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EVOLUTIONARY AND PHYLOGENIC RELATIONSHIPS OF WILD AND CROP SPECIES OF IRANIAN SAFFRON BY DNA BARCODING

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Abstract

DNA barcoding method was applied to identify and study the phylogenic relationships existing between 13 species of saffron collected in Iran including 4 crop and 9 wild species. PCR amplifications were performed using primers designed on the nucleotide sequence of three plastid barcode genes, comprising two protein encoding genes (rbcL and matK) and an intragenic spacer (trnH-psbA), and a nuclear region (ITS). A total of 52 sequences were obtained and registered in NCBI database. In particular, 21 of these sequences were not present in the scientific library. Nucleotide polymoprhic sites were counted for each barcode gene (rbcL, n = 16; matK, n = 15; trnH-psbA, n = 46; ITS, n = 71). Each sample could be distinguished from the others in the phylogenic trees developed based on the data obtained by single barcode gene. In addition, a phylogenic tree based only on plastid information (trnH-psbA + rbcL + matK) and another created on the data resulting from both nuclear and plastid genomes (trnH-psbA + rbcL + matK + ITS) was also generated. In general, ITS sequence, indicating high resolution at the genus and species level, appeared as the best barcode sequence of the present study. Phylogenic analysis demonstrated the genetic relationship between crop saffron and wild *Crocus* species. According to the results of this study, among 13 available sample, the wild species are *Crocus cancellatus* L. and *Crocus* sp. Eslamabad were hypothesized as the closest species to the Iranian saffron. The present investigation also indicated that the different ecotypes of *C. sativus* L. may have evolved through independent events probably due to geographic and environmental pressures.

Introduction

Crocus L. 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, picked up manually, is desiccated for the production of saffron (the world's most expensive spice) (Zubor *et al*. 2004). Other species of saffron have attracted attention as garden plants and collection species (Petersen *et al*. 2008). In 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). 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 (matK, maturase K; rbcL, RuBisCO large subunit; trnH-psbA, intragenic spacer between tRNAHisGUG gene and thylakoid membrane protein of photosystem II of Mr 3200 gene; ITS, internal transcription spacer of nuclear ribosomal DNA) has been successfully used to identify and classify several plant species (Gismondi *et al*. 2012, 2013).

Petersen *et al*. (2008), for the first time, performed phylogenetic analysis on *Crocus* genus using three nucleotide sequences encoding proteins (ndhF, accD, and rpoCl) and two other non-

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encoding regions (trnH-psbA and rpl36-rps8) of the plastid genome. Harpke *et al.* (2013) analyzed phylogenic relationships and evolution samples of *Crocus*, via trnL-F, ITS, and pCOSAt103 barcodes, demonstrating ITS/trnL-F data resulted in a monophyletic genus *Crocus*, and cloning of pCOSAt103 resulted in the detection of homoeologous copies in about one third of the taxa of section Nudiscapus, indicating an allotetraploid origin of this section. Gismondi *et al*. (2013) studied Italian and Spanish saffron species, through ITS, rbcL matK, and trnH-psbA barcodes demonstrating independent event of generation of *Crocus sativus* species in Spain and in Italy. The purpose of this study is to study the best barcode for identifying *Crocus* species and evolution as well as phylogenic relationships between some wild and crop species of Iranian saffron by DNA barcoding approach.

Materials and Methods

The plant material was collected from 9 wild *Crocus* species including *C. cancellatus, C. michelsonii, C. speciosus* deylaman, *C. speciosus* roudbar, *C. caspius, C. almehensis, C.* sp. Eslamabad-e Gharb, *Crocus* sp. harsin, and *C.* sp. sonqor, as well as four crop species including *C. sativus* fars, *C. sativus* khorasan razavi, *C. sativus* nehbandan, and *C. sativus* zabol, in different regions of Iran. Detailed information is presented in Table 1. For each specimen, 10 samples were considered and analyzed. These plant species cover most wild and crop species in Iran. The experiment was conducted at the Center of Agricultural Biotechnology, University of Zabol, Iran.

Table 1. Scientific name, specification and place of collection of saffron species used in this study.

Species	Flowering time	County	Altitude	Latitude	Longitude	Number of samples
C. cancellatus	Autumn	Kermanshah	2055	34.39	46.56	10
C. caspius	"	Rasht	161	37.44	49.96	10
C. speciosus	"	Roudbar	1340	36.50	49.43	10
C. speciosus	"	Deylaman	1573	36.52	49.05	10
C. sp.	"	Harsin	1542	34.11	47.27	10
C. sp.	"	Sonqor	1805	34.39	47.41	10
C. sp.	"	Eslamabade Gharb	1350	34.07	43.36	10
C. michelsonii	Spring	Bojnurd	1376	37.27	57.18	10
C. almehensis	"	Hamedan	1850	34.75	48.53	10
C. sativus	Autumn	Torbat-e Heydarieh	1354	35.20	59.22	10
C. sativus	"	Estahban	1720	29.07	54.02	10
C. sativus	"	Nehbandan	1100	31.48	60.01	10
C. sativus	"	Zabol	475	31.04	61.53	10

DNA was extracted from *Crocus* sp. leaves by Dellaporta method (Dellaporta *et al*. 1983). Purified DNA was amplified using PCR method in a reaction 50 µl mixture containing 2 µl of DNA template, 2 µl of each primer (Table 2) with a concentration of 10 picomolar and 25 µl of Master Mix 2x (Ampliqon, Germany). For the final volume, deionized distilled water was used. DNA was replicated using BioRad ICycler. The PCR products were separated on agarose gel 1%, using buffer TAE 1X by adding Fluoro Vue Nucleic Asid Gel Stain 2 µmolar 10000X (Smobio) and visualized under UV light. All PCR reactions were performed at least in triplicate.

All amplicons were sequenced by Macrogene (South Korea). The sequence quality was evaluated using software DNASTAR (Schwei 2015) SeqMan. The sequences were visualized by BioEdit v7.0.5 program. Barcode genes of each sample were compared using Clustal W2-Multiple sequence alignment individually (rbcL, matK, trnH-psbA, and ITS) or in combination (trnH-psbA

+ rbcL + matK; rbcL + matK + trnH-psbA + ITS). All detectable variable sites were included in the analysis. In order to certify the amplifications, the identity of each amplified sequence was compared to that of those already registered in GenBank (NCBI) using sequence alignment search tool BLAST. The results of this study resulted in the registration of 52 sequences for *Crocus* species on NCBI site (Table 3). Phylogeny analysis was performed through MEGA5 software (Tamura *et al*. 2011). In software MEGA5, all necessary parameters were set as reported in Huang *et al*. (2016). The genetic tree was drawn applying Tamura-Nei model, maximum likelihood method, where 1000 bootstrap validation system and genetic indices were examined using DNA SP. An orchid case (*Orchis mascula*, ID: JN896032, JN893527, HG800547, AY351379), extrapolated by NCBI database, was also added to this analysis as out-group species.

Table 2. Primer pair names (F: forward; R: reverse), their sequences, target gene and relative Tm (°C) used in PCRs.

Primer pair name	Sequence[a] (5'–3')	Amplified gene	Tm (°C)
rbcL F	ATGTCACCACAAACAGAGACT	rbcL	57.4
rbcL R	TGTCCATGTACCAGTAGAAGA		
matK F	GTTCTAGCACAAGAAAGTCGA	matK	56
matK R	CTCAGATTATGATATTATTGA		
trnH-psbA F	CGCGCATGGTGGATTCACAATCC	trnH-psbA	57.4
trnH-psbA R	GTTATGCATGAACGTAATGCTC		
ITS F	TCCTCCGCTTATTGATATGC	ITS	57.5
ITS R	CCTTATCATTTAGAGGAAGGAG		

[a]References: Gismondi *et al*. (2012, 2013).

Table 3. The results of this study resulted in the registration of 52 sequences of ITS, rbcL, matK and trnH-psbA for *Crocus* species on NCBI site.

ITS	rbcL	matK	trnH-psbA
KY860629	MF034878	MF066223	KY990029
KY860628	MF034877	MF066222	KY990028
KY860627	MF002371	MF066216	KY990024
KY828970	MF002366	MF066211	KY923248
KY797651	MF034876	MF066221	KY962510
KY797650	MF034875	MF066220	KY990022
KY886374	MF034874	MF066219	KY990025
KY886373	MF034873	MF066218	KY990026
KY886372	MF002369	MF066214	KY996723
KY797649	MF002372	MF066217	KY990023
KY797648	MF002370	MF066215	KY990027
KY860626	MF002368	MF066213	KY923250
KY614361	KY695236	KY695238	KY645945

The concatenated alignment of rDNA regions had a length of 293 bp (Table 4) and was the most variable dataset with 20 parsimony informative sites (PIS), while the concatenated chloroplast data (721 bp) had the fewest PIS (8). Aligning the four concatenated loci resulted in the alignment of 1014 bp length with 28 PIS (Table 4). The results of conserved DNA regions of

the ITS, chloroplast data, and combined gene in the *Crocus* family were 0.79, 0.98 and 0.93, respectively. Conservation threshold region revealed a minimum conservation length of 61, 39, and 53 bases respectively, and sequence conservation of 0.694, 0.886, and 0.836, respectively (Table 4). These conserved regions included a small part of the sequence of the above gene, 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.

Table 4. Characteristics of the included data partitions.

Character partition	Alignment length	S	H	Hd	Pi	Eta	K	C	MWL	CT
ITS	293	71	9	0.945	20	85	17.505	0.694	61	0.79
trnH-psbA + rbcL + matK	721	77	11	0.934	8	83	13.437	0.886	39	0.98
Combined	1014	148	13	0.989	28	168	30.978	0.836	53	0.93

S: The number of polymorphic positions, H: number of haplotypes, Hd: Haplotype (gene) diversity, Pi: Parsimony informative sites, Eta: total number of mutations, K: number of nucleotide differences between population or species (nucleotide divergence), C: Sequence conservation, MWL: Minimum conservation Length, CT: Conservation threshold.

Results and Discussion

The success rate of rbcL gene amplifications was calculated as 85% in *Crocus* plant samples. Also, 80% of them were successfully sequenced. The length of each amplicon was verified on agarose gel (Fig. 1). Then, all sequences were verified by BLASTn, with each one of them completely fitting other rbcL sequences of crocus species registered in GenBank. After searching the database, it was found that 9 rbcL sequences, of 13 submitted to NCBI, had not been previously recorded in the database and, therefore, they were first recorded in NCBI database (MF034876, MF034875, MF034874, MF034873, MF002369, MF002372, MF002370, MF002368 and KY695236). In the phylogenetic tree obtained from rbcL barcode data, the species related to saffron genus (*C. sativus*) were separated from the others (Fig. 2). The number of variable sites (SNPs) was counted as 16 using rbcL sequences in this study.

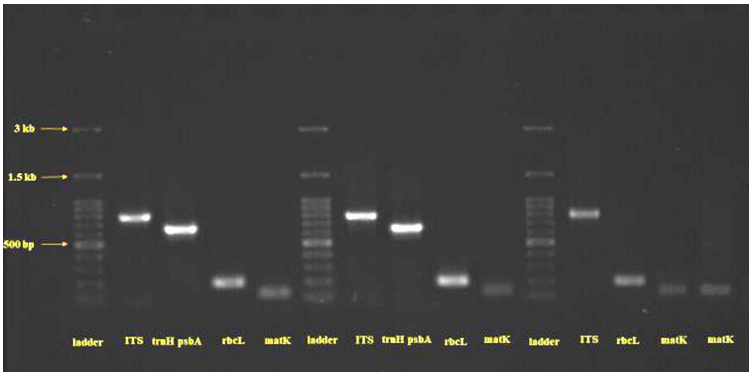

Fig. 1. DNA extracted from a saffron sample, amplified by PCR and visualized, by UV light, after separation on 1% agarose gel. trnH-psbA, rbcL, matK and ITS barcode genes were shown. Molecular weight markers were also reported (ladder lane).

The success rate of trnH-psbA gene amplification was calculated to be 92%. Here, 85% of the amplicons were successfully sequenced. The length of each amplification was verified on agarose gel (Fig. 1). The results obtained by the sequence alignment with Nucleotide-NCBI database by BLASTn confirmed the genetic identity of trnH-psbA sequences of crocus species. Here, 13 sequences of trnH-psbA for these species were deposited in the NCBI site. After searching the databases, it was found that 5 of them had not already been registered in the database (KY990023, KY990027, KY990025, KY990026 and KY996723). According to trnH-psbA sequences, the resulted phylogenic tree revealed a relationship in *Crocus* genus as reported in Fig. 3. The number of variable sites was counted to be 46 in this case.

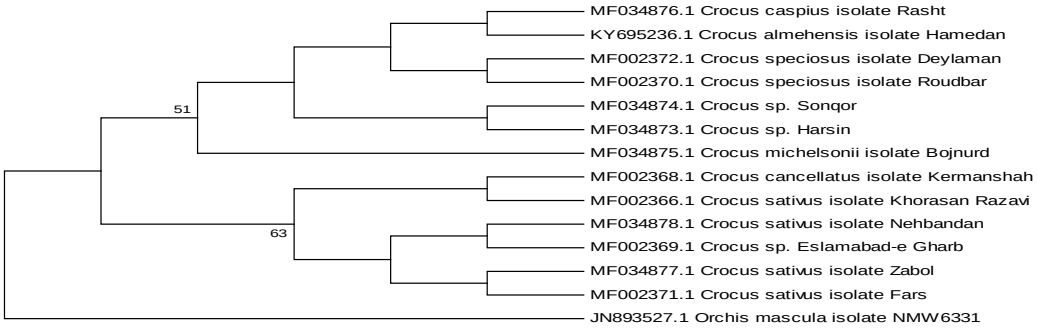

Fig. 2. Phylogenic tree showing the relationship constructed from rbcL sequences of *Crocus* species.

The matK gene amplification success rate was found to be 80% in the studied crocus samples, where 73% of them were effectively sequenced. Each amplification length was authenticated on agarose gel (Fig. 1). In order to verify the identity of the matK sequences resulting from Crocus samples, the obtained sequences were certified by employing BLASTn and through GenBank accessions. The results of the present study led to the registration of 13 matK sequences for these species on NCBI site. Having investigated the database, it was realized that 3 matK sequences observed in the present research had not been previously registered in the database and were accordingly deposited in the NCBI database (MF066214, MF066218 and MF066219). Furthermore, a phylogenic tree was produced according to the matK data (Fig. 4). Using matk sequences, the number of variable sites was counted to be 15 (SNPs).

Fig. 3. Phylogenic tree showing the relationship constructed from trnH-psbA sequences of *Crocus* species.

The ITS region amplification success rate was measured to be 94% in the examined *Crocus* plant samples, where 93% of them were efficaciously sequenced. The amplicons length was verified on agarose gel (Fig. 1). All the sequences were examined by using BLASTn and through GenBank database and all of them were attributed to other ITS sequences of *Crocus* species which had already been submitted to NCBI. The results of the present study corresponded to 13 ITS sequences available on NCBI site. After analyzing the database, the researchers observed that 4 ITS sequences found in the present study had not been recorded in the database. Therefore, these sequences were successfully deposited in the database (KY797650, KY886374, KY886373 and KY886372). According to the ITS barcode data, crocus genus species were strongly separated in the phylogenic tree (Fig. 5). It was measured that there were 71 variable sites for the specific ITS sequences obtained in this research (SNPs).

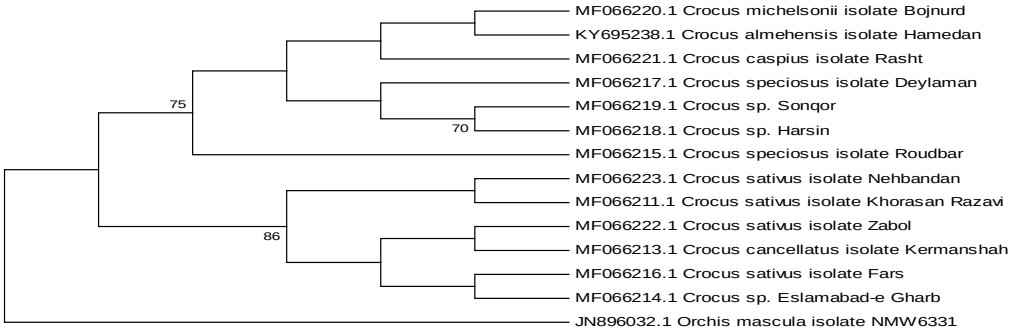

Fig. 4. Phylogenic tree showing the relationship constructed from matK sequences of *Crocus* species.

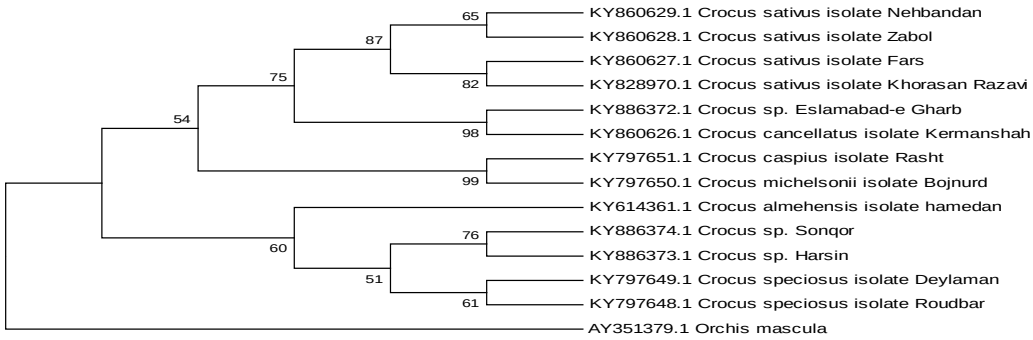

Fig. 5. Phylogenic tree showing the relationship constructed from ITS sequences of *Crocus* species.

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). Genetic identification and registration of various plant cultivars is crucial for adequate conservation and utilization of genetic resources; a monitoring which is hard to be performed by morphological features for most plants in the early stages of development (Asadi *et al*. 2015). According to the literature, a barcode should show a good resolution power at interspecific levels (Hebert *et al*. 2003).

The ITS nuclear barcode used in this study, which has high resolution at both genus and species levels, has been widely used in literature 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. Further, as it is present in multiple copies per cell, it represents a good candidate for plants' barcoding (Kress *et al*. 2005). 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).

RbcL barcode was reported to be informative in identifying *Crocus* plant samples, showing low evolution rate but high-resolution level during alignment with related genera (Kress *et al.* 2009). Although rbcL has low resolution at the species level, most studies emphasize that it can strongly contribute to interspecific studies in combination with other plant barcodes (Kress *et al*. 2005).

Considering the high success rate of amplification and sequencing of trnH-psbA barcode, this specific region was suggested as a strategic tool for plant species classification. The disadvantages of trnH-psbA sequence, even evidenced in this study, are presence of repetitive positions and inappropriate sequence alignment due to its variable length among samples. In the study of Kress *et al*. (2005), this genome site has been introduced along with ITS as a good candidate for plant barcoding.

Due to the high evolutionary rate and extensive presence among plants, matK gene has been extensively used in phylogenic studies (Rohwer 2000, Kim *et al*. 2007). At the same time, this sequence has always indicated high amplification and sequencing rate, suggesting it as an optimal target for genetic studies (Wolfe *et al*. 1987). This phylogenetic reconstruction seemed to enjoy greater discrimination power compared to rbcL and trnH-psbA data previously produced in this work.

In crop species of saffron, which are replicated through basal maternal, the plastids transmitted through the maternal cytoplasm should play an important role. Indeed, using a combination of plastid barcodes, more relevant results should be obtained. In this regard, Peterson *et al*. (2008), for the first time, performed the sequencing of five plastid genes (trnH-psbA, rp136-rps8, ndhF, accD, and rpoC1) on *Crocus* samples and used all of them to solve specific genetic questions about this genus. Similarly, Hollingsworth *et al*. (2009) successfully used a combination of plastid genes (rbcL, matK, trnH-psbA, and rpoC1) on angiosperms and gymnosperms plant groups. In particular, in order to provide more accurate information, a phylogenetic tree, including the entire trnH-psbA+rbcL+matK data was created (Fig. 6).

The phylogenic hypothesis that is based solely on the plastid sequences cannot be totally complete, as in cases of hybridization it only reflects the plastid evolution typical of only one parent (Petersen *et al*. 2008). Rohwer (2000) also states that diversity in the sequence of one or two plastid genes is not sufficient to determine the genetic linkage among species, since in some plant’s hybridization and back-crossing also occur (Okuyama *et al*. 2005).

According to all this evidence, in this study, three plastid sites including two protein encoding genes (rbcL and matK) and one intergeneric spacer (trnH-psbA), as well as a nuclear sequence, ITS, were used to reconstruct phylogenetic events in *Crocus* genus, using plant materials taken from Iran. These sites were selected because of the relatively high ability to diversify *Crocus* species (Gismondi *et al*. 2013). Meanwhile, all these sites have also been proposed as barcoding candidates for plant organisms (Kress *et al*. 2005; Peterson *et al*. 2008). Accordingly, we performed our analysis using the three plastid genes and joined them to the ITS sequence (Fig. 7).

In the present result, a dual separation can be seen. Obviously, *Orchis mascula*, as expected, positioned itself far away from all other saffron species, being the outgroup. *Crocus* genus appeared monophilic according to the present data. Accepting this hypothesis, a basic branching divided all samples in two clades, which were poorly supported and poorly sustained (bootstrap values of 52 and 63%), respectively.

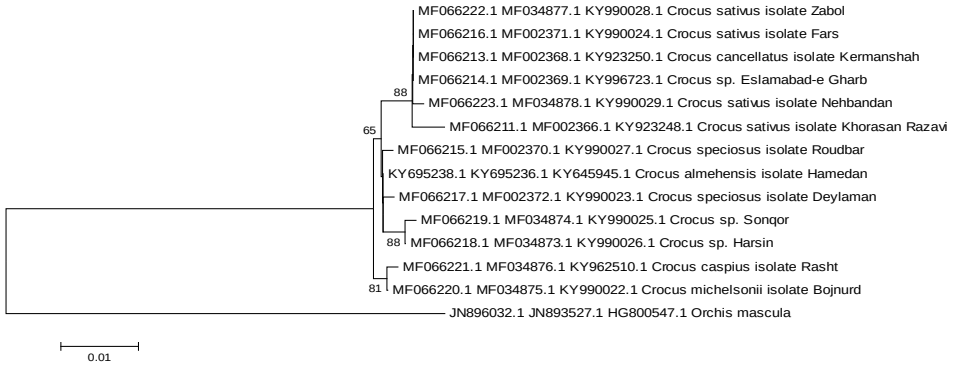

Fig. 6. Phylogenic tree showing the relationship constructed from trnH-psbA+rbcL+matK sequences of *Crocus* species.

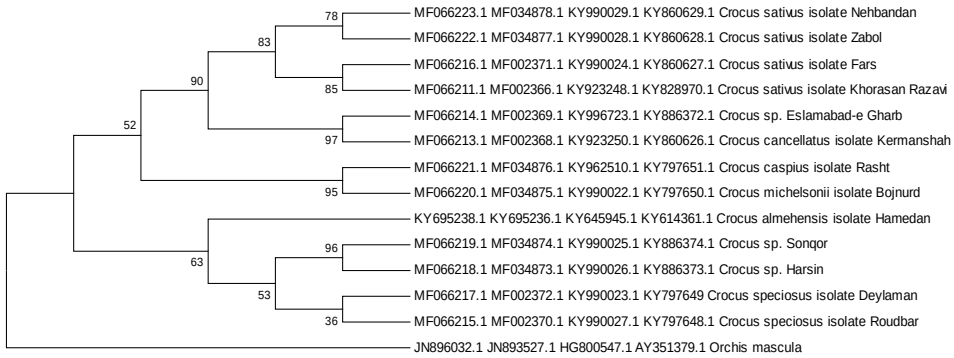

Fig. 7. Phylogenic tree showing the relationship constructed from trnH-psbA+rbcL+matK+ITS sequences of *Crocus* species.

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

The first part was made up of a strongly supported clade (bootstrap value of 90%) which included all *C. sativus* samples, with the exceptional addition of *C. cancellatus* and another unidentified *C.* species. Specifically, *Crocus sativus* series formed a single group strongly supported (Bootstrap 83%) which could be divided in media and strongly sustained clusters (bootstrap values 78 and 85%, respectively), including *C. sativus* isolate Nehbandan and *C. sativus* isolate Zabol (1st cluster), and *C. sativus* isolate Fars and *C. sativus* isolate Khorasan Razavi (2nd cluster). Since both these last clades included crop species which were replicated asexually, 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 by Gismondi *et al*. (2013). Indeed, *C. sativus* is thought to be a hybrid evolved by breeding between *C. cartwrightianus* and another *Crocus* species (Zubor *et al*.

2004). The series belonging to *Nudiscapus* and *Reticulati* subgenus (*C.* sp. eslamabad-e Gharb and *C. cancellatus*) were included into this clade with strong validation (bootstrap value of 97%).

The second portion of the first clade, presenting species from *Orientales* and *Biflori* series (*C. michelsonii* and *C. caspius*, respectively), was characterized by a strong bootstrap value (95%). Morphologically, *C. caspius* is not a typical member of *Biflori* series. Consequently, its position in the phylogenetic tree was not so surprising. *Crocus caspius* and *Orientales* series have the same unusual behavior, with capsules reaching the ground; thus, their close genetic relationship can be easily explained.

Similarly, the second clade was separated two branches including a species *C. almehensis* samples of *Biflori* series, and another poorly supported clade (Bootstrap 53%). In particular, two autumn flowering species *C. speciosus* isolate Deylaman and *C. speciosus* isolate Roudbar, belonging to *Speciosi* series, are in a very poorly supported clade (bootstrap value of 36%) associated with *C.* sp. Sonqor and *C.* sp. Harsin, according to high bootstrap values (96%).

Therefore, this last evidence could not be fully confirmed. Indeed, Petersen *et al*. (2008) stated that Reticulati and Biflori groups have revealed some of the most challenging taxonomic problems in this genus. Since *Crocus* sp. Sonqor and *Crocus* sp. Harsin samples are autumn flowering species which were collected in a geographical area close to Kermanshah and have a high bootstrap value (96%), thus they are in the same branch.

The analysis indicated a close relationship between crop saffron and four wild species of *Crocus caspius*, *Crocus michelsonii*, *C.* sp. Eslamabad-e Gharb and *C. cancellatus*. According to this study among 13 available sample, the wild species *C. cancellatus* and *C.* sp. Eslamabad-e Gharb were considered as the closest species to saffron in Iran (*C. sativus*), based on genetic distance and dendrogram obtained by analyzing four barcode genes (trnH-psbA + rbcL + matK + ITS) separately and all together.

However, according to IRAP data (Alavi-Kia *et al*. 2008), this analysis revealed a close relationship between crop saffron and three wild species, *C. almehensis, C.michelosnii,* and *C. cancellatus*. Accordingly, this study considered *C. almehensis* and *C. michelosnii* as the closest relatives of modern saffron and possible ancestors of this species. It implies the involvement of a geographical relationship in the production of these three species. 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 series *Crocus*, making an autotriploid origin of *C. sativus* from *C. cartwrightianus* very likely.

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