Industrial Crops and Products 32 (2010) 147–151

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Intersimple sequence repeat markers for molecular characterization of *Crocus cartwrightianus* cv. *albus*

Angela Rubio Moraga, Almudena Trapero-Mozos, Lourdes Gómez-Gómez, Oussama Ahrazem*

Departamento de Ciencia y Tecnología Agroforestal y Genética, ETSIA, Universidad de Castilla-La-Mancha, Campus Universitario s/n, Albacete E-02071, Spain

ARTICLE INFO

Article history:
Received 9 March 2010
Received in revised form 9 April 2010
Accepted 16 April 2010

Keywords:
Crocus cartwrightianus cv. albus
Crocus sativus
Taxonomy
HPLC–ESI-MS
ISSR

ABSTRACT

The *Crocus* genus, which comprehends approximately 80 species, is known mainly for the triploid sterile plant *C. sativus*, whose dried stigmas provide us with the spice called saffron. Among the species belonging to the *C. sativus* L. aggregate, *C. cartwrightianus* cv. *albus* shares morphological and cytological characters with both *C. sativus* and *C. cartwrightianus*, although its positions seem to be uncertain. Due to the complexity of the genus, few studies have revised the classification proposed by Mathew in 1982. In the present study, intersimple sequence repeat (ISSR) and HPLC–ESI-MS analyses have been used in an attempt to clarify the taxonomical position of cv. *albus*. No differences were found between *C. sativus* and *C. cartwrightianus* cv. *albus* using 12 ISSR primers. Results on apocarotenoid contents from HPLC analysis showed some quantitative differences. According to our findings, *C. cartwrightianus* cv. *albus* is more related to *C. sativus* than to *C. cartwrightianus* and may be an albinic saffron. These data agree with other authors who reached the same conclusion using different techniques. The results obtained in this work may be used in improving programme of saffron spice.

1. Introduction

The *Crocus* genus includes approximately 80 species but is known mainly for the cultivated species *C. sativus*, commonly called saffron, which is used mostly as a spice and food colourant and, less extensively, as a textile dye or perfume (Negbi, 1999). Due to its analgesic and sedative properties, herbal folk medicines have also used saffron to treat numerous illnesses over the centuries (Abdullaev, 1993). Other species belonging to this genus are highly prized for their colourful flowers and used extensively in specialized gardening, e.g. varieties of *C. vernus*, *C. versicolor* and *C. aureus*, among others.

These plants grow from corms and are mainly hardy perennials, being found throughout a wide range of habitats, including woodland, scrub and meadows. *Crocus* taxonomy has been based primarily on morphology, while also taking chromosome numbers into consideration (Mathew, 1982). The genus *Crocus* is divided into two subgenera, subgenus *Crociris* containing *C. banaticus* and the subgenus *Crocus* comprising the remaining species. The subgenus *Crocus* is further divided into two sections: section *Crocus* and section *Nudiscapus*, and each is again divided into Series a–f and g–o, respectively. The taxonomy of *Crocus* is extremely complicated due to the lack of clear distinctive characters, the wide range of habitats and the heterogeneity of the morphological traits and cytological data (Nørbæk et al., 2002). Among the species belonging to the *C. sativus* L. aggregate, we find *C. cartwrightianus* cv. *albus*, which shares morphological and cytological characters with *C. sativus* and *C. cartwrightianus* (Grilli-Caiola, 1995), although its positions seem to be uncertain.

The diploid species *C. cartwrightianus* is more similar to *C. sativus* from both a morphological and cytological standpoint (Pathak, 1940; Brighton, 1977; Mathew, 1977, 1982; Heywood and Brighton, 1983). According to Grilli-Caiola (1995), *C. cartwrightianus* cv. *albus* bears white flowers with intensive golden yellow anthers; each corm produces many flowers after the appearance of the leaves and flowers are usually smaller than in *C. cartwrightianus*. Mathew (1982) described some white flowered *C. sativus* which occur together with the lilac form in a same population; these phenotypes are instable along the time and are reversible to the lilac form. These colour alterations seem to be due to viruses' infections.

In the literature there are few articles using molecular and biochemical approaches in an attempt to classify and clarify the systematic and phylogeny of this genus (Frello and Heslop-Harrison, 2000; Nørbæk et al., 2002; Castillo et al., 2005; Frizzi et al., 2007; Peterson et al., 2008; Seberg and Peterson, 2009). PCR-based approaches are in demand because of their simplicity and also because they can be carried out with only small quantities of sample DNA. Intersimple sequence repeats (ISSR) have become an accepted DNA molecular marker for authenticating species and researching populations of the same species. This method has several benefits

---

* Corresponding author. Tel.: +34 967599200; fax: +34 967599238.
E-mail address: oussama.ahrazem@uclm.es (O. Ahrazem).

0926-6690/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.indcrop.2010.04.012

Table 1
Sources of species studied.

Species	Code number	Geographic origin	Suppliers	No. of individuals
C. sativus	C340	Cult. ex Cambridge UK	Dr. U. Jacobsen	3
C. sativus	C395	Cult. ex China Yunan	Dr. U. Jacobsen	3
C. cartwrightianus cv. *albus*	C337	Cult. ex Cambridge UK	Dr. U. Jacobsen	3
C. cartwrightianus cv. *albus*	Cal	Without locality	Pottertons Nursery	3
C. cartwrightianus	12627	Nomos Atiki, Greece	Dr. U. Jacobsen	3
C. cartwrightianus	12629	Nomos Atiki, Greece	Dr. U. Jacobsen	3
C. kotschyanus	Cko	Without locality	Pottertons Nursery	3

over other techniques: it is able to discriminate between closely related genotypes (Nagaoka and Ogihara, 1997; Korbin et al., 2002; Hodkinson et al., 2002; Galvan et al., 2003) and it can also detect polymorphisms without any previous knowledge of the crop's DNA sequence. Hence, ISSR is an efficient procedure to use when investigating the molecular diversity of *Crocus*.

The aim of this study was to clarify the doubtful position of *C. cartwrightianus* cv. *albus* and to determine whether it is a *C. sativus*, *C. cartwrightianus* or a different species by using the ISSR method along with chemical stigma patterns.

2. Materials and methods

2.1. Source of experimental material

For this study, we included 7 *Crocus* isolates. Specimens were obtained from Dr. U. Jacobsen of the Agricultural University of Denmark and from private collections in the UK (Pottertons Nursery). The details of the accessions and their geographic origin are listed in Table 1. Three individuals representing each population were used. Plant tissues were independently harvested, frozen in liquid nitrogen and stored at −80 °C until required.

2.2. DNA extraction

DNA was extracted from 150 to 300 mg of leaf material using a modified Doyle and Doyle (1987) method. Leaf material was then ground to a fine powder in liquid nitrogen and placed in a microcentrifuge tube with 2 ml of extraction buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 0.01% proteinase K) plus 40 μl of 2-mercaptoethanol. Following incubation at 65 °C for 30 min, 1.4 ml of chloroform:isoamyl alcohol (24:1) was added, mixed and centrifuged at 8000 rpm for 30 min; the supernatant was transferred to a new tube and then repeated 3 times. DNA was precipitated with isopropanol (2/3 volume of supernatant), then centrifuged at 8000 rpm for 30 min, the supernatant discarded and the pellet washed in 70% ethanol containing 10 mM ammonium acetate for 20 min. The pellet was dissolved in 100 μl of TE buffer (10 mM Tris–HCl pH 7.4, 1 mM EDTA) and the DNA was reprecipitated with 0.5 volume of ammonium acetate 3 M and 2.5 volumes of ethanol. After centrifuging at 8000 rpm for 30 min, the pellet was redissolved in TE buffer with 10 μg/ml RNase and incubated at 30 °C for 30 min. The extracted DNA was quantified with a spectrophotometer and diluted to 30 ng/μl in TE. The DNA was stored at −20 °C for further analyses.

2.3. DNA amplification

15 and 30 ng of genomic DNA were amplified in a volume of 25 μl containing 10 mM Tris–HCl pH 9.0, 1.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, dTTP, 0.4 μM primer, and 1 unit of Taq DNA polymerase by means of a thermal cycler (MJ-Mini, BioRad). The cycling programme began with an initial 2 min at 94 °C followed by 45 cycles at 94 °C for 45 s, 48–62 °C for 45 s and 72 °C for 2 min plus a final 10 min at 72 °C and storage at 4 °C. A negative control was added in each run to test contamination. The sequences of primers are shown in Table 2. Amplification products were separated by electrophoresis in 2% agarose gel containing 1 μg/ml ethidium bromide and TAE buffer. Ten microlitres of amplified DNA were mixed with 3 μl sample buffer (1.2 mg/ml; 125 mg/ml Ficoll) and 10 μl was applied in each well of the gel. DNA molecular weight markers (1 kb, Promega) were then added to each gel. The gels were run at a current of 50 mA until the bromophenol had migrated 10 cm from the well. The bands were then visualized under UV light and photographed. To ensure the reproducibility of the method, the procedure was repeated 3 times for each concentration of genomic DNA and primer.

2.4. Data analysis

The amplification products were scored in terms of a binary code as present (1) or absent (0), each of which was treated as a unit character regardless of its intensity. Bands with the same migration distance were considered homologous. Jaccard's similarity coefficients were then calculated and accessions were grouped by using the unweighted pair group method with arithmetic averages (UPGMA). The dendrogram construction utility was from DendroUPGMA, S. Garcia-Vallve, Biochemistry and Biotech-

Table 2
Features of ISSR primers used for ISSR-PCR of the studied species and the amplified DNA products. Key to symbols: R = A + G, Y = C + T, H = A + T + C, V = G + A + C.

Name	Sequence	Annealing temperature (°C)	No. of amplified bands	No. of polymorphic bands	% of polymorphic bands
ISCS12	$(TTG)_6C$	55.0	3	2	66.6
ISCS17	$DBDBCA(CCA)_4C$	61.0	13	9	69.2
ISCS19	$HVHGT(GGT)_4G$	61.0	8	3	37.5
ISCS20	$DHB(CGA)_5$	62.0	6	5	83.3
ISCS23	$HBDB(GACC)_4$	63.0	7	3	42.8
ISCS32	$(AC)_8YG$	52.0	10	7	70.0
ISCS33	$(TG)_8RT$	52.0	5	3	60.0
ISCS35	$(TG)_8RA$	50.0	7	6	85.7
ISCS38	$(GT)_8YC$	50.0	7	6	85.7
ISCS43	$(GAA)_6$	50.0	7	6	85.7
ISCS49	$(CCCT)_4$	60.0	7	6	85.7
ISCS55	$(CT)_8RC$	50.0	3	2	66.6