Plant Syst Evol (2013) 299:1859–1863
DOI 10.1007/s00606-013-0841-7

ORIGINAL ARTICLE

Crocus sativus L. genomics and different DNA barcode applications

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Received: 12 December 2012 / Accepted: 11 May 2013 / Published online: 23 May 2013

Abstract *Crocus sativus* L. is a sterile species (3*n*) whose origin has not been yet clarified. A lot of morphological studies supported the theory that it would have been originated from the evolution, or the hybridization, of other *Crocus* exemplars, especially *C. thomasii*, *C. hadriaticus* and *C. cartwrightianus. Crocus sativus* stigmas are saffron raw source but, because of their high economic value, sometimes this spice is adulterated. By the application of the DNA barcode technique, we analyzed different *Crocus* species genomes and we partially clarified some aspects of the phylogeny of this genus: in particular, *C. sativus* possible genetic derivation was elucidated. Our results also showed that different *C. sativus* species might have evolved by independent events, probably due to several geographical pressures. We demonstrated that barcoding method, usually adopted for interspecific taxonomic identification, could be also applied to intraspecific and population studies. Finally, we proposed this molecular approach as scientific tool able to discriminate and certificate saffron authenticity.

Keywords Adulteration · *Crocus sativus* L. · DNA barcode · Phylogeny · Saffron

Introduction

Crocus is a monocotyledon herbaceous plant genus belonging to the Iridaceae family. It includes about 85 species distributed between Mediterranean Europe and Western Asia (Mathew 1982). The most important species of this group is *Crocus sativus* L. because its dried stigmas represent the raw source of the saffron spice. *Crocus sativus* is known as a cultivated plant; in fact, it only propagates vegetatively by corms because of the incapacity to produce fertile pollen and, therefore, seeds. The infertility of this plant is due to an irregular meiosis process caused by its triploid genome (Chichiriccò 1999; Gresta 2007; Grilli Caiola 2004, 2005). In order to determine *C. sativus* botanical origin, for a long time, scientists essentially based their hypothesis on morphological observations. Only a few molecular approaches were applied to this question that remains still unresolved. However, literature data support the theory according to which *C. sativus* species might have been generated by the hybridization between *C. cartwrightianus* and *C. hadriaticus* or by the evolution (triploid mutation) of a *C. cartwrightianus* or a *C. thomasii* (Grilli Caiola and Canini 2010). The flower structure of this species is made up by a perigonium of six violet tepals, an androecium of three stamens with yellow sagittate anthers and a gynoecium that presents a characteristic red stigma divided in three filaments. The plant blooms only once a year and the harvest of stigmas, by manual picking, should be performed within very short time; for these reasons, saffron is the most expensive spice in the world (Sano and Himeno 1987). *Crocus sativus* stigmas are characterized by

Electronic supplementary material The online version of this article (doi:10.1007/s00606-013-0841-7) contains supplementary material, which is available to authorized users.

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the presence of sugars, minerals, fats, vitamins and secondary metabolites. Terpenes, flavonoids, anthocyanins and carotenoids only determine spice color, aroma and taste (Lonzano et al. 1999; Sampathu et al. 1984). The use of saffron dates back to ancient Egypt, Greece and Rome where it was used as dye, perfume, spice for culinary purposes and traditional drug in the treatment of numerous illnesses (Abdullaev et al. 2004; Tavakkol-Afshari et al. 2008). Since the middle ages, the high economic value of saffron has induced its adulteration with natural or synthetic substances (Carmona and Alonso 2003; Maish 1885). To avoid these fraudulent practices and for the valorization of this spice, international standards (ISO/Technical Specification 3632 2003) were applied to define saffron quality. According to these rules, spectrophotometric and chromatographic analyses scientifically determine spice color, taste and aroma, although they do not allow a complete identification of contaminants and counterfeits (Caballero-Ortega et al. 2007; Sabatino et al. 2011; Zalacain et al. 2005). Recently, alternatively to empiric and imprecise morphological study, new molecular techniques were used to recognize authentic plant exemplars, with respect to other species. In particular, DNA barcode method employs standard nucleotide sequence analysis (maturase K, *mat*K; ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit, *rbc*L; intragenic spacer between tRNAHisGUG gene and photosystem II thylakoid membrane protein of *M*r 32.000 gene, *trn*H-*psb*A; internal transcribed spacer of nuclear ribosomal DNA, ITS) for botanical species identification (Gugerli et al. 2005; Gismondi et al. 2012; Kress and Erickson 2007, 2008; Seberg and Petersen 2009; Yao et al. 2009). On this basis, focal point of this work was *C. sativus* genetic characterization, to clarify and determine further the possible origin of this infertile species. On the other hand, we also proposed two different scientific applications of DNA barcode technique: intraspecific discrimination and saffron authenticity determination.

Materials and methods

Plant material

Crocus sativus corms were obtained from Civitaretenga cultivars (AQ, Italy) and grown in the Botanical Garden of the University of Rome "Tor Vergata". Other *C. sativus* L. (ES), *Merendera pyrenaica* (Pourr.) P. Fourn., *C. carpetanus* Boiss. & Reuter and *C. serotinus* Salisb. plants, used for the genetic study, were collected in Spain. *C. cartwrightianus* Herb., *C. hadriaticus* Herb. and *C. thomasii* Ten. species derived, as the Italian *C. sativus* (IT), from the Botanical Garden of the University of Rome "Tor Vergata". For each species 15 exemplars were considered and analyzed. Plant species were chosen according to their probable evolutionary relationship with *C. sativus* (Grilli Caiola and Canini 2010).

DNA extraction

DNA was extracted from *Crocus* flowers (or from saffron commercial sachet) by NucleoSpin Plant II kit (Macherey–Nagel). Briefly, vegetal material (100 mg) was homogenized, using mortar and pestle in liquid nitrogen, resuspended in kit lysis buffer (containing CTAB, cetyl trimethylammonium bromide and RNAse A) and incubated for 30 min at 65 °C. Then, sample was filtered and DNA was precipitated, by adding a solution with ethanol and guanidine hydrochloride. Nucleic acid was bound to a specific membrane, washed twice with isopropanol and ethanol and finally eluted.

PCR amplification and gel electrophoresis

PCR-based amplification of the purified DNA was carried out in a 50-μL reaction mixture; it contained 50–200 ng template DNA, 2.5 U JumpStart REDAccu Taq LA DNA polymerase (for high fidelity PCR, Sigma-Aldrich), 20 μM of each primers (Table 1, Sigma-Aldrich), 0.2 mM each dNTP, 1X Taq LA DNA polymerase buffer, 3 mM $MgCl_2$ and 5 % (v/v) DMSO. Amplification of the DNA was performed using a Biorad (ICycler) thermocycler with following parameters: (a) initial denaturation at 95 °C for 4 min; (b) 35 cycles of denaturation at 95 °C for 1 min, primer annealing at the adequate temperature for each primer pair (below described) for 1 min and extension at 68 °C for 2 min; (c) final extension at 68 °C for 15 min. The reaction was stored at 4 °C. PCR products were fractionated on 1 % (w/v) agarose gel, using 1X TAE buffer (40 mM Tris; 1 mM EDTA; 20 mM acetic acid; pH 8.5) containing 10 mg/mL ethidium bromide, and visualized and photographed under UV light (Gel Doc 2000 BIO-RAD). All PCR reactions were performed in triplicate.

Sequencing and data analysis

PCR products (3 μL) were treated with ExoSAP-IT (Affymetrix) for 15 min at 37 °C, to remove unused primers and nucleotides. 1.5 μL of BigDye (Applied Biosystems) and 1.5 μL of forward primer were added to samples. Then, they were subjected to PCR (25 cycles of denaturation at 96 °C for 10 s, annealing 50 °C for 5 s and final extension at 64 °C for 4 min). DNA was precipitated in samples by adding 50 μL of 95 % ethanol and 2 μL of 3 M sodium acetate pH 5.2. After incubation at room temperature for 15 min, samples were centrifuged for 30 min at 2,500 rpm/min.

Table 1 Primer pair names (F: forward; R: reverse), their sequences, target gene, size (in base pairs) of amplified regions and relative T_m (°C) used in PCRs

Primer pair name amplified region (BP)	Sequence[a] (5′–3′)	Amplified gene	T_m (°C)
*rbc*L F	ATGTCACCACAAACAGAGACT	*rbc*L	53
*rbc*L R	TGTCCATGTACCAGTAGAAGA		
*mat*K F	GTTCTAGCACAAGAAAGTCGA	*mat*K	52.3
*mat*K R	CTCAGATTATGATATTATTGA		
*trn*H-*psb*A F	CGCGCATGGTGGATTCACAATCC	*trn*H-*psb*A	53.9
*trn*H-*psb*A R	GTTATGCATGAACGTAATGCTC		
ITS F	TCCTCCGCTTATTGATATGC	ITS	52.9
ITS R	CCTTATCATTTAGAGGAAGGAG		

[a] References: Gugerli et al. (2005), Gismondi et al. (2012), Kress and Erickson (2007, 2008), Seberg and Petersen (2009), Yao et al. (2009)

Pellets were resuspended in 150 µL of 70 % (v/v) ethanol and then centrifuged for 10 min at 13,000 rpm/min. Finally, DNA was resuspended in 20 µL of 100 % formamide. Sequencing analysis was performed using 3100 Avant Genetic Analyzer (HITACHI, Applied Biosystem). The sequences obtained from the 15 exemplars of the same species did not show nucleotide differences. Sequences (for each different sample) were visualized by BioEdit v7.0.5 program and the combination of all analyzed barcode regions (*rbc*L + *mat*K + *trn*H-*psb*A + ITS) were aligned using ClustalW2-Multiple Sequence Alignment. All detected variable sites were included in the analysis and no ambiguously aligned region was revealed. Phylogenetic analysis was performed by phylip-3.69 software: in particular, DNAPARS program for Maximum Parsimony study (Templeton et al. 1992) and SEQBOOT for the application of Bootstrap statistic method on results (1,000 replicas) (Efron and Tibshirani 1986). Strict consensus phylogenetic tree, obtained by phylip-3.69, was visualized with TreeView32 program. Sequence identity percentage and nucleotide sequence comparison with scientific nucleotide database were performed using Basic Local Alignment Search Tool (Blast).

Results and discussion

Crocus genus and, in particular, *C. sativus* species, whose dried stigmas are the source of saffron, have captured human attention since the ancient past. This spice increased its economic value along the time and, nowadays, it is produced worldwide with a million dollar commercial cost (Negbi 1999). *Crocus sativus* triploidy and infertility motivated scientists to research its genetic origin. Many morphological studies, but only a few molecular approaches (RAPD and AFLP), were conducted to clarify *Crocus* phylogeny: no absolute and clear results were obtained (Grilli Caiola and Canini 2010), except for Petersen et al. (2008) that, for the first time, performed the sequencing of five plastid genes on *Crocus* samples to solve this question. To confirm and clarify further *C. sativus* putative ancestors, DNA barcode method was re-applied in the present work, this time analyzing other plastidial (*rbc*L, *mat*K and *trn*H-*psb*A) and also nuclear (ITS) barcode regions, and new information were gained. Hispanic *C. sativus* (ES), *C. carpetanus* and *C. serotinus* plants and Italian *C. cartwrightianus*, *C. hadriaticus*, *C. thomasii* and *C. sativus* (IT) species were subjected to genetic characterization. Total DNA was extracted from samples and used, as template, for PCR amplifications. Barcode genes were analyzed in all samples. Resulting sequences were aligned and compared to create a partial

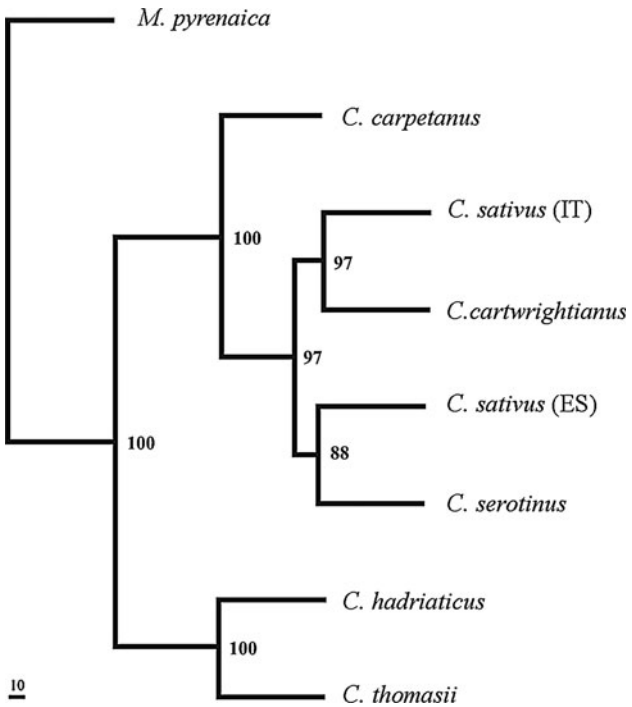

Fig. 1 Strict consensus phylogenetic tree, based on maximum parsimony principle, describing partial *Crocus* genus phylogeny. Genetic relationships were obtained analyzing, for each sample, the addition of all studied barcode genes (*rbc*L + *mat*K + *trn*H-*psb*A + ITS). Bootstrap value was also reported to each ramification

phylogram on *Crocus* genus (Fig. 1). An Iberian *M. pyrenaica* individual was also added to this analysis and considered as outgroup species. Phylogenetic tree clearly showed the early separation of *C. hadriaticus* and *C. thomasii* from the other specimens, as suggesting a very ancestral origin of these two species. Subsequently, *C. carpetanus* turned away from the rest of *Crocus* group that could be divided in two sub-clades: the first one including *C. sativus* (IT) and *C. cartwrightianus* and the second one containing *C. sativus* (ES) and *C. serotinus*. Very high bootstrap values (>88 %) supported these results giving them an elevated reliability. Phylogenetic relations, found in this analysis (Fig. 1), suggested that *C. sativus* (IT) would have had a close common forefather with *C. cartwrightianus*. These data supported the principal scientific theory, also demonstrated by Petersen et al. (2008), that *C. sativus* (IT) would have come from *C. cartwrightianus* but rejected the possibility that *C. thomasii* and *C. hadriaticus* could be *C. sativus* progenitors. Contrary to literature data (Petersen et al. 2008), *C. carpetanus* appeared genetically more distant from *C. thomasii* and *C. hadriaticus* than remaining *Crocus* species. Moreover, this study hypothesized that *C. cartwrightianus* would not have been *C. sativus* (ES) precursor because they were not closely associated, unlike *C. sativus* (IT): so, both *C. sativus* species would have been generated by two independent evolutionary processes, probably dictated by different geographical pressures. This important result suggested the possibility, never supposed, that some *C. sativus* species would have had different origins. DNA barcode technique is a very accurate species-level discriminating tool, in molecular biology, because of the high target sequence mutation rate during the evolution (Gismondi et al. 2012). Moreover, this research was very innovative because it demonstrated the existence of barcode gene intraspecific nucleotide divergences, contrary to literature reports (Shneer 2009). Italian (IT) and Spanish (ES) *C. sativus* species were specifically compared, by the single alignment of their amplified barcode sequences, to identify possible intraspecific differences at nucleotide level. Nucleotide identity percentages of each analyzed gene, for these two taxa, were reported in Table 2. *Rbc*L and *mat*K sequences proved to be highly conserved (100 % of identity); conversely, *trn*H-*psb*A and ITS regions presented some genetic divergences, respectively, of 1 and 10 %. Genetic differences, found between *C. sativus* IT and ES plants, might be due to different habitat selections, although they belonged to the same species. In particular, ITS resulted the most variable and informative region in *C. sativus* species direct comparison (Table 2) but also in the previous study on *Crocus* genus (data not shown). These results supported the hypothesis that DNA barcode approach could also be used for molecular intraspecific discriminating analysis (i.e. population studies). The high economic value of saffron induced traders to adulterate it. Because of the absence of a specific technique able to discriminate saffron authenticity, in alternative to Javanmardi et al. (2011) suggestion (the use of RAPD technique) and as also recently proposed for the discrimination of other plant products (Guo et al. 2011; Kumar et al. 2011), we suggest the DNA barcode method as scientific tool to certificate and trace saffron origin. We extracted DNA from a saffron commercial sachet and used it as template to amplify a barcode region (in this case, for example, we have amplified ITS sequence) (Fig. 2). Pure saffron should only present *C. sativus* DNA; the sequencing of the ITS amplicon, previously obtained in this experiment, allowed us to discriminate and identify the authenticity of saffron sample from possible contaminants, by its matching with plant sequence accessions registered in the scientific nucleotide database. We observed, in fact, the maximum identity (98 %) of the ITS sequence only with *C. sativus* accessions (Electronic Supplementary Material). The positive result, reported in this preliminary study, invited us to divulgate the application of DNA barcoding with the aim to reveal and denote spice illegal adulterations.

Table 2 Identity percentages of Italian and Spanish *Crocus sativus* barcode sequences

	*rbc*L	*mat*K	*trn*H-*psb*A	ITS	Total
C. sativus (IT) vs. *C. sativus* (ES)	100 %	100 %	99 %	90 %	97.25 %

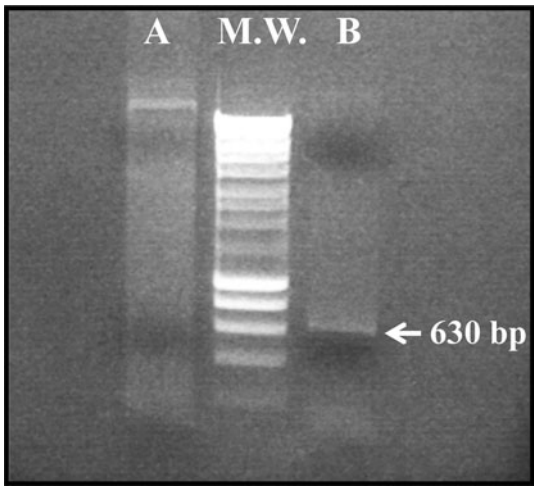

Fig. 2 DNA extracted from a saffron commercial sachet and visualized, by UV light, after separation on 1 % agarose gel (**a**). It has been used as template for ITS barcode gene amplification (**b**). *MW* molecular weight

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