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RAPD analysis in *Crocus sativus* L. accessions and related *Crocus* species

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Abstract

In the present paper a Random Amplified Polymorphic DNA (RAPD) investigation was carried out on DNAs from five *Crocus sativus* L. (saffron) accessions cultivated in different countries and on six closely related *Crocus* species. Aims of the study are to check whether cultivated saffron has maintained a constant genomic organisation and to clarify its relationships with possible ancestor species. For the fifteen primers, which produced positive results, DNAs of saffron corms from different accessions present the same amplification pattern, in accordance with the similar DNA content and base composition pointed out in previous studies. The amplification of the seven *Crocus* species DNAs with twenty-one primers provided 217 repeatable and interpretable fragments, which were scored for presence/absence and employed for a cluster analysis. Results indicated that *C. sativus* is very closely related to *C. cartwrightianus* and also similar to *C. thomasii*. This result, concurring with part of the previous evidence, would rule out the hypothesis of close relationships between *C. sativus* and *C. pallasii*.

Additional key words: DNA analysis, saffron ancestors.

Introduction

Archeological and historical sources (Tammaro 1987, Negbi 1999) indicate that saffron (*Crocus sativus* L., *Iridaceae*) is a very old cultivation dating back to 2500 - 1500 BC, probably originated in Iran, Asia Minor or Greece and later widespread in India, China, the Mediterranean basin and Eastern Europe. Saffron, unknown in the wild state (Grilli Caiola *et al.* 2001), is a triploid (2n=24; x=8) male-sterile species that is reproduced via corms selected by man for improving saffron production.

Whether saffron has undergone modifications along its millenarian cultivation and whether it has one or more ancestors is still uncertain. Brighton (1977) reported that the karyotype of *C. sativus* from different countries was always 2n=24, but in the literature other karyotypes appeared (Mather 1932, Karasawa 1940, 1943, Pathak 1940, Pogliani and Del Grosso 1971). Phenotype differences, such as flowers with a larger number of styles, branches and stamens, have been described by Piccioli (1932) in field. Recently, Estilai (1978) discovered in Iranian cultivation new variants of saffron with increased number of stigmas, maintaining 2n=24, although the frequency of the rare types was only 1.2×10^{-6} flowers.

In spite of numerous cytological, morphological and karyological contributions dealing with the problem, the ancestral species and the mechanisms of speciation which led to the origin of *C. sativus* are still matter of controversy (Mather 1932, Karasawa 1933, Pathak 1940, Feinburn 1958, Brighton 1977, Mathew 1977, 1982, 1999, Estilai 1978, Ghaffari 1986, Tammaro 1990).

Morphological comparison of saffron flowers from corms obtained from Italy, Israel, Spain, Holland cultivation (Grilli Caiola *et al.* 2001) revealed some differences in flower colour intensity and the presence of lobed tepals in some accessions, other than differences in pollen size and viability. Cytofluorimetric analyses (Brandizzi and Grilli Caiola 1998) of these accessions indicated that *C. sativus* in spite of phenotypical differences have the same DNA amount and base pair

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composition. These data may suggest that saffron has an unique ancestor and that it has been spread by man in different cultivation sites. Moreover the same cytometric studies indicated that the genome of *C. sativus* is more similar to that of *C. cartwrightianus* rather than to that of *C. thomasii*.

In the last years technological advances in DNA techniques were made available to systematists, opening new insights on taxonomic analysis. Among the techniques available, RAPD (Random Amplified Polymorphic DNA) analysis was successfully applied to the study of closely related species and hybrids (*e.g.*, Marsolais *et al.* 1993, Millan *et al.* 1996, Gehrig *et al.* 1997, Purps and Kadereit 1998, Bartish *et al.* 1999, Schaffer and Arnholdt-Schmitt 2001, Latha *et al.* 2002, Ranade *et al.* 2002, Samal *et al.* 2003) in spite of limitations in applicability and in interpretation of results (Thormann *et al.* 1994).

In the present paper, RAPD analysis was used to investigate variability of saffron plants from different countries and the relationship between *C. sativus* and the diploid, autumn-flowering *Crocus* species supposedly related to it, belonging to the series of *C. sativus*.

Materials and methods

Source of experimental material: *Crocus sativus* L. from two sites in Italy [Piano di Navelli, near L'Aquila and Sardinia], from Israel, Spain and Holland were cultivated at the University of Rome "Tor Vergata" in 1998. For each accession five individuals were tested (Table 1). The other species used in this study, belonging to the same *Crocus* series (Table 2), were supplied by the *Jacques Amand Ltd.* nursery (Stanmore, UK) for *C. cartwrightianus* and by the *Monocot Nursery* (Clevedon, UK) for the other taxa. In both cases, these companies propagated *Crocus* from seeds collected in nature. The various accessions were all cultivated at the University of Rome "Tor Vergata" in 1998. *C. thomasii* was collected at Castel del Monte (Bari, Italy) where it occurs in nature. For each species 5 individuals were tested.

Table 1. *Crocus sativus* L. material used for a survey of RAPD variation; five individuals for each accession were analysed.

	Origin	Label
Crocus sativus L.	Italy (L'Aquila)	CsIA
Crocus sativus L.	Italy (Sardinia)	CsIS
Crocus sativus L.	Israel	CsIs
Crocus sativus L.	Spain	CsSp
Crocus sativus L.	Holland	CsNL

Table 2. *Crocus* species studied, chromosome number as reported in literature, source of studied samples and label used in this research. Five individuals for each species were analysed.

Species	Chromosome number	Source	Label
C. asumaniae Mathew	2n=26 x=13	cultivated	Ca
C. cartwrightianus Herb.	2n=16 x=8	cultivated	Cc
C. hadriaticus Herb.	2n=16 x=8	cultivated	Ch
C. oreocreticus Burtt.	2n=16 x=8	cultivated	Co
C. pallasii Goldb.	2n=14 x=7	cultivated	Cp
C. sativus L.	2n=24 x=8	cultivated	CsIt
C. thomasii Ten.	2n=16 x=8	wild	Ct

DNA extraction, amplification and electrophoresis: DNA was extracted from 1 g of leaf tissue according to Dellaporta *et al.* (1985). PCR were carried out in a volume of 0.025 cm^3 containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at room temperature), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 10pM primer, 5 - 20 ng genomic DNA (depending on the primer, after several initial experiments aimed at optimising conditions), and 1 unit of *Taq* DNA polymerase (*Pharmacia Biotech*, New Jersey, USA). A control PCR tube containing all components, but no template, was run with each primer to check for contamination. DNA amplification was carried out in a *Perkin-Elmer 9600* (Shelton, USA) thermocycler, with the following conditions: 94 °C (4 min) initial denaturation, 45 cycles of 94 °C (30 s), 36 °C (1 min), 72 °C (2 min), 72 °C (7 min) final extension.

An initial set of 40 decamer primers (OPB 1-20, OPC 1-20 *Operon Technologies*, Alameda, USA) was employed. After preliminary tests aimed to find evident, repeatable and polymorphic patterns, various primers were excluded. For the intraspecific RAPD analysis of saffron, fifteen oligonucleotide (10-mer) primers gave evident bands (Table 3) and were used for the subsequent investigations. For the interspecific investigation (Table 4) a total of twenty-one decamer primers was used.

Amplification products were analysed by gel electrophoresis in 2 % agarose in TAE 1× and detected by staining with ethidium bromide. The *AmpliSize*™ *Molecular Ruler*, range 50 - 2000 bp, (*BioRad*, Milan, Italy) was used to estimate the molecular weights of DNA fragments separated on gels. Gels were photographed under UV radiation with Polaroid film 667.

Scoring of RAPD bands and data analysis: RAPD bands were scored for presence (1) or absence (0). Only

bands present in two replicated PCRs were considered.

Calculation of Nei and Li's (1979) distance and cluster analysis (*UPGMA*) were carried out with the RAPD software package (Armstrong *et al.* 1994) and the Neighbor program of the *PHYLIP* software package (Felsenstein 1993), respectively.

Results

Twenty-one primers were used to amplify random sequences from total *C. sativus* DNA extracted from five individuals of each of the accessions studied. No amplification products were obtained using the following six primers: OPC03, OPC04, OPC10, OPC11, OPC17, OPC19. The other fifteen primers used resulted in forty-four interpretable RAPD bands. Primers produced between one and seven bands (Table 3). The bands obtained were in a molecular mass range of 0.2 - 2 kb. For each primer all samples presented an identical pattern of amplification products, independently of the cultivation site (Fig. 1).

Table 3. Primers used for RAPD analysis of individual plants of *Crocus sativus* and number of bands obtained by random amplification.

Primer	Number of generated markers
OPC 01	1
OPC02	2
OPC03	-
OPC04	-
OPC05	3
OPC06	7
OPC07	3
OPC08	2
OPC09	4
OPC10	-
OPC11	-
OPC12	4
OPC13	2
OPC14	2
OPC15	3
OPC16	2
OPC17	-
OPC18	1
OPC19	-
OPC20	6
OPB01	2
OP total	44

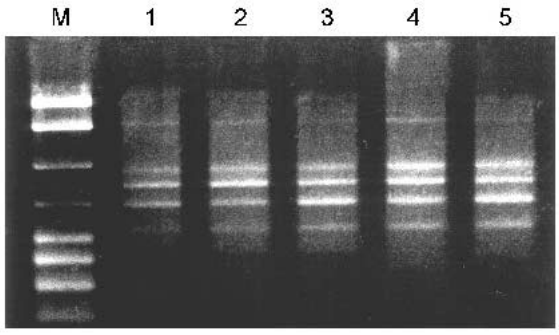

Fig. 1. RAPD-PCR fragment profiles of 5 different *Crocus sativus* accessions, generated by random primer OPC07. *Lane 1* - CsIA, *lane 2* - CsIs, *lane 3* - CsSp, *lane 4* - CsIS; *lane 5* - CsNL. M denotes the lane of the molecular mass markers.

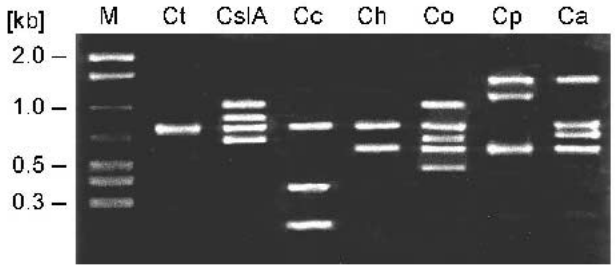

Fig. 2. DNAs of seven *Crocus* species amplified by primer OPB 11. *Lane M* contains molecular mass markers and the size of fragments in kb are on the left. Labels designating the *lanes* are: Ct - *C. thomasii*, CsIA - *C. sativus* l'Aquila, Cc - *C. cartwrightianus*, Ch - *C. hadriaticus*, Co - *C. oreocreticus*, Cp - *C. pallasii*, Ca - *C. asumaniae.*

Table 4. Primers used and number of DNA fragments amplified from each *Crocus* species studied.

Primer	Ct	CsIt	Cc	Ch	Co	Cp	Ca
OPB01	1	3	2	2	4	2	4
OPB03	2	1	2	6	2	3	7
OPB06	4	6	2	7	5	2	4
OPB07	3	2	4	4	2	4	2
OPB08	2	3	3	2	1	5	5
OPB10	5	3	3	5	3	4	4
OPB11	1	4	3	2	5	3	4
OPB12	4	5	5	5	5	3	5
OPB13	4	4	1	1	2	1	2
OPB14	3	3	5	4	6	2	5
OPB15	3	3	2	3	4	5	5
OPB16	1	1	3	1	1	1	2
OPB18	3	4	2	2	4	3	4
OPB20	1	2	4	1	2	1	1
OPC08	2	2	2	1	3	1	1
OPC09	3	4	3	1	2	2	4
OPC13	1	2	2	3	1	6	1
OPC14	2	2	2	2	2	4	4
OPC15	2	3	3	3	2	3	2
OPC16	4	2	1	1	1	2	2
OPC18	2	1	1	1	2	1	4

In different *Crocus* species only twenty-one primers gave easily interpretable amplification bands. The five individuals investigated for each species were identical in terms of banding pattern for each random primer used. An example of RAPD markers produced with primer OPB11 and DNA of the seven *Crocus* species in study is shown in Fig. 2 (given the intraspecific identity of our samples, only one individual per species is shown). A total of 217 PCR products were scored and included in the cluster analysis (Table 4).

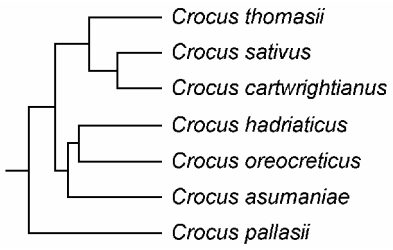

Fig. 3. UPGMA dendrogram for the *Crocus* species in study.

Primer sequences and number of amplified DNA products for each species and primer are listed in Table 4. Among the 217 fragments recorded, 5 (2.3 %) were common to all species and 86 (39.7 %) were shared by at least two species but no by all of them. The number of unique fragments was 126 (58.0 %), distributed as follows: 28 (corresponding to 38.8 % of the total number of fragments found) in *C. asumaniae*, 14 (25.4 %) in *C. cartwrightianus*, 15 (26.3 %) in *C. hadriaticus*, 25 (43.1 %) in *C. pallasii*, 14 (23.7 %) in *C. oreocreticus*, 18 (30.0 %) in *C. sativus*, 12 (22.6 %) in *C. thomasii*.

The *UPGMA* phenogram obtained from the Nei and Li's distance matrix (Table 5, Fig. 3) clearly indicates that *C. cartwrightianus* and *C. thomasii* are the two species that share the largest amount of RAPD fragments with *C. sativus*; in particular, *C. cartwrightianus* is most closely related to saffron. *C. hadriaticus*, albeit not exceedingly distant from *C. sativus*, is clearly more related *to C. oreocreticus* and to *C. thomasii*. *C. asumaniae* and *C. pallasii* show the most divergent RAPD pattern.

Table 5. Nei and Li's distances between the *Crocus* species in study.

PCR products	Distances						
Ct	0.000000	0.525424	0.592593	0.621622	0.681416	0.767857	0.669291
Cslt	0.525424	0.000000	0.474576	0.636364	0.626016	0.770492	0.722628
Cc	0.592593	0.474576	0.000000	0.639640	0.663717	0.750000	0.653543
Ch	0.621622	0.636364	0.639640	0.000000	0.586207	0.634783	0.630769
Co	0.681416	0.626016	0.663717	0.586207	0.000000	0.743590	0.606061
Cp	0.767857	0.770492	0.750000	0.634783	0.743590	0.000000	0.709924
Ca	0.669291	0.722628	0.653543	0.630769	0.606061	0.709924	0.000000

Discussion

Although some phenotypic features, such as lobated tepals, seem to be characteristic of some accessions of *C. sativus*, such as those from Israel (CsIs) and from Sardinia (CsIS), and although pollen size and viable pollen percentage resulted substantially variable (Grilli Caiola *et al.* 2001), RAPD analysis did not identify any location-specific differences, and this despite the fact that *C. sativus* is a triploid species which may have undergone multiple origins. Probably, as today *C. sativus* exists only as a cultivated species due to its high male-sterility (Zanier and Grilli Caiola 2001), selection for the best corms for saffron production may have influenced the amount of variation in *C. sativus*.

The results reported in this study confirm those on DNA content and base composition obtained by cytofluorimetric analysis (Brandizzi and Grilli Caiola 1998). High homogeneity of DNA content and composition among *C. sativus* cultivation sources with different geographical environments was found. Cytofluorimetric and RAPD data are in accordance with the karyotype similarity for saffron cultivated in different areas as reported by Brighton (1977).

Referring now to the ancestry of *C. sativus*, the results obtained here would suggest that *C. sativus* originated from an ancestor which was similar to present-day *C. cartwrightianus*. Previous experiments which document the production of seeds in *C. sativus* after artificial pollination with pollen of *C. cartwrightianus* (Grilli Caiola 1999, Grilli Caiola *et al.* 2001) or *C. thomasii* (Chichiriccò 1990) would confirm the close relationships described in the present paper.

According to our findings, *C. pallasii*, which was suggested as a possible ancestor of *C. sativus* (*e.g.*, Tammaro 1990), is not involved in the immediate

ancestry of cultivated saffron.

The results of the present analysis confirm Mathew's hypothesis (1982, 1999) on the origin of *C. sativus*. In fact, the phenogram obtained here (Fig. 3) and cytofluorimetric studies (Brandizzi and Grilli Caiola 1998) are compatible with a direct origin of *C. sativus* from a gene pool similar to that of *C. cartwrightianus*, by means of autopolyploidy (Karasawa 1933). This would be in accordance with intraspecific RAPD analysis of saffron carried out in this paper, which suggested a unique ancestral line for *C. sativus*. An issue that should be addressed in this regard, however, is the presence of unique RAPD bands in all the investigated samples. In particular, the agamic *C. sativus* shows 18 unique fragments (*i.e.*, 28.1% of total number of bands not shared by the presumably unique direct ancestor *C. cartwrightianus*). A possible hypothesis which can be made to justify the comparatively high proportion of unique fragments in *C. sativus* would suggest that the genetic variation of *C. cartwrightianus* (and/or of some of the other taxa involved) was not entirely sampled in this study, and therefore some unique bands which were observed for *C. sativus* are shared with some unsampled population of *C. cartwrightianus*. Artificial selection on saffron, and successive somatic mutations, may also have determined accumulation of other unique fragments. Otherwise, it would be necessary to suppose that *C. sativus* originated from some presently extinct *Crocus* species or population, which was very similar to *C. cartwrightianus*, and possibly vicariated the latter in Italy.

As far as the other species are involved, it may be worth noting that also *C. asumaniae* and *C. pallasii* show an unusually high proportion of unique fragments (38.8 and 43.1 %, respectively), and, according to this, may be regarded as comparatively more isolated species.

In conclusion, *C. sativus* very likely originated from *C. cartwrightianus* (or from a genetic pool very similar to that of the latter species), probably in Greece. The species was successively selected by man who contributed to its distribution (and variation) in different countries by means of cultivation.

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