS barcode gene was shown. Molecular weight markers were also reported (ladder lane).

These levels were lower for purine bases and were obtained as 16.31 and 9.46% for the adenine-guanine and guanine-adenine conversions, respectively. The results of this study are consistent with the results of other studies reported frequent cases of pyrimidine substitutions. These changes probably result from cytosine methylation (Ghanbari *et al*., 2018).

The concatenated alignment of rDNA regions had a length of 693 bp (Tab. 6). After investigating the genetic (using DNA SP software) indices of ITS region of the *Crocus* family, a total of 114 mutations were identified which were distributed differently throughout the genome. A number of polymorphic positions was observed in 97 positions (Tab. 6) indicating the process of positive selection of this nuclear region (ITS). The results of conserved DNA regions of ITS region in the *Crocus* family showed a conservation threshold region of 0.86, a minimum conservation length of 56 bases, and sequence conservation of 0.761 (Tab. 7). These conserved regions included a small part of the sequence in the above region, suggesting a different differentiation of this position as well as its susceptibility to nucleotide changes and mutations among different varieties, culminating in variability among the variants.

The numerical value of the dN / dS ratio (using HIV databases) was equal to 1:01 (Tab. 8) for the *Crocus* family indicating the positive selection of ITS region among the *Crocus* varieties during evolution. This type of selection has resulted to the emergence of new varieties on one hand and stabilization of better purification for their performance during the evolution on the other hand resulting from the conversion of noncoding gene regions to gene coding regions (Ghanbari *et al*., 2018). The determination of haplotype groups (using NETWORK software) is one of the important steps in determining the geographical location of the studied breeds compared to other breeds. As shown in Fig. 3, high-abundance and low-abundance haplotypes are shown with bigger and smaller circles, respectively. All 41 samples were placed in Haplotype group A, also all the samples obtained in Iran were placed in Haplotype group B. This haplogroup is the largest haplogroup in a variety of different species worldwide. Research results showed that, haplogroup A is commonly found in all continents, and haplogroup B may also have originated from Asia, as confirmed in the study by Ghanbari *et al*. (2018).

TABLE 4

Abundance of nucleotides derived from a nucleotide sequence of ITS region in the Crocus **family**

Nucleotide	A	T	C	G
Abundance	18.0	19.84	31.14	31.03

TABLE 5

Estimation matrix of the nucleotide substitution pattern of the ITS region in the *Crocus* family

From/To	A	T	C	G
A	-	2.31	3.63	***16.31***
T	2.10	-	***31.09***	3.62
C	2.10	***19.81***	-	3.62
G	***9.46***	2.31	3.63	-

S: Each input may be substituted from one base (row) to another base (column). In this table, the percentage of transition mutations (purine-purine substitution, pyrimidine-pyrimidine substitution) and transversion mutations (purine-pyrimidine substitution and vice versa) are shown in bold and italics, respectively.

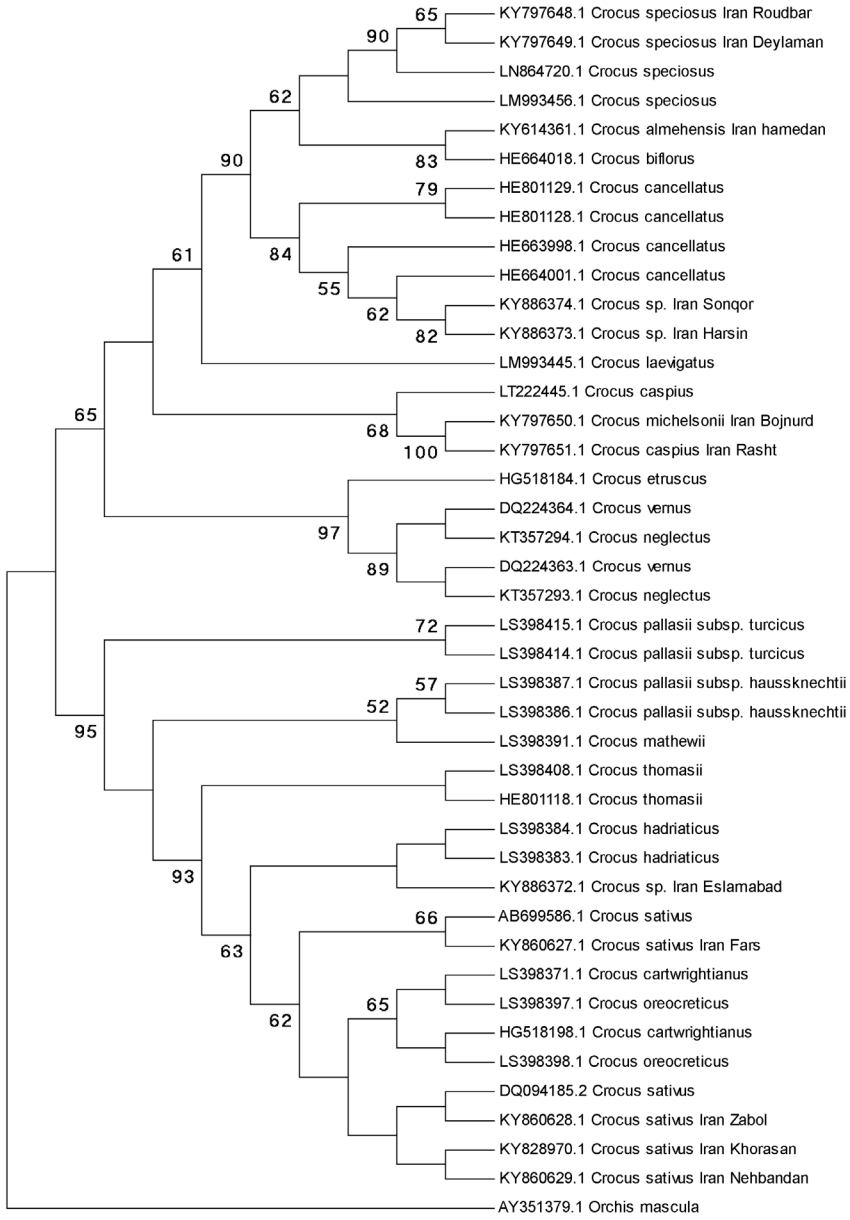

FIGURE 2. Phylogenic tree showing the relationship constructed from ITS sequences of *Crocus* species, using the maximum likelihood method (Mega7 software). Numbers above nodes indicate bootstrap estimate for 1000 replications. NCBI GenBank identification number of each accession are presented.

TABLE 6

Location of ITS region polymorphism in the *Crocus* family

Population	alignment	S	H	Hd	Pi	Eta	K
Crocus	693	97	27	0.976	0.05128	114	26.665

S: The number of polymorphic positions, H: number of haplotypes, Hd: Haplotype (gene) diversity, Pi: nucleotide diversity, Eta: total number of mutations, K: number of nucleotide differences between population or species (nucleotide divergence).

TABLE 7

Conserved DNA regions of the ITS region in the *Crocus* family

Population	C	MWL	CT
Crocus	0.761	56	0.86

C: Sequence conservation, MWL: Minimum conservation Length, CT: Conservation threshold.

TABLE 8

Identifying the natural selection process for the ITS region in the *Crocus* family

Parameter	Numerical value
ds	0.0715
dn	0.0729
dn/ds*	1.01

* The numerical value of dn/ds represents the natural selection process

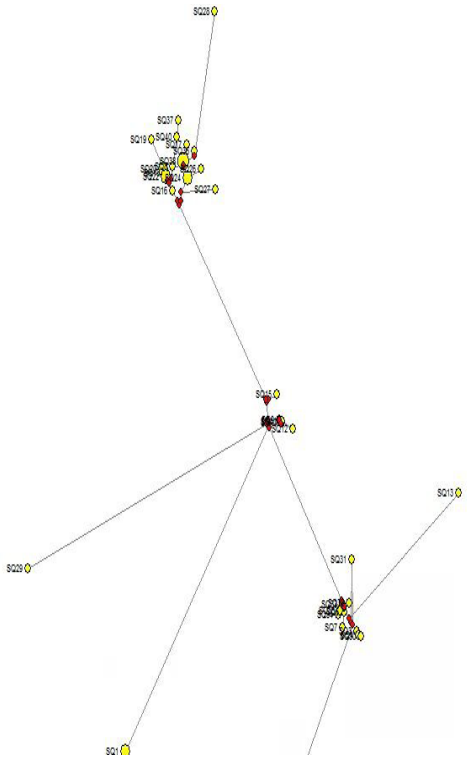

FIGURE 3. The interconnected grid of the studied varieties of the *Crocus* genus using the ITS region (NETWORK 4600 software).

Discussion

Different cultivars of herbal species and their wild relatives constitute a large part of the valuable plant samples of each country. Today, various hazards such as drought, overgrazing, and over-harvesting have endangered genetic reserves of these plants (Rahimmalek *et al*., 2009). The low level of nucleotide diversity in this research is probably due to a sharp decline in the effective population of varieties belonging to crocus family and the confirmation of the risk and extinction of these varieties (Tab. 6). Genetic identification and registration of various plant cultivars is crucial for adequate conservation and utilization of genetic resources; this monitoring is hard to perform by morphological features for most plants in the early stages of development (Asadi *et al*., 2015). In case of molecular biology, DNA barcode technique is a highly accurate scientific tool for taxonomic identification at species level, which is based on the high rate of mutations occurring in target sequences during the evolution (Gismondi *et al*., 2012). Previous studies showed that, a barcode should show a good resolution power at interspecific levels (Hebert *et al*., 2003). It contains sufficient information to assign a name to unknown species, classifying it in a specific taxonomic category (Mahadani and Ghosh, 2013). In fact, advancements in scientific techniques using barcoding-DNA tools provide promising avenues to understand the diverse array of organisms and to elucidate the confusing taxonomic troubles (Bchir *et al*., 2019). The ITS nuclear barcode used in this study, possessing high resolution at both genus and species levels has been widely used in the previous studies as testified by the elevated number of accessions registered in scientific database under this name. Although it has different lengths among plant organisms, ITS barcode has been adopted as its flanking regions are strongly conserved and allow the use of generic primers. Furthermore, as it is present in multiple copies per cell, it represents a good candidate for plants' barcoding (Kress *et al*., 2005; Chase *et al*., 2007). Compared to chloroplast genome, ITS does not have a single parent inheritance and possesses recombination events (Curyis and Clegg, 1984). Indeed, nuclear genome deals with more changes, which is more informative than plastid genome, and therefore, it is more useful in inter-species studies (Kim and Misra, 2007). Okuyama and Kato (2009) used ITS region with great success as a barcode for identification of *Asimitellaria* genus species from the related species. Kerndorff *et al*. (2017) postulated new species in Turkey and Iran, *Crocus* adamii GAy, using morphological traits and ITS nuclear sequence.

In the present result (Fig. 2), a dual separation was found. Obviously, *Orchis mascula*, as expected, positioned itself far away from all other *Crocus* species, as the out group. *Crocus* genus appeared monophilic according to our data. Accepting this hypothesis, that a basic branching divided all samples in two clades, then the two clades were poorly supported and strongly sustained (bootstrap values of 65 and 95%), respectively.

The first clade had a two-part separation in the base

In particular, four autumn flowering species including *C. speciosus* Iran Deylaman, *C. speciosus* Iran Roudbar, *C. speciosus* (LN864720) ,and *C. speciosus* (LM993456), belonging to Speciosi series are categorized in the supported clade (bootstrap value of 45%) associated with cluster *C. almehensis* Iran Hamedan and *C. biflorus*, samples of Biflori series according to high bootstrap values (83%).

Another part of autumn flowering species, including four species of *C. cancellatus*, appeared to group together with other autumn flowering species, namely *C. sp*. Iran Sonqor and *C. sp*. Iran Harsin, according to high bootstrap values (84%). Since *Crocus sp*. Iran Sonqor and *Crocus sp*. Iran Harsin samples are autumn flowering species and were collected in geographical area of Kermanshah, the high bootstrap value (82%), aggregated from the habitat *C. cancellatus*, suggested that probably these two *Crocus sp*. are *C.*

cancellatus exemplars based on morphological features and dendrogram obtained by analyzing ITS region.

The portion of clade, presenting species from Orientales and Biflori series (*C. michelsonii* Iran Bojnurd and *C. caspius* Iran Rasht, respectively), was characterized by a strong bootstrap value (100%) associated with *C. caspius* (LT222445). Morphologically, *C. caspius* is not a typical member of Biflori series. Consequently, its position in the phylogenetic tree was not very surprising. *C. caspius* and Orientales series have the same unusual behavior, with capsules reaching the ground; thus, their close genetic relationship can be easily explained. The second portion of the first clade, namely *C. vernus* and *C. neglectus* forms a group categorized in a strongly supported clade (bootstrap value of 89%) associated with *C. etruscus*, according to high bootstrap values (97%). Petersen et al. (2008) also showed the same results in their research.

The second part was made up of a strongly supported clade (bootstrap value of 95%) including all *C. sativus* samples, with the addition of *C. pallasi*, *C. mathewii*, *C. thomasii*, *C. hadriaticus*, *C. cartwrightianus*, *C. oreocreticus*, and unidentified species *C. sp*. Iran Eslamabad. Specifically, *Crocus sativus* series formed a supported group (Bootstrap of 62%) which could be divided into two sustained clusters, including *C. sativus* (AB699586) and *C. sativus* Iran Fars (1st cluster), and *C. sativus* (DQ094185), *C. sativus* Iran Zabol, *C. sativus* Iran Khorasan, and *C. sativus* Iran Nehbandan (2nd cluster). Since both of these last clades included crop species replicated asexually, thus this important result suggested that some *C. sativus* species might have been generated by different and independent evolutionary events, probably due to different geographic and environmental pressures, as already proposed in the study by Gismondi *et al.* (2013). Indeed, *C. sativus* is thought to be a hybrid evolved by breeding between *C. cartwrightianus* and another *Crocus* species (Grilli Caiola *et al*., 2004). *C. sp*. Iran Eslamabad and *C. hadriaticus* were included in a cluster.

The analysis indicated a close relationship between crop saffron Iran, two crop saffron species of NCBI (AB699586 and DQ094185) and two wild species of *C. cartwrightianus* and *C. oreocreticus*. Accordingly, the wild species of *C. cartwrightianus* and *C. oreocreticus* were considered as the closest relatives of saffron (*C. sativus*) and probably *C. cartwrightianus* the ancestor of this cultivated species, based on genetic distance and dendrogram obtained by analyzing ITS nuclear sequences. Nemati *et al*. (2018) analyzed sequences of two chloroplast (trnL-trnF, matK-trnK) and three nuclear (TOPO6, ribosomal DNA ETS, and ITS) marker regions to infer phylogenetic relationships among series *Crocus*, making the probability for the existence of an autotriploid origin of *C. sativus* from *C. cartwrightianus* very likely, also stated that *C. oreocreticus*, is the closest relative of *C. cartwrightianus* and *C. sativus.* Schmidt *et al.* (2019), by comparative FISH of six Crocus species from 11 accessions, indicated that *C. sativus* is an autotriploid hybrid derived from heterogeneous *C. cartwrightianus* cytotypes. Finally, Nemati *et al.* (2019), in Phylogenetic and population assignment analyses together with chloroplast polymorphisms indicated the *C. cartwrightianus* population in the vicinity of Athens as most similar to *C. sativus*. They concluded that the *C. sativus* is an autotriploid that has evolved in Attica by combining two different genotypes of *C. cartwrightianus*.

Conclusions

In general, ITS sequence, indicating high resolution at the species level is suitable for phylogenetic studies in the *Crocus* genus. Phylogenetic analysis demonstrated the genetic relationship between crop saffron and wild *Crocus* species. The results of this study revealed that, the wild species of *C. cartwrightianus* and *C. oreocreticus* are considered as the closest relatives of the saffron. The results of this study also indicated that, the different ecotypes of *C. sativus* may have evolved through independent events probably due to the geographic and environmental pressures.

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References

Alsayied, NF, Fernández JA, Schwarzacher T, Heslop-Harrison JS (2015). Diversity and relationships of *Crocus sativus* and its relatives analysed by inter-retroelement amplified polymorphism (IRAP). *Annals of Botany* **116**: 359-368.

Asadi F, Dezhsetan S, Ghahramanzadeh R, Razmjou J, Alebrahim MT (2015). DNA barcoding of some medicinal plants. *Crop Biotechnology* **10**: 31-40.

Bandelt HJ, Forster P, R€ohl A (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37-48.

Bchir R, Djellouli AS, Zitouna N, Aurelle D, Pergent G, Pergent-Martini C, Langar H (2019). Morphology and Genetic Studies of Cymodocea Seagrass Genus in Tunisian Coasts. *Tech Science Press* **88**: 171-184.

Casiraghi M, Labra M, Ferri E, Galimberti A, De-Mattia F (2010). DNA barcoding: a six-question tour to improve users' awareness about the method. *Briefings in Bioinformatics* **11**: 440-453.

Chase MW, Cowan RS, Hollingsworth PM, van-den-Berg C, Madriñán S, Petersen G, Seberg O, Jørgsensen T, Cameron KM, Carine M, Pedersen N, Hedderson TAJ, Conrad F, Salazar GA, Richardson JE, Hollingsworth ML, Barraclough TG, Kelly L, Wilkinson M (2007). A proposal for a standardised protocol to barcode all land plants. *Taxon* **56**: 295-299.

Curtis SE, Clegg MT (1984). Molecular evolution of chloroplast DNA sequences. *Molecular Biology and Evolution* **1**: 291-301.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA mini preparation: version II. *Plant Molecular Biology Reporter* **1**:

19-21.

Erol O, Harpek D, Yildirim (2015). A new *Crocus* L. (Iridaceae) species from SE Turkey, based on morphological and molecular data. *Phytotaxa* **239**: 223-232.

Erol O, Kaya HB, Sik L, Tuna M, Can L, Tanyolac MB (2014). The genus *Crocus*, series *Crocus* (Iridaceae) in Turkey and 2 East Aegean islands: a genetic approach. *Turkish Journal of Biology* **38**: 48-62.

Ghanbari S, Fakheri BA, Naghavi MR, Mahdinezhad N (2018). Evaluating phylogenetic relationships in the Lilium family using the ITS marker. *Journal of Plant Biotechnology* **45**: 236-241.

Gismondi A, Fanali F, Labarga JMM, Caiola MG, Canini A (2013). *Crocus sativus* L. genomics and different DNA barcode applications. *Plant Systematics and Evolution* **299**: 1859-1863.

Gismondi A, Rolfo MF, Leonardi D, Rickards O, Canini A (2012). Identification of ancient *Olea europaea* L. and *Cornus mas* L. seeds by DNA barcode. *Comptes Rendus Biologies* **335**: 472-479.

Grilli Caiola M, Caputto P, Zanier R (2004). RAPD analysis in *Crocus sativus* L. accessions and related *Crocus* species. *Biologia Plantarum* **48**: 375-380.

Gu W, Song J, Cao Y, Sun Q, Yao H, Wu Q, Chao J, Zhou J, Xue W, Duan J (2013). Application of the ITS2 region for barcoding medicinal plants of Selaginellaceae in Pteridophyta. *PLoS One* **8**: e67818.

Hall A (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Harpke D, Meng S, Rutten T, Helmut K, Frank B (2013). Phylogeny of *Crocus* (Iridaceae) based on one chloroplast and two nuclear loci: ancient hybridization and chromosome number evolution. *Molecular Phylogenetics and Evolution* **66**: 617-627.

Hebert PDN, Ratnasingham S, Dewaard JR (2003). Barcoding animal life: cytochromec oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences* **270**: 96-99.

Kerndorff H, Pasche E, Harpke D (2017). *Crocus adamii Gay* (Liliiflorae, Iridaceae) and some of its relatives in Iran. *STAPFIA* **107**: 3-10.

Kim S, Misra A (2007). SNP genotyping: technologies and biomedical applications. *Annual Review of Biomedical Engineering* **9**: 289-320.

Kress WJ, Erickson DL (2008). DNA barcodes: genes, genomics, and bioinformatics. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 2761-2762.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 8369-8374.

Kumar S, Stecher G, Tamura K (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**: 1870-1874.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Larsen B, Orabi J, Pedersen C, Ørgaard M (2015). Large intraspecific genetic variation within the Saffron-*Crocus* group (*Crocus* L., Series *Crocus*; Iridaceae). *Plant Systematics and Evolution* **301**: 425-437.

Lemey P, Salemi M, Vandamme AM (2009). The Phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing. Cambridge University Press Cambridge.

Librado P, Rozas J (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452.

Mahadani P, Ghosh SK (2013). DNA Barcoding: a tool for species identification from herbal juices. *DNA Barcodes* **1**: 35-38.

Mathew B (1982). The *Crocuses*: a revision of the genus *Crocus* (Iridaceous). BT Batsford, London.

Nemati Z, Blattner FR, Kerndorff H, Erol O, Harpke D (2018). Phylogeny of the saffron-*Crocus* species group, *Crocus* series *Crocus* (Iridaceae). *Molecular Phylogenetics and Evolution* **127**: 891-897.

Nemati Z, Harpke D, Gemicioglu A, Kerndorff H, Frank R, Blattner FR (2019). Saffron (*Crocus sativus*) is an autotriploid that evolved in Attica (Greece) from wild *Crocus cartwrightianus*, *Molecular Phylogenetics and Evolution* **136**: 14-20.

Okuyama Y, Kato M (2009). Unveiling cryptic species diversity of flowering plants: successful biological species identification of Asian *Mitella* using nuclear ribosomal DNA sequences. *BMC Evolutionary Biology* **9**: 105.

Petersen G, Seberg O, Thorsoe S, Jorgensen T, Mathew B (2008). phylogeny of the genus *Crocus* (Iridaceae) based on sequence data from five plastid regions. *Taxon* **57**: 487-499.

Rahimmalek M, Bahreininejad B, Khorrami M, Tabatabaei BES (2009). Genetic variability and geographic differentiation in *Thymusdaenensis* subsp. *daenensis*, an endangered medicinal plant, as revealed by inter simple sequence repeat (ISSR) markers. *Biochemical Genetics* **47**: 831.

Schmidt T, Heitkam T, Liedtke S, Schubert V, Menzel G (2019). Adding color to a century-old enigma: multi-color chromosome identification unravels the autotriploid nature of saffron (*Crocus sativus*) as a hybrid of wild *Crocus cartwrightianus* cytotypes. *New Phytologist* **222**: 1965-1980.

Schwei T (2015). DNASTAR Lasergene 12.2 Software Achieves High Accuracy (Press release). DNASTAR.

Seberg O, Petersen G (2009). How many Loci does it take to DNA Barcode a *Crocus*? *PLoS One* **4**: e4598.

Tammaro F (1987). Notizie storico- conturaii sullo zafferano (*Crocus Sativus* L.), nell, area mediterranea. *Micologia e Vegetazione Mediterranea* **2**: 44-59.

Vavilov NI (1951). Phytogeographic basis of plant breeding-The origin, variation, immunity and breeding of cultivated plants. *Chronica Botanica* **13**: 1-366.

Xu JY, Xu GB, Chen SL (2009). A new method for SNP discovery. *BioTechniques* **46**: 201-208.