



Phylogeny of the saffron-crocus species group, *Crocus* series *Crocus* (Iridaceae)

Zahra Nemati^a, Frank R. Blattner^a, Helmut Kerndorff^{a,b}, Osman Erol^c, Dörte Harpke^{a,*}

^a Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany

^b São Romão, São Brás de Alportel, Portugal

^c Department of Botany, Faculty of Science, Istanbul University, Istanbul, Turkey

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ABSTRACT

Phylogenetic relationships among the taxa of *Crocus* series *Crocus* are still unclear, preventing the understanding of species diversity and the evolution of the important spice saffron (*Crocus sativus*). Therefore, we 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*. Our phylogenetic analyses resolved the relationships among all taxa of the series. *Crocus hadriaticus* and the former *C. pallasii* subspecies appeared polyphyletic. The latter deserve elevating the subspecies to species rank, while for *C. hadriaticus* a detailed study of species boundaries is necessary. Multi-locus and also genome-wide single nucleotide polymorphism data obtained through genotyping-by-sequencing placed *C. sativus* within *C. cartwrightianus* with no indication that other *Crocus* species contributed to the evolution of the triploid. Our analyses thus made an autotriploid origin of *C. sativus* from *C. cartwrightianus* very likely.

1. Introduction

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), consists of 16 autumn-flowering species. Among them is the triploid *C. sativus*, whose dried, long three-lobed stigmas are used to produce the spice saffron. The other species of this group are *C. asumaniae*, *C. cartwrightianus*, *C. hadriaticus* (with three subspecies), *C. haussknechtii*, *C. kofudagensis*, *C. macedonicus*, *C. mathewii*, *C. moabiticus*, *C. naqabensis*, *C. oreoreticus*, *C. pallasii* (with four subspecies), and *C. thomasi* (Mathew, 1982; Kerndorff and Pasche, 1994; Al-Eisawi, 2001; Mathew et al., 2009). This group was confirmed to be monophyletic by molecular phylogenetic analyses (Petersen et al., 2008; Harpke et al., 2013), but molecular investigations suggest that also *C. baytopiorum*, which was included in series *Verni* by Mathew (1982), belongs to series *Crocus* (Harpke et al., 2013, 2015). Recent investigations in the genus *Crocus* showed that several subspecies represent independent evolutionary lineages (Harpke et al., 2014, 2016) and that the subspecies of *C. pallasii* possess a rather large genetic diversity (Erol et al., 2014; Larsen et al., 2015). Therefore, Rukšans (2017) ranked these taxa as species (i.e. *C. dispathaceus*, *C. haussknechtii*, *C. turcicus*) instead of subspecies.

The high interest in the origin of saffron resulted in several

phylogenetic studies carried out within series *Crocus*. Still, relationships among the taxa of series *Crocus* are not yet clarified because either not all known taxa were investigated within single analyses (Grilli Caiola et al., 2004; Zubor et al., 2004; Tsaftaris et al., 2011; Namayandeh et al., 2012; Erol et al., 2014; Nemati et al., 2014; Alsayed et al., 2015), too low variability was found within the analyzed sequence regions (Petersen et al., 2008), only single/very few individuals per species were included (Petersen et al., 2008; Harpke et al., 2013), or the application of marker methods was not appropriate to uncover relationships among distantly related taxa within the series (RAPD: Grilli Caiola et al., 2004; AFLP/SSR: Erol et al., 2014; Nemati et al., 2012, 2014; Larsen et al., 2015; IRAP: Alsayied et al., 2015). Thus, the origin of saffron is not yet clear. Most often *C. cartwrightianus* (Grilli Caiola et al., 2004; Harpke et al., 2013; Larsen et al., 2015), *C. hadriaticus* (Tsaftaris et al., 2011), *C. pallasii* (Harpke et al., 2013; Erol et al., 2014; Alsayed et al., 2015), and/or *C. thomasi* (Tsaftaris et al., 2011) have been hypothesized to be its closest wild relative or progenitor.

Generally, the genus *Crocus* is known for variable chromosome numbers. With the exception of the triploid *C. sativus* ($2n = 3x = 24$), which is male sterile and only propagated vegetatively (Brighton, 1977; Mathew, 1977), the species in series *Crocus* are probably all diploid. The most common chromosome numbers within the series are $2n = 16$ (*C. cartwrightianus*, *C. hadriaticus*, *C. haussknechtii*, *C. oreoreticus*, *C.*

* Corresponding author.

E-mail address: harpke@ipk-gatersleben.de (D. Harpke).

thomasi) and $2n = 14$ (*C. dispathaceus*, *C. moabiticus*, *C. naqabensis*, *C. pallasii*), respectively (Brighton et al., 1973; Karamplianis et al., 2013). Although *C. asumaniae* ($2n = 26$) and *C. mathewii* ($2n = 70$) have higher chromosome numbers (Brighton et al., 1973) their genome sizes suggest that they might be still diploids (Erol et al., 2014). *Crocus turcicus* is reported with $2n = 12$ chromosomes and *C. haussknechtii* seems to occur also with this chromosome number (Brighton, 1977; Sanei et al., 2007).

To obtain a resolved phylogeny we analyzed two chloroplast and three nuclear markers in all taxa of series *Crocus* with the aim to answer the following questions. (i) What are the phylogenetic relationships among the taxa of series *Crocus*? (ii) Do the *C. pallasii* subspecies, which were recently elevated to species rank, represent independent evolutionary lineages? For saffron and its closest relatives genotyping-by-sequencing (GBS; Elshire et al., 2011) was carried out to address the question (iii) who are the closest relatives and potential progenitors of saffron, *C. sativus*?

2. Materials and methods

2.1. Taxon sampling

Fifty two individuals, representing 16 species of series *Crocus* and the two outgroup species *C. vernus* and *C. cf. tauri* were included in the multi-locus analysis. Based on this analysis, 14 individuals comprising six *C. cartwrightianus*, two *C. hadriaticus*, three *C. oreoreticus* and three *C. sativus* were included in a GBS analysis to better define the position of *C. sativus* among its closest relatives. For seven of them the samples included in the multi-locus analysis could be used, while some different individuals had to be chosen when not enough DNA was available for

the GBS analysis. Vouchers of the analyzed taxa are stored in the herbaria GAT, ISTF, MAIC or University of Niš (Table 1).

2.2. Molecular methods

The extraction of genomic DNA and amplification of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) and external transcribed spacer (ETS), and the chloroplast *trnL-trnF* and *matK-trnK* regions were carried out according to Harpke et al. (2014). As a nuclear single-copy marker we included the topoisomerase 6 (TOPO6) subunit B intron 11 (Blattner, 2016) that was obtained using the PCR primers *Croc_Top6_11_f* (5'-CAGTTTCTTCAACACGAATTT-3') and *Croc_Top6_12-r* (5'-ATGGATTCTAACTATTTGTTGGG-3). All nuclear markers were direct Sanger sequenced. PCR products were cloned and sequenced [six clones per individual; following Harpke et al. (2015)] where direct sequencing revealed polymorphic sequence positions or length differences. All ITS sequences were checked for the presence of pseudogenes according to Harpke and Peterson (2008a,b). All newly obtained sequences were submitted to the EMBL nucleotide database and are accessible through sequence accession numbers LT991589-LT991638, LT991641-LT991747, LS398293-LS398342, LS398365-LS398415.

To obtain genome-wide single-nucleotide polymorphisms (SNPs), genotyping-by-sequencing (GBS) analyses were conducted for 14 individuals comprising *C. cartwrightianus*, *C. hadriaticus*, *C. oreoreticus* and *C. sativus*. For the library preparation 200 ng of genomic DNA were used and cut with the two restrictions enzymes *Pst*I-HF (CTGCAG, NEB Inc., Ipswich, UK) and *Msp*I (CCGG, NEB Inc.). Library preparation and individual barcoding followed Wendler et al. (2014). The libraries were sequenced on an Illumina HiSeq 2000/2500 (100 bp single-end reads).

Table 1
Studied plant materials.

Taxon	Origin and sample ID ^a	Herbarium; ID
Series <i>Crocus</i> L.		
<i>C. asumaniae</i> B. Mathew & T. Baytop	Turkey, Antalya (cultivated); cr1366	GAT; 47,271
	Turkey, Antalya; cr2976, cr2977	ISTF; 41,072
<i>C. baytopiorum</i> B. Mathew	Turkey, Denizli (cultivated); cr2070- cr2072	GAT; 7187
<i>C. cartwrightianus</i> Herb.	Greece, Crete; cr1909, 2663 ^b	GAT; HKEP1517
	Greece, Crete; cr1936, cr3269 ^b	GAT; HKEP1525,
	Greece, Syros; cr2635	GAT; HKEP1443
	Greece, Paros; cr1265, cr2783 ^b	GAT; HKEP1445
	Greece, Anafi; cr2938, cr2939 ^b	GAT; HKEP1601
	Greece, Astypalaia; cr2821	GAT; HKEP1609
<i>C. dispathaceus</i> Bowles	Turkey, Mersin; cr2966, cr2999	ISTF; 40,663
	Turkey, Mersin; cr2970, cr2971	ISTF; 40,664
<i>C. hadriaticus</i> Herb.	Greece, Pindus (cultivated); cr2420, cr2421	GAT; 47,247
	Greece, Peloponnese; cr2644-cr2647	GAT; 7220
<i>C. haussknechtii</i> (Boiss. & Reut. ex Maw) Boiss.	Iran, Sanandaj (cultivated); cr1567-cr1569	–; Kv140
<i>C. kofudagensis</i> Rukšāns	Turkey, Antalya (cultivated); cr3335	GAT; JJJ-024
<i>C. macedonicus</i> Rukšāns	Greece, Macedonia; cr1344, cr1346	GAT; 20,314
<i>C. mathewii</i> Kernd. & Pasche	Turkey, Antalya; cr2984	ISTF; 40,183
<i>C. moabiticus</i> Bornm.	Jordan (cultivated); Rik11; cr1717, cr1719	–; Rik11
<i>C. naqabensis</i> Al-Eisawi & Kiswani	Jordan, Ma'an Governorate (cultivated); cr2108, cr2109	GAT
<i>C. oreoreticus</i> B.L. Burt	Greece, Crete; cr1373, cr1374	GAT; KR23
	Greece, Crete; cr2674, cr2579 ^b , cr2675 ^b	GAT; HKEP1585
<i>C. pallasii</i> Goldb.	Ukraine, Crimea (cultivated); cr1575-cr1577	GAT; 47,264
	Macedonia, Kumanovo; cr795-cr797	Niš; s.n.
<i>C. sativus</i> L.	Iran (cultivated); cr1573	GAT
	Spain (cultivated); cr3330	GAT; 163
	France (cultivated); cr3327	GAT; 160
<i>C. thomasi</i> Ten.	Croatia, Dalmatia; cr1350	–; IS0501
	Italy, Apulia; cr527	GAT; HKEP1326
	Italy, Apulia; cr3091-cr3093	GAT; HKEP1614
<i>C. turcicus</i> (B. Mathew) Rukšāns	Turkey, Gaziantep; cr2970, cr2971	ISTF; 40,665
Outgroups		
<i>C. cf. tauri</i> Maw	Turkey, Upper Euphrates; cr38	GAT; 7143
<i>C. vernus</i> Hill	Switzerland, Airolo; cr305	GAT; 30,292

^a Accessions indicated as 'cultivated' were regrown from seeds or corms collected in the wild.

^b DNA substitute for GBS analyses.

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