Molecular Biological Approach of the Systematics of *Crocus sativus* L. and its Allies

Ákos A. Zubor[1], Gyula Surányi[2], Zoltán Győri[1], George Borbély[2] and József Prokisch[1]
[1]University of Debrecen, Agricultural Centre
Department for Food Science and Quality Assurance
H-4032, Debrecen, Böszörményi u. 138
[2]University of Debrecen, Faculty of Science
Department of Botany
Hungary

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Abstract

The hay saffron (*Crocus sativus* L.) is a sterile triploid plant, known in human culture only, with no fertile seeds produced. The origin of saffron is still a mist, however it is assumed to be an autopoliploid mutant or a hybrid. The recent classification and most of the former taxonomic publications define *C. sativus* to be derived from *C. cartwrightianus*, a wild species. Because of the sterility of hay saffron it seemed to be reasonable to apply molecular biological methods to complete classical taxonomic studies in examining its relations. The DNA polymorphism based AFLP method has confirmed the close relationship between these species.

INTRODUCTION

Crocus sativus provides the most expensive spice in the world. The plant itself can be found in human culture only. It is sterile and triploid. These features all above makes this tiny geophyte a very interesting subject of taxonomic research. The most recent taxonomic publications (Mathew in Negbi, 1999) and most of the former classifications (Mathew in Tutin, 1980; Mathew, 1982) arrange *C. sativus* and *C. cartwrightianus* to each other. Either as *C. sativus* to be a subspecies of *C. cartwrightianus* either as a variety or a mutated derivative. Some gardening books even confuse the two species (RHS, 1997). However similar they are, there is a need to find evidence to this relationship and to search for the origin of saffron. Experiments of cross pollinations turned *C. thomasii* out to produce some seeds with *C. sativus* (Grilli, 2003; Chichiriccò, 1989). Further studies also defined *C. cartwrightianus* and *C. thomasii* to be the closest to hay saffron (Grilli, 1998). Present study attempts to contribute DNA polymorphism based molecular biological approach to the theme by applying AFLP method (Vos et al., 1995).

The systematic status of *C. sativus* within the genus *Crocus* is remarkable. Not just type species it is of the series that it is classified into but is the type species of the section and subgenus as well.

Taxonomy of *C. sativus* and its allies according to Mathew in Negbi (1999):

The genus *Crocus*

1. Subgenus *Crocus*. Type species: *C. sativus* L.
   - A. Section *Crocus.* Type species: *C. sativus* L.
   - (a) Series *Verni* Mathew. Type species: *C. vernus* Hill.
   - (b) Series *Scardici* Mathew. Type species: *C. scardicus* Kos.
   - (c) Series *Versicolores* Mathew. Type species: *C. versicolor* Ker-Gawl.
   - (d) Series *Longiflori* Mathew. Type species: *C. longiflorus* Raf.
   - (e) Series *Kotschyani* Mathew. Type species: *C. kotschyanus* Koch.
   - (f) Series *Crocus.* Type species: *C. sativus* L.
   - B. Section *Nudiscapus* Mathew. Type species: *C. reticulatus* Stev. Ex Adams
   - (g) Series *reticulati* Mathew. Type species: *C. reticulatus* Stev.ex Adams
   - (h) Series *Biflori* Mathew. Type species: *C. biflorus* Mill.
   - (i) Series *Orientales* Mathew. Type species: *C. korolkovii* Regel ex Maw.
   - (j) Series *Flavi* Mathew. Type species: *C. flavus* Weston

(k) Series *Aleppici* Mathew. Type species: *C. aleppicus* Baker
(l) Series *Carpetani* Mathew. Type species: *C. carpetanus* Boiss. & Reut.
(m) Series *Intertexti* (Maw) Mathew. Type species: *C. fleischeri* Gay
(n) Series *Speciosi* Mathew. Type species: *C. speciosus* M. Bieb.
(o) Series *Laevigati* Mathew. Type species: *C. laevigatus* Bory & Chaub.

2. Subgenus *Crociris (Schur)* Mathew. Type species: *C. banaticus* Gay

Series *Crocus* includes *Crocus sativus* and its allies.

Species comprising series *Crocus:*

1. *C. cartwrightianus* Herbert
2. *C. sativus* L.
3. *C. moabiticus* Bornm. & Dinsm.
4. *C. oreocreticus* B. L. Burtt
5. *C. pallasii* Gold.
6. *C. thomasii* Ten.
7. *C. hadriaticus* Herbert
8. *C. asumaniae* B. Mathew & T. Baytop
9. *C. mathewii* Kerndorff & Pasche

The aim of this study is to compare the available *Crocus* species and to establish a similarity system amongst them with the use of AFLP method, which tries to give percentage estimations to set the similarity between the species.

MATERIALS AND METHODS

For the comparision of genetical polymorphism of *C. sativus* and its allies and other species of the genus *Crocus* a modified AFLP method was applied on total genomic DNA samples.

DNA Samples

DNA samples of the following species were purchased from the DNA bank of Jodrell Laboratory, Royal Botanic Gardens, Kew (London, UK):

1.C. sativus
2.C. cartwrightianus
3.C. oreocreticus
4.C. hadriaticus
5.C. pallasii ssp pallasii
6.C. thomasii

The next DNA samples were taken from living plant materials of private collections:

7.C. nudiflorus
8.C. boryi
9.C. goulimyi
10. C. clusii
11. C. reticulatus

DNA samples were extracted from living plant material using Zenogene Plant DNA Extraction Kit (Zenon Bio Ltd., Szeged, Hungary). All DNA samples were stored at -18°C until use.

AFLP

For this method EcoRI and Tru1I (equivalent with MseI) restriction endonuclease enzymes, Y^+/Tango^{TM} buffer, T4 Ligase, Ligation Buffer and PCR Mastermix (all from Fermentas, Lithuania) were used. EcoRI and Tru1I adapters and primers were synthesised at Bio Science Ltd (Budapest, Hungary).

Enzymatic digestion was carried out in ~10µl volumes. 2µl total genomic DNA sample was digested with 1µl (10U) EcoRI restriction enzyme in 2µl Y^+/Tango^{TM} buffer and 5µl H_2O. Incubated at 37°C for 1h, then 1µl (1U) Tru1I restriction enzyme added and incubated at 65°C for 20 min.

Ligation was carried out in a 20µl total volume adding 5µl double digested DNA,

1-1µl (10 pmol) EcoRI and 1-1µl (100 pmol) Tru1I adapter lower and upper strain, 1µl T4 Ligase, 2µl Ligation Buffer and 8µl H_2O. The incubation took place at 20°C for 2h.

The method was simplified by using only two AFLP primers with three extension bases (ACA of EcoRI and CAC of Tru1I) respectively and no preselective amplification. PCR reactions were carried out in 25µl total volume adding 2µl ligated DNA template, 2.5µl (10pmol/µl) EcoRI, 2.5µl (100pmol/µl) Tru1I primers, 12.5µl PCR Mastermix and 5.5µl H_2O. Thermocycles were set as follows: first a 2 min. 94°C denaturing step, then the cycles: 94°C 30 sec, 65°C 30 sec (decreasing temperature at every cycle by 0.7°C), 72°C 1 min for 12 cycles, then 94°C 30 sec, 56°C 30 sec, 72°C 1 min for 23 cycles and a final annealing at 72°C for 2 min.

Gel Analysis

Because of the available short (20cm) polyacrylamide gel kit, a modified, 5-18% gradient acrylamide gel was used for the PCR product separation with 1% TBE running buffer, at 20 mA current for approximately 3h. A 100 bp DNA Ladder (Fermentas Co.) was applied as a molecular marker (range: 1000-80 bp).

AFLP DNA fragment patterns were developed by applying silver staining (Sammons et al.,1981). The gels were digitalised with Gene Genius Bio Imaging System, and GeneSnap software (Syngene).

DNA band positions were determined using Genetools software (Syngene).

Statistical Analysis

DNA band patterns in the acrylamide gel show specific polymorphism, similarities and differences between the given samples. These patterns were statistically analysed with the use of Jaccard index, which is the estimated possibility of two samples being the same at least in one variable of any of the two samples (Podani,1997). The Jaccard index values are determined using SPSS for WindowsMS statistical software (SPSS Inc.), and arranged in a proximity matrix and dendrogram, applying single linkage cluster.

RESULTS

Results of two different gels are discussed here. The first is made up purely of the six *Crocus* DNA samples from the DNA Bank of Jodrell Laboratory, RBG, Kew, because these samples are trusted to be extracted from the species that are indicated. The second gel is made up from the former six species plus five more of different origin. Both of the results are repeated at least three times.

AFLP method on total genomic DNA samples resulted 18-26 polymorphic DNA bands per sample (Figure 1A, 2A). Most of the bands were commmon within all the samples. All of the samples had unique molecular bands specific for the given species. There were common but distinctive bands of two or more species as well (Figure 1B). In the first gel the statistical analysis resulted in a proximity matrix of Jaccard indices that determined the possibility of *C. sativus* to be similar to *C. cartwrightianus* up to 85%, and 75% to *C. thomasii*. The similarity between other species range from approx. 52% to 68% which is a relatively high value regarding the average similarity between other plant species does not usually exceed 50% (Table 1). This fact can be explained with the closely related taxonomic group of series *Crocus*. In the second gel the statistical analysis gave different results. *C. sativus* was possibly similar to both *C.cartwrightianus* and *C. thomasii* to approx. 62%, with a similarity range 25-61.9% of the other species (Table 2). In both results the similarities between *C. sativus*, *C cartwrightianus* and *C. thomasii* were the highest. The overall results are illustrated in dendrograms using single linkage cluster (Figure 1C, 2B).

DISCUSSION

The origin of *C. sativus*, a sterile triploid plant that produces the spice saffron, is still not completely revealed. Many classical botanical studies including anatomical, floristical and reproduction biological works conclude *C. sativus* being the most similar to

C. cartwrightianus (Mathew, 1982; Mathew in Negbi, 1999; Grilli in Negbi, 1999;). Beside these classical taxonomical studies the DNA polymorphism based taxonomy with the use of AFLP method has provided further results to confirm the closest relative amongst the allies of *C. sativus* to be *C. cartwrightianus*.

However one must take into consideration the remarkable proximity of *C. thomasii* to both *C. sativus* and *C. cartwrightianus*. Grilli (1998) had similar results with the use of flow cytometry, quantitative and qualitative DNA analysis and mathematical DNA base pair estimation. Chichiriccò (1989) reported about seed development in *C. sativus* after fertilization with *C. thomasii* pollen that presume very strong relation between these species.

The introduction of AFLP in the taxonomic research of *C. sativus* provided results that ascertain percentage similarities between the species. Out of six species from series *Crocus* it was *C. cartwrightianus* and *C. thomasii* that showed similarity to *C. sativus* and to each other above 70%. This value presumes the closest relationship between these three species from the members of genus *Crocus* that were available in this study. In the future an enhanced investigation should be favourable to examine as many species as possible from the genus to look for other evidence regarding the origin of hay saffron.

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Tables

Table 1. Proximity matrix of Jaccard indices of Gel I samples

Proximity Matrix

	Matrix File Input					
Case	C.SATIV	C.CARTWR	C.OREOCR	C.HADRI	C.PALSP	C.THOMA
C.SATIV	1,000	,850	,682	,652	,609	,750
C.CARTWR	,850	1,000	,583	,560	,520	,714
C.OREOCR	,682	,583	1,000	,773	,652	,565
C.HADRI	,652	,560	,773	1,000	,560	,609
C.PALSP	,609	,520	,652	,560	1,000	,565
C.THOMA	,750	,714	,565	,609	,565	1,000

Table 2. Proximity matrix of Jaccard indices of Gel II samples.

Proximity Matrix

	Matrix File Input										
Case	C.SATK	.CARTW	.OREOC	.HADRI	.PALSP	.THOMI	.NUDIF	C.BORY	.GOULI	C.CLUSI	C.RETIC
C.SATK	1,000	,619	,611	,591	,476	,619	,333	,455	,429	,545	,333
C.CART	,619	1,000	,476	,423	,375	,565	,360	,308	,333	,500	,250
C.OREO	,611	,476	1,000	,600	,556	,550	,526	,381	,421	,550	,563
C.HADR	,591	,423	,600	1,000	,619	,542	,522	,400	,500	,480	,409
C.PALSI	,476	,375	,556	,619	1,000	,435	,476	,292	,611	,375	,421
C.THOM	,619	,565	,550	,542	,435	1,000	,308	,478	,455	,565	,429
C.NUDIF	,333	,360	,526	,522	,476	,308	1,000	,280	,250	,478	,400
C.BORY	,455	,308	,381	,400	,292	,478	,280	1,000	,304	,478	,474
C.GOUL	,429	,333	,421	,500	,611	,455	,250	,304	1,000	,280	,300
C.CLUS	,545	,500	,550	,480	,375	,565	,478	,478	,280	1,000	,429
C.RETIC	,333	,250	,563	,409	,421	,429	,400	,474	,300	,429	1,000

Figures

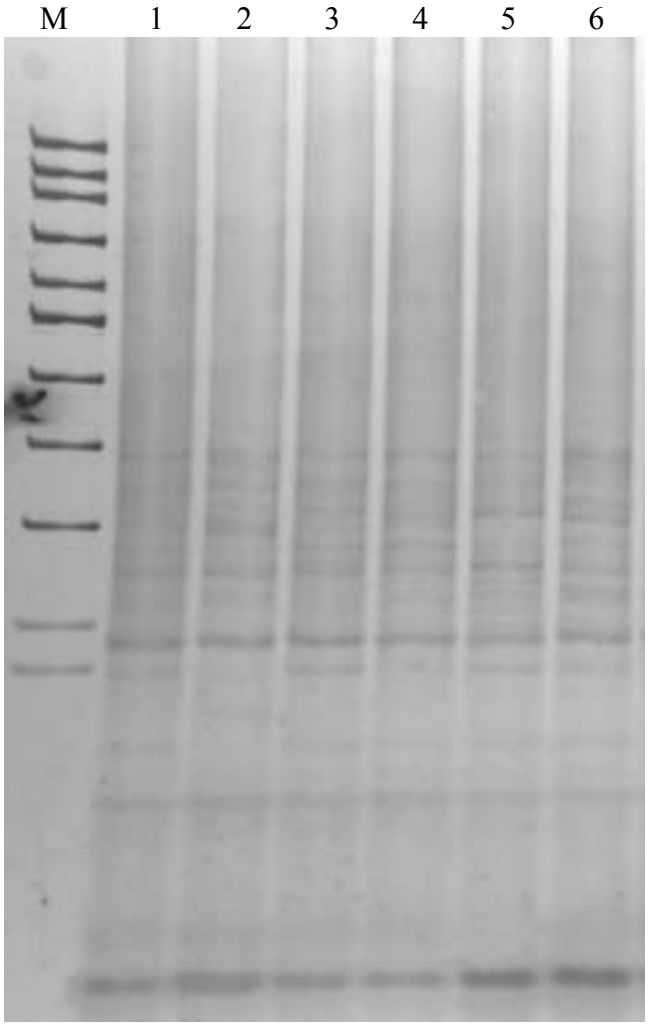

Fig. 1. A. AFLP pattern of six *Crocus* DNA samples. M: molecular size marker, 1. *C. sativus*, 2. *C. cartwrightianus*, 3. *C. oreocreticus*, 4. *C. hadriaticus*, 5. *C. pallasii ssp pallasii*, 6. *C. thomasii*

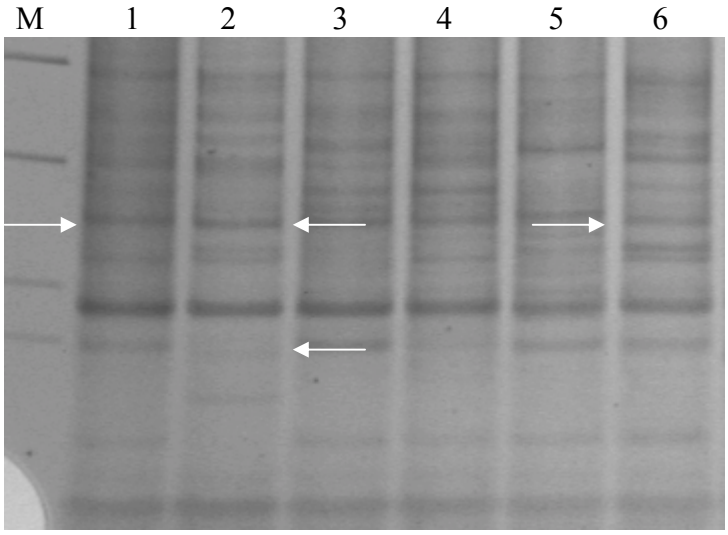

Fig. 1. B. Enlarged AFLP pattern of six *Crocus* DNA samples. (For samples name see Figure 1a) Lower arrow indicates unique band, specific for sample 2. Upper arrows indicate specific double bands for samples 1, 2 and 6.

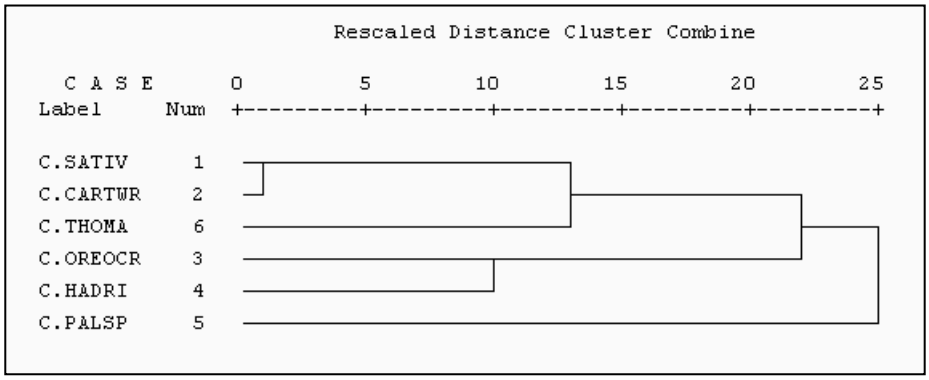

Fig. 1. C. Dendrogram of Jaccard indices of Gel I samples using single linkage clusters

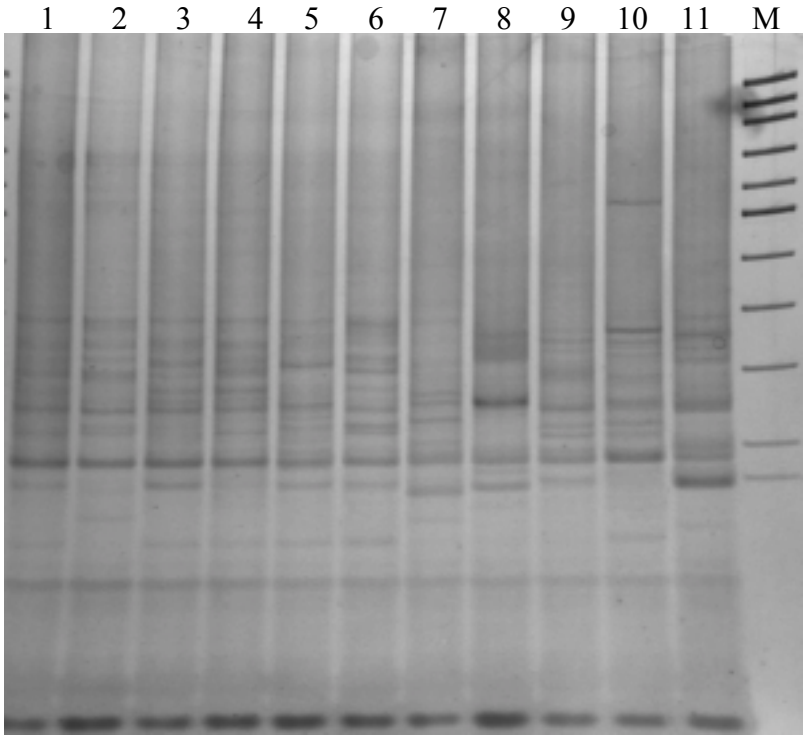

Fig. 2. A. AFLP pattern of eleven *Crocus* DNA samples. M: molecular size marker, 1. *C. sativus*, 2. *C. cartwrightianus*, 3. *C. oreocreticus*, 4. *C. hadriaticus*, 5. *C. pallasii ssp pallasii*, 6. *C. thomasii. 7. C. nudiflorus, 8. C. boryi, 9. C. goulimyi, 10. C. clusii, 11. C. reticulatus*

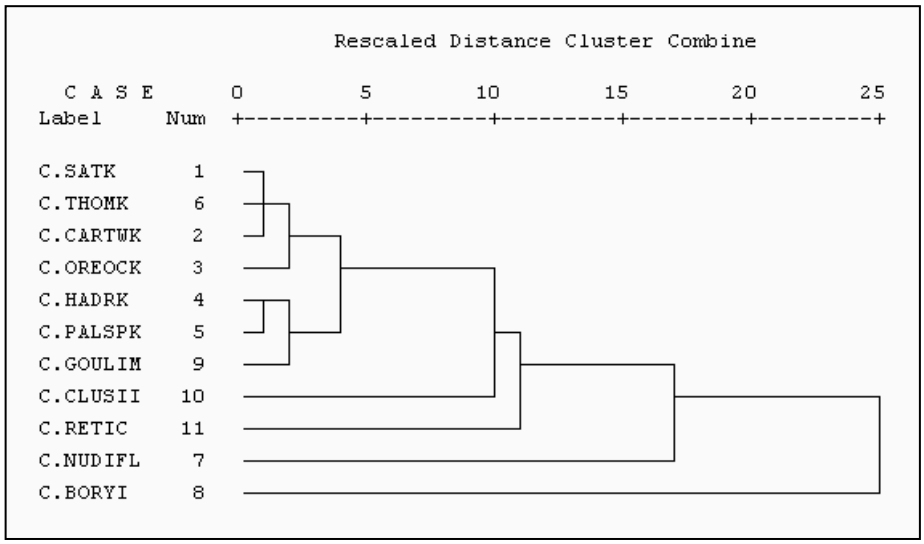

Fig. 2.B. Dendrogram of Jaccard indices of Gel II samples using single linkage clusters