Research Article

Identification of *Crocus sativus* and its Adulterants from Chinese Markets by using DNA Barcoding Technique

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Background: Saffron (*Crocus sativus* L.) is a common but very expensive herbal medicine. As an important traditional medicine, it has an outstanding effect in treating irregular and painful menstruation. Recently, the over-demand tendency of saffron results in an unusual phenomenon in the medicinal markets. Adulterants and saffron-like substitutes are intentionally mixed into medicinal markets and pharmacies or online stores, affecting drug safety and food quality.
Objectives: Our study aimed to identify saffron from its adulterants via DNA barcoding.
Materials and Methods: Samples (13 saffron + 4 others containing *Carthamus tinctorius* or *Chrysanthemum x morifolium*) obtained from 12 different provinces of China. Through DNA barcoding, samples were compared using three candidate markers, *trn*H-*psb*A, *rbc*L-a and ITS2.
Results: *trn*H-*psb*A and *rbc*L-a were capable of distinguishing different accessions. ITS2 could identify samples even at intra-specific level. According to these three barcodes, four samples were identified saffron-like substitutes.
Conclusions: The adulterant rate in Chinese markets reaches as high as 33.33% that may cause health risks and further may reduce saffron efficacy once is being used as herbal remedy. In order to make a distinction between *C. sativus* with other genera as adulterants, DNA barcoding is suggested.

Keywords: Adulterant identification; Classification; *Crocus sativus*; DNA barcoding

1. Background

Crocus sativus L., commonly known as saffron, or *Zang-Hong-Hua* in Chinese, is a perennial stemless herb of Iridaceae (1). Saffron originally grew in Iran, India, Spain, Greece. It has been also successfully cultivated in various places in China for many centuries (2). Since ancient times, the dried stigmas of *C. sativus* have been considered as the most precious and expensive medicine (3, 4). In folk, it is commonly used for its analgesic and/or sedative properties. In traditional Chinese medicine (TCM), it has been used as an anti-anginal (2). The other pharmacological activities of saffron, such as anti-cancer, anti-inflammatory, and anti-atherosclerotic activities have also been reported (5, 6). The major active constituents responsible for these biological activities are two natural carotenoids, crocin and crocetin (7). For example, crocetin, an antioxidant, has memory enhancing effects in aged mice (8) and crocin demonstrated to be useful in pharmacological alleviation of cognitive defects (9). Even saffron odor may be effective in treating menstrual distress (10). In addition, people use saffron to season their food for its flavor and taste.

However, due to the limited production and labor-intensive process of harvesting, it is sold at an extremely high price around $2,000/kg. Due to its high demand and price, adulterations are common with the crude drugs of *Carthamus tinctorius*, *Chrysanthemum x morifolium*, *Zea mays* and *Nelumbo nucifera* (11-14). These plants have fiber-type structure similar to *C. sativus* anthers that makes the differentiation rather impossible by just morphological investigation. However, the contents of the active compounds of these adulterants are distinct from that of saffron that obviously reduce the efficacy of *C. sativus*. Thus, for the benefits of consumers and the quality control of

saffron, its correct identification is a must.

Although herbal medicine has a great sayings in pharmalogical sciences, it suffers from contaminations that may occur through the processing steps during collection, drying and grinding (15). To check the authenticity of a medicine, physical, chemical, and molecular marker techniques are amongst the suggested methods. Although physical methods such as microscopy are easy to operate, the amount of helpful data to check the purity of product is not enough. Chemical methods such as chromatography or spectroscopic analysis are time-consuming and cost-effective and may be strongly influenced by the experimental conditions. DNA barcoding on the other hand that takes advantage of using particular nuclear or organellar genome sequences as genetic markers seems a way forward in determination any impurities. This technique is rapid, sensitive, accurate and simple and in our case is efficient for species identification (16-18).

Recently, this technique has been widely applied to discriminate medicinal herbs of TCM, such as *Codonopsis Radix*, *Paeoniae Radix Rubra*, *Clematis chinensis*, using ITS2 (19-21). *Sabia* species and *Radix astragali* were correctly identified by the combined DNA barcodes (22, 23). Therefore, use of DNA barcodes in identification of medicinal herbs is recommended.

According to previous saffron identification studies, most materials came from wild or garden species (24, 25). The authentication of saffron from medicinal markets or drugstores was rarely tested (26). In this study, we employed three DNA barcodes, including *trn*H-*psb*A intergenic spacer (*trn*H-*psb*A), large subset of ribulose-bisphosphate carboxylase (*rbc*L−a) and nuclear internal transcribed spacer 2 (ITS2), to differentiate saffron from its adulterants by sequences diversity analysis.

2. Objectives

Our objectives were not only to investigate and detect the market substitution of saffron, but also to identify what the adulterants were as well as to remind people to be cautious to select.

3. Materials and Methods

3.1. Plant Materials

All 13 samples were called *Zang-Hong-Hua* in Chinese, which meant they had been used or sold as saffron (*C. sativus*). They were collected from 12 different provinces. Among them, only two samples were identified as *C. sativus* (S1) and *Carthamus tinctorius* (C1) respectively, based on their morphological characteristics. Other 11 samples unidentified, labeled saffron were bought from medicinal markets and drugstores during 2012-2013 (Table 1). These samples were from commercial products, except the one from Nanjing Botanical Garden Mem. Sun Yat-Zen. The familiar adulterants, such as *Carthamus tinctorius* (C2), *Nelumbo nucifera* (NN), *Crysanthemum x morifolium* (CM) and *Zea mays* (ZM), their relevant gene sequence numbers downloaded from Genbank were also listed in Table 1.

3.2. DNA Isolation, Amplification and Sequencing

Total genomic DNA was isolated from the dried stigma (20 mg each). The stigma of each sample was immersed in liquid nitrogen and crushed into a fine powder. Plant Genome DNA Kit (DP305, Beijing, China) was used for DNA extraction. Quality of the extracted DNA was determined using gel electrophoresis.

Amplifications of the three loci *trn*H-*psb*A, *rbc*L-a, and ITS2 were obtained. The primer pair names, primer sequences, and reaction conditions (27-30) used are listed in Table 2.

Polymerase chain reaction (PCR) amplifications of the three candidate DNA barcode loci carried out in a T100 Thermal Cycler (Bio-Rad, United States) were performed in a 25 μL reaction mixture containing 2.5 μL of 10× PCR buffer, 2.0 μL of 25 $mmol.L^{-1}$ $MgCl_2$, 2.5 μL of 2.5 $mmol.L^{-1}$ dNTPs, 0.5 μL of each primer (10 $mmol.L^{-1}$), 0.5 U of Taq DNA polymerase, 1 μL of genomic DNA (~30 ng) and ddH_2O. PCR products were examined using 1% agarose gel electrophoresis in 1×TAE buffer at 100 Volt for about 30 min. Gel images were obtained using Gel documentation imaging system (Bio-Rad, United States). Purifying and sequencing were completed by Biosune Co., Ltd (Beijing, China).

3.3. Sequence Alignment and Analysis

Sequences were assembled and aligned by Clustal X software (Version 1.83) and adjusted manually in CodonCode Aligner (Version 4.2.3). The nucleotide sequences data of the partial *trn*H-*psb*A spacer, *rbc*L-a and ITS2 genes were submitted to Genbank. Genetic distance was computed using MEGA5.2 by Kimura two-parameter (K2P) model (31). Based on each locus, maximum parsimony trees were built with bootstrap testing of 1000 replicates. The DNA sequences were then deposited in Genbank.

Table 1. Plant samples used in the study

Latin name	Sample ID	Origin	Genbank No.
Crocus sativus	S1	Nanjing Botanical Garden Mem.	KF88648, KF88658, KF886671
		Sun Yat-Sen, Jiangsu	
(To be determined)	S2	Shanghai Plantation, Shanghai	KF886649, KF886659, KF886672
		Beijing Tongrentang Pharmacy,	
(To be determined)	S3	Beijing	KF886650, KF886660, KF886673
		Lhasa Pharmacy, Xizang	
(To be determined)	S4	Bozhou medicinal market, Anhui	KF886651, KF886661, KF886674
(To be determined)	S5	Chengdu medicinal market,	KF886652, KF886662, KF886675
(To be determined)	S6	Sichuan	KF886653, KF886663, KF886676
		Nanjing Pharmacy, Jiangsu	
(To be determined)	S7	Dalian, Liaoning	KF886654, KF886664, KF886677
(To be determined)	S8	Baoding, Hebei	KF886665
(To be determined)	S9	Yantan, Shandong	KF886666, KF886678
(To be determined)	S10	Zhangzhou, Fujian	KF886667, KF886679
(To be determined)	S11	Kunming, Yunnan	KF886655, KF886668, KF886680
(To be determined)	S12	Urumqi, Xinjiang	KF886656, KF886669, KF886681
Carthamus tinctorius	C1	----	KF886657, KF886670, KF886682
Carthamus tinctorius	C2	----	GQ435089, GU990409, GU724317
Nelumbo nucifera	NN	----	AB331309, JN407324, JF977132
Chrysanthemum x	CM		GU575275, JF949972, FJ539128
morifolium		----	
Zea mays	ZM		GU575286, M16836, DQ683012

Table 2. Primers and reaction conditions used in this study

Locus	Primer name	Primer sequence (5'-3')	Reaction conditions
*trn*H-*psb*A	PA	GTTATGCATGAACGTAATGCTC	94°C 5 min
	TH	CGCGCATGGTGGATTCACAATCC	94°C 1 min, 55°C 1 min,
			72°C 1.5 min, 35 cycles
			72°C 7 min
*rbc*L-a	R-F	ATGTCACCACAAACAGAGACT	95°C 2 min
	R-R	TCGCATGTACCTGCAG-TAGC	94°C 1min, 55°C 30 s,
			72°C 1 min, 34 cycles
			72°C 7min
ITS2	ITS3F	ATGCGATACTTGGTGTGAAT	94°C 5 min
	ITS2R	GACGCTTCTCCAGACTACAAT	94°C 30 s, 56°C 30 s,
			72°C 45 s, 40 cycles
			72°C 10 min

*trn*H-*psb*A: trnH-psbA intergenic spacer; rbcL-a: ribulose-1,4-bisphosphate carboxylase large subunit; ITS2: internal transcribed spacer 2.

4. Results

Three genes from nuclear and chloroplast were selected to discover genetic variations and to carry out analysis on the identification of 11 samples retailed as saffron in different markets. A total of 39 DNA sequences from three DNA barcodes (*trn*H-*psb*A, *rbc*L-a, and ITS2) were generated (Table 3). They were able to differentiate authentic saffron from the substituted ones with inter-specifically variable sites among 11 commodity samples from S2 to S12. The three DNA barcodes were successfully amplified in the order of *trn*H-*psb*A (13/13) > *rbc*L-a (11/13) > ITS2 (10/13). By combining a portion of *trn*H-*psb*A gene with ITS2 gene (Figure 1), it was demonstrated

Table 3. Properties of the three DNA barcoding regions of *C. sativus* and its adulterants. Numbers about the polymorphic sites are their positions in the multiple sequence alignment.

Property	Sample	*trn*H-*psb*A	*rbc*L-a	ITS2
Sequence length		431	655	240
avg. (bp)		1.04	0.58	1.27
All inter-specific		105bp-112bp	347bp-354bp	127bp-134bp
distance (K2P)	S1	TCGGGAAA	CCCCCTGC	TCCGGCTT
Selected variable	S2	TCGGGAAA	CCCCCTGC	TCCGGCTT
sites	S3	TCGGGAAA	CCCCCTGC	TCCGGCTT
	S4	TCGGGAAA	CCCCCTGC	TCCGGCTT
	S5	TCGGGAAA	CCCCCTGC	TCCCCCTC
	S6	TCGGGAAA	CCCCCTGC	TCCGGCTT
	S7	TCGGGAAA	CCCCCTGC	TCCGGCTT
	S8	TCGGGAAA	–	–
	S9	GTAGGATT	–	–
	S10	GTAGGATT	CCTCCTGC	–
	S11	GTAGTATT	CCTACTGC	CTAAAGAC
	S12	TTAGTAT-	CCTACTGC	TTAAAAAC
	C. tinctorius	GTAGTATT	CCTACTGC	CTAAAGAC
	C. x *morifolium*	GTACTAT-	CCTACTGC	TTAAAAAC
	N. nucifera	ATTTTATT	CCTCCTGC	GTGTGACC
	Z. mays	TTTGCGAT	GTCCCTGT	CCCGGCGC

that both sequence variations can distinguish *C. sativus* from different sources at intra-species taxa. Based on the genetic analysis of *rbc*L-a region, C1 and C2 were separated into two different clades with 67% supporting rate and clustered C1, S11, S12 and CM (*C. x morifolium*) into one clade with bootstrap support of 98%.

Maximum parsimony tree constructed by either single locus or combined loci suggested that the samples were divided into two major clades (Figures 1 and

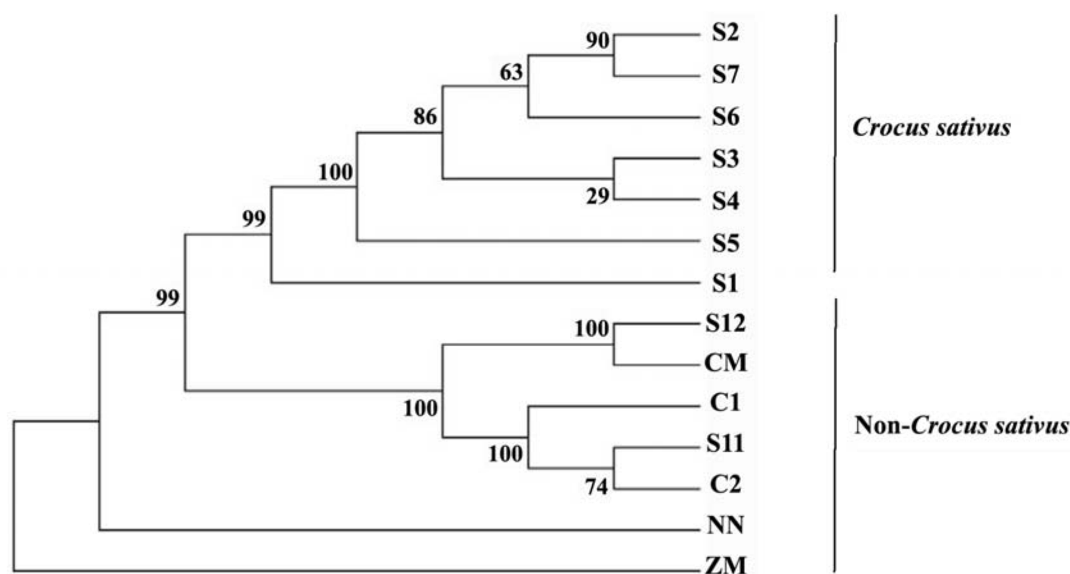

Figure 1. Classification tree of combining *trn*H-*psb*A with ITS2 gene using the Maximum Parsimony (MP) method

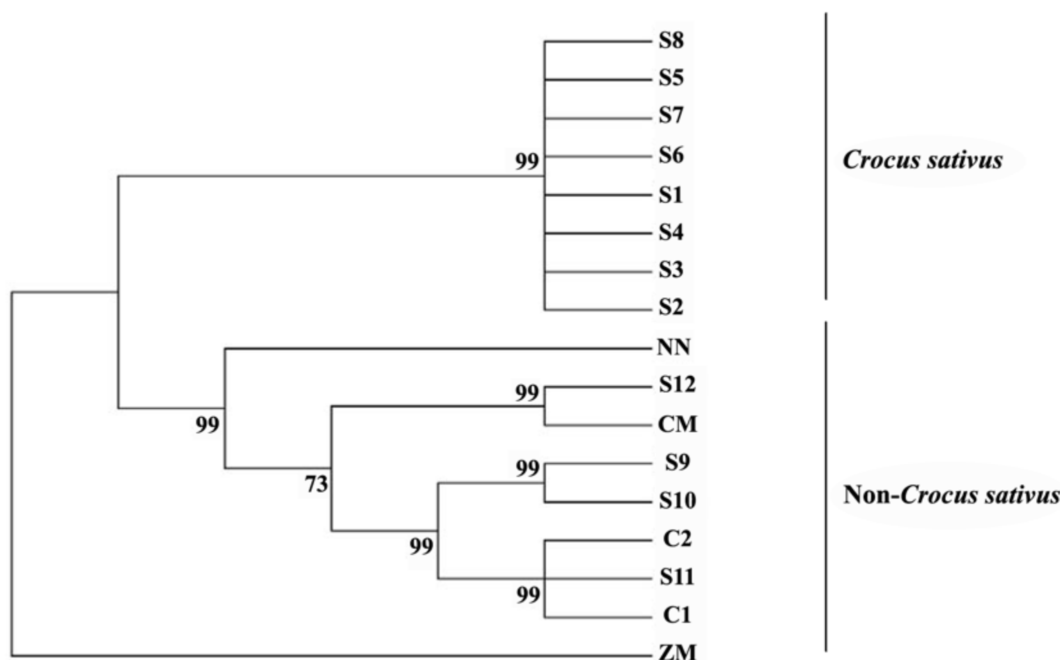

Figure 2. Classification tree of partial *trn*H-*psb*A gene using the Maximum Parsimony (MP) method

2). Both of the phylogenetic trees demonstrated that S2 to S8 were authentic saffron among market samples, while S9 (Hebei), S10 (Shandong), S11 (Fujian) and S12 (Yunnan) were not with 99% bootstrap support (BS). The accessions including S9, S10, S11, S12, C1, C2 and CM were divided into two sub-clusters in the *trn*H-*psb*A tree. As for the relationship among species, S11 and S12 were closer to *C. tinctorius* (99% BS) and *C. x morifolium* (97% BS), respectively through phylogenetic analysis. From Figure 1, S9 and S10 were inferred to be the same species (99% BS) and could belong to *Carthamus*. Thus, S9 (Hebei), S10 (Shandong), S11 (Fujian) and S12 (Yunnan) were proved not saffron, but its adulterants.

5. Discussion

The result showed that the *trn*H-*psb*A region could place an unidentified specimen into a family and even genus (32), while *rbc*L-a may be effective in identifying specimen at family level. As one of the most common regions used for phylogenetic analyses, the ITS2 region was selected as a barcode candidate that can distinguish accessions from different geographical origin at the species level (33, 34). But one of potentially negative factor for sequencing ITS2 is the presence of poly-G, poly-C, and poly-A repeats (35). Moreover, multi-locus approach may be a much more effective strategy for species identification. As *C. sativus* and its adulterants are completely in different families, each of the three loci is adequate when used at the family level. In addition, DNA integrity and purity are the major concerns in DNA barcodes. Poor DNA quality and quantity may lead to the unsuccessful amplification of DNA barcodes in some of the commodity samples. When materials are highly processed or stored for a long time, total DNA is highly degraded or contaminated (36) that leads to the DNA amplification and sequencing difficulties.

When presented with a completely unknown sample, it would be highly desirable to place it in a smaller group of taxa (*i.e.* within one genus) (37). DNA based methods maybe more useful in quickly and efficiently discriminate adulterated or substituted raw materials (38). By using DNA barcoding method, total DNA of species can be achieved from fresh and dried plant parts, processed herbal drugs, as well as tablets and capsules. For its easy operation, DNA barcodes will also be powerful tool for non-professional users. Nowadays, with the exponential growth of international market and increasing demand of high quality medicinal materials, the adulterated or substituted source materials have sprung up and could confuse the wholesalers, retailers and consumers (39). Thus, correct identification by DNA barcoding technique has become an essential task for the regulatory authorities and related industries in order to ensure drugs safety

and fair trade. As a result, adverse health problems can be avoided and negative effects such as purchasing products mixed with adulterants or protected species marketed as unprotected species can be reduced (40, 41). Though many substitutions have been disclosed by researchers, still lots of adulterants have remained undiscovered in the medicine market.

Through our research, we found the adulterant rate of saffron from Chinese markets reached as high as 33.33% (excluding the sample we collected from the botanical garden). Saffron can be identified by a unique DNA barcode or a combination of multiple DNA barcodes. This technology is useful in providing a reliable and effective means for the differentiation of saffron from its substitutes or adulterants. With the adoption of barcoding as an authentication tool, perhaps it will be possible to discourage medicinal plants substitution in the marketplace. As a high-valued product, the official authentication and monitoring of saffron become very important in China for safety reasons. And DNA barcode tool is highly recommended.

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