Amplified Fragment Length Polymorphism (AFLP) Analysis in *Crocus* spp. Collected from Northern Jordan

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ABSTRACT

This study was initiated to assess the genotypic relatedness of *Crocus hyemalis* and *Crocus moabiticus* accessions collected from two areas in northern Jordan. Amplified Fragment Length Polymorphism (AFLP) analysis was carried out on DNAs from ten *Crocus hyemalis* accessions from Ajloun area and five *Crocus moabiticus* accessions from Al-Mafraq area. Four different combinations of AFLP primers were used to analyze genotypic relatedness. A total of 869 markers were produced from all samples of which 93.09% were with polymorphic presence–absence and matrices were processed with NTSYS-PC software. Similarity relationships were described graphically by a dendrogram which revealed two main clusters. The number of polymorphic loci detected by single primer combination for each variety was calculated. AFLP banding patterns were transformed into binary clusters of data. The first cluster consisted of all samples belonging to *Crocus hyemalis* with a similarity coefficient equal to 61.5%. The second cluster consisted of all samples belonging to *Crocus moabiticus* with a similarity coefficient equal to 83%. The presented results indicate that AFLP method is an effective approach for resolving the genetic variation of *Crocus*.

Keywords: AFLP analysis, *Crocus* spp., Northern Jordan.

INTRODUCTION

Crocus is a genus belonging to the family *Iridaceae. Crocus* includes about 90 species all of which are distributed in the old world, ranging from Portugal to Morocco to China (Grilli-Caiola *et al*., 2004). *Crocus sativus* L. is the most common *Crocus* species, also known as saffron (Rios *et al*., 1996). *Crocus* is a herbaceous perennial plant, adapted to withstand a dry dormant period as an underground corm. Corms are subterranean, covered with the expanded bases of the leaves; tunics. Tunics are membranous, coriaceous or fibrous. Tunics are produced annually, the older ones being pushed outward, extending at the apex to build a persistent neck in some cases. The corms live for one year, being replaced by new ones during the growth period (Zohari, 1986). Leaves are basal. The blade is linear and narrow, distinguished by a white mid rip on the upper surface (Mathew, 1982). Flowers are either autumnal or vernal, bisexual with a count ranging from one to several ones per corm (Zohari, 1986).

The yellow dye of saffron is obtained from the stigma of *Crocus sativus* L. About 4000 stigma are required to produce one ounce (28.3 g) of the dye. Saffron is commonly used in dyeing and flavoring of

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Received on 20/7/2009 and Accepted for Publication on 14/6/2010.

foods. Also, it is used as a stimulant; stomachic, aphrodisiac and antispasmodic (Dhar and Mir, 1997). It was recorded that saffron may be used against different kinds of tumors and liver, kidney, stomach and spleen cancers (Rois *et al.,* 1996). It has also been reported that saffron has a uterine sedative property and is an anticholesterol and atherosclerosis reducer (Dhar and Mir, 1997).

Eight species of saffron were reported in Jordan, these are: *C. aleppicus, C. cancellatus, C. cartwrightianus, C. hermones, C. hyemalis, C. moabiticus, C. naqabensis* and *C. pallasii* (Al-Eisawi, 2003). Urbanization activities as well as overgrazing represent the most endangering factors on *Crocus* in Jordan (Al-Eisawi, 1986).

Conservation of genetic resources of wild plants is of great importance in agriculture (Swanson, 1996). Phenotypic variation and genetic variation among and within plant species are studied in order to determine the center of diversity of such plants and their ecological status (Arafeh *et al*., 2002).

Amplified Fragment Length Polymorphism (AFLP) is a Polymerase Chain Reaction (PCR) based fingerprinting technology (Vos *et al*., 1995). In its most basic form, AFLP involves the restriction of genomic DNA, followed by the ligation of adaptors complementary to the restriction sites and selective PCR amplification of a subset of the adapted restriction fragments. These fragments are visualized on denaturing polyacrylamide gels or using the capillary electrophoresis technique (CE). The availability of many different restriction enzymes and corresponding primer combinations provides a great deal of flexibility, enabling the direct manipulation of AFLP fragment generation for defined applications (e.g. polymorphism screening, genetic mapping) (Liu and Cordes, 2004).

The present study was initiated to investigate the genetic diversity among and within *Crocus* spp. in Al-Mafraq and Ajloun areas in northern Jordan using AFLP technique.

MATERIALS AND METHODS

Plant Material and DNA Extraction

Three populations from two locations (Ajloun and Al-Mafraq) were sampled to analyze genetic variation. *Crocus hyemalis* samples were collected from Ajloun with two populations (Rhaba and O'saim) and *Crocus moabiticus* samples were collected from Al-Mafraq with one population (Al-Khanasri). Five samples were collected randomly from each population during the peak of flowering season (December, 2005 and January, 2006). The sites are described in Table (1).

Table 1. Eco-geographical parameters and description of the three sites sampled in this study.

Site	Code	Species	Latitude Longitude	Altitude (m)	Rainfall (mm)	Description
Rhaba	Rh	*C. hyemalis*	32º 24' 06" N 35º 49' 23" E	1099	600 mm	Mountainous, cultivated land, urban area
O'saim	Os	*C. hyemalis*	32º 24' 09" N 35º 48' 29" E	1019	600 mm	Mountainous, very steep slope, cultivated land, urban area, large *Crocus* population
Khanasri	Kh	*C. moabiticus*	32º 24' 37" N 36º 03' 45" E	678	150 mm	Hilly, non-cultivated, non-urbanized, range land, small *Crocus* population

Genomic DNA was isolated from the leaves following the procedure described in the technical manual provided by Wizard® Genomic Purification Kit (Promega, Madison, WI., USA cat. #A1125).

AFLP Procedure

The AFLP technique was carried out as described by Vos *et al*. (1995). Genomic DNA (0.5 µg) was double-digested using both *Eco*RI and *Mse*I enzymes and oligonucleotide adapters were ligated to the obtained fragments (Table 2). An amount of five microliters of template DNA from a 1:1 diluted ligation mixture was used for PCR pre-amplification with primers carrying one selective nucleotide (*Eco*RI+A and *Mse*I+C). Twenty six cycles were carried out at 94ºC for 60 sec, 56ºC for 60 sec and 72ºC for 60 sec in a PTC - 200 DNA Thermal Cycler (MJ Research, USA). The pre-amplification products were diluted (1:10) and used as template for hot selective amplification. Four combinations of selective primers with three additional nucleotides were used: (*Eco*RI+A+AC and *Mse*I+C+AC), (*Eco*RI+A+AG and *Mse*I+C+AG), (*Eco*RI+A+AT and *Mse*I+C+AT) and (*Eco*RI+A+CA and *Mse*I+C+CA). Table (2) illustrates the sequences of adaptors, preselective and selective primers used in this study.

Table 2. Sequences of adaptors, preselective and selective primers used in this study.

Sequence	Code name	Description
5-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA	*Eco*RI	Adaptor
5-GACGATGAGTCCTGAG TACTCAGGACTCAT	*Mse*I	Adaptor
GACTGCGTACCAATTC+A	*Eco*RI+A	Preselective primer
GATGAGTCCTGAGTAA+C	*Mse*I+C	Preselective primer
GACTGCGTACCAATTC+A+AC	*Eco*RI+A+AC	Selective primer (1)
GATGAGTCCTGAGTAA +C+AC	*Mse*I+C+AC	Selective primer (1)
GACTGCGTACCAATTC+A+AG	*Eco*RI+A+AG	Selective primer (2)
GATGAGTCCTGAGTAA +C+AG	*Mse*I+C+AG	Selective primer (2)
GACTGCGTACCAATTC+A+AT	*Eco*RI+A+AT	Selective primer (3)
GATGAGTCCTGAGTAA +C+AT	*Mse*I+C+AT	Selective primer (3)
GACTGCGTACCAATTC+A+CA	*Eco*RI+A+CA	Selective primer (4)
GATGAGTCCTGAGTAA +C+CA	*Mse*I+C+CA	Selective primer (4)

The following PCR conditions were used: first cycle at 94ºC for 30sec; 65ºC for 30 sec; 72ºC for 60 sec. The annealing temperature was then reduced every cycle by 0.7ºC and after 12 cycles it reached the optimal

annealing temperature of 56ºC. Twenty three additional cycles were done at these temperatures (94ºC for 30 sec; 56ºC for 30 sec; 72ºC for 60 sec) to complete the second amplification. The selectively amplified products were run on a 6% polyacrylamide gel.

DNA samples were prepared for preheating by adding 4 µl stop buffer (98% Formamide [24.5 ml], 10 mM EDTA (pH 8) [500 µl], 0.05% bromophenol blue [12.5 µl] and 0.05% xylene cyanol [12.5 µl]) to 1 µl of the DNA template, then the DNA was heated to 94º C for 3 minutes before putting on ice.

To visualize DNA bands in the gel, silver staining was applied on the gel using SILVER SEQUENCE™ DNA Sequencing System (Promega, Madison, WI., USA) according to the instruction manual and as described by Vos *et a*l. (1995).

Data Analysis

For each primer combination, the numbers of polymorphic and monomorphic bands were determined. The bands were analyzed using the Nei similarity index (Nei and Li, 1979), which excludes common negative data. Bands were scored as (1) for presence and (0) for absence and entered into a data matrix. The matrix of similarity was analyzed by the Unweighted Pair-Group Method (UPGMA) and the dendrogram was obtained using NTSYSpc software version 2.1 by (Rohlf, 1998).

RESULTS

Collected *Crocus* plants were genetically analyzed using four combinations of selective AFLP primers. A total of 869 markers were produced from all samples. Of these markers, 809 (93.09%) were polymorphic and the remaining 60 (6.90%) were monomorphic. As shown in Table 3, Primer combination *Eco*RI+A+AG / *MseI*+C+AG revealed the highest number of markers (265), 15 (5.66%) of which were monomorphic, with an average of 17.67 markers per sample. Primer combination *Eco*RI+A+AC / *Mse*I+C+AC revealed 257 polymorphic markers, with an average of 17.13 markers per sample. Primer combination *Eco*RI+A+CA / *Mse*I+C+CA revealed 248 loci, 45 (18.14%) of which were monomorphic, with an average of 16.53 markers per sample. The last primer combination, *Eco*RI+A+AT / *Mse*I+C+AT revealed 99 polymorphic markers, with an average of 6.6 markers per sample.

Table 3. Four primer combinations, with their monomorphic and polymorphic markers.

Item / Item number	Primer combination	Number of markers	monomorphic	%	polymorphic	%	average of markers per sample
1	*Eco*RI+A+AG/ *MseI*+C+AG	265	15	5.66%	250	94.34	17.67
2	*Eco*RI+A+AC / *Mse*I+C+AC	257	-	-	257	100	17.13
3	*Eco*RI+A+CA / *Mse*I+C+CA	248	45	18.14%	203	81.85	16.53

Item / Item number	Primer combination	Number of markers	monomorphic	%	polymorphic	%	average of markers per sample
4	*Eco*RI+A+AT / *Mse*I+C+AT	99	-	-	99	100	6.6
Total	4	869	60	6.90%	809	93.09%	57.93

The similarity coefficients among all possible pairs of samples ranged from 2% (between samples from different species) to 95% (between samples from the same species and location). The average similarity of *C. hyemalis* was 45.5% and that of *C. moabiticus* was 87.13%. The overall similarity was 50.27%. The dendrogram constructed by UPGMA cluster analysis (Figure 1) revealed two main clusters. The first cluster consisted of all samples belonging to *C. hyemalis* with a similarity coefficient equal to 61.5%. The second cluster consisted of all samples belonging to *C. moabiticus* and it was subdivided into two sub-clusters with a similarity coefficient equal to 83%.

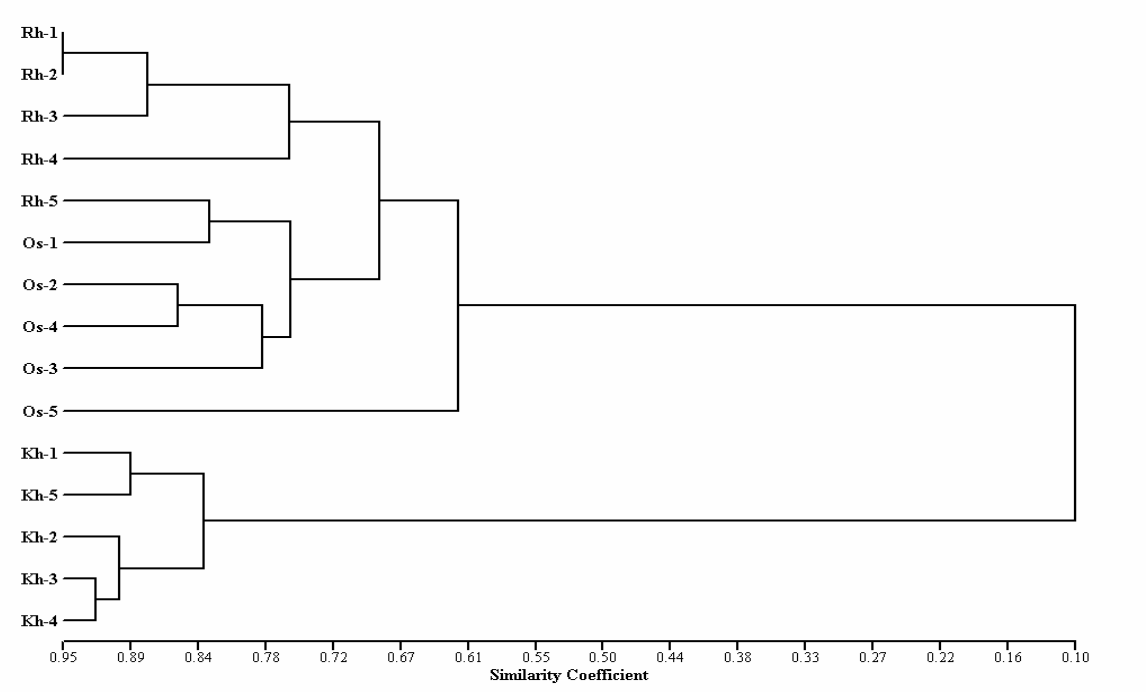

Figure 1. Dendrogram generated by UPGMA method on the basis of four selective primer combinations.

DISCUSSION

The success of any genetic conservation program is dependent on understanding the amount and distribution of the genetic variation present in the genetic pool.

Morphological traits were used to describe such genetic variation by describing vegetative and reproductive organs along with classical agronomic assessment (Lowe *et al*., 1996).

AFLP markers along with four selective primer combinations were used to characterize *Crocus* collected from Jordan at the molecular level. The total number of markers scored for the four selective primer combinations was 869.

The large number of polymorphic markers produced with the four selective primer combinations is explained by the comparison between *Crocus* plants from two different species, indicating a high level of polymorphism between them. The comparison between *C. moabiticus* samples from Al-Khanasri indicated a high level of similarity between samples ranging from 78% to 92%. Comparison between *C. hyemalis* from Rhaba indicated a high level of similarity ranging from 71% to 95%. *C. hyemalis* samples from O'saim indicated also a high level of similarity but to a lower degree than those from Rhaba with percentages ranging from 58% to 85%. In addition, the average similarity of *C. hyemalis* was 45.5% and for *C. moabiticus* it was 87.13%. Similar to that, the DNA polymorphism based AFLP method had confirmed the close relationship between *C. sativus* and *C. cartwrightianus* (Zubor et al., 2004). On the other hand, inter-retrotransposon amplified polymorphism was used to analyze the genetic diversity and phylogenetic relationships in *Crocus* genus of Iran; the result of this study documented *C. almehensi* and *C. michelosnii* as the closest relatives of saffron and probably the possible wild ancestors of this cultivated species (Alavi-Kia et al., 2008). RAPD and ISSR marker systems were used to estimate genetic diversity of 56 individuals representing 19 *Crocus* taxa from Western Turkey. RAPD and ISSR banding matrices were calculated to construct a dendrogram (Ward) by a cluster analysis performed by JMP software (Levent et al., 2008).

The overall similarity was 50.27%. These results agree with the findings of Grilli-Caiola *et al.* (2004) which could not identify any location-specific differences for *C. sativus* accessions collected from Italy, Israel, Spain and Holland. However, these data do not agree with the findings of Lamote *et al*. (2002) who unexpectedly detected a high degree of genetic diversity in two hydrologically isolated populations of *Iridaceae* family related genus *Iris pseudacorus*. Our results were unexpected, since *Iris pseudacorus* is known to be clonally propagated. Our results suggest that *Crocus* samples from O'saim location are richer in genetic diversity than those from Rhaba.

The cluster analysis separated the samples into two main clusters; the first cluster consisted of all samples belonging to *C. hyemalis* with a similarity coefficient equal to 61.5% consisting of three sub-clusters. The first sub-cluster consists of the first four samples, the second sub-cluster consists of the second five samples and the third sub-cluster consists only of one sample with the lowest similarity to the other samples (61.5%). The second cluster consisted of all samples belonging to *C. moabiticus* and was further subdivided into two sub-clusters with a similarity coefficient equal to 83%. Results showed an abroad genetic diversity among samples Rh-1, Os-5 and Kh-1 which produced the highest numbers of unique markers (2 unique markers each).

The results show that AFLP technique is a powerful tool for evaluating genetic diversity among *Crocus* spp. and this agrees with other reports in studying and evaluating genetic diversity in date palms (Cao and Chao, 2002), olives (Resta *et al*., 2002; De la Rosa *et al*., 2002) and sweet cherries (Boritzki, 2000).

This study recommends studying other locations throughout Jordan to get a clear image of the diversity for other *Crocus* species.

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استخدام مضاعفات الحامض النووي غير المتماثلة لدراسة الاختلافات الوراثية لنبات الزعفران في شمال الأردن

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ملخـص

تم إجراء هذه الدراسة لدراسة التباين الوراثي بين صنفي نبات الزعفران (*C. hyemalis* و*C. moabiticus*) من منطقتين في شمال الأردن. تم استخدام تقنية المعلمات الوراثية (AFLP) لدراسة التركيب الوراثي لنبات الزعفران، حيث تم أخذ عينات من المادة الوراثية (DNA) من عشر عينات من الصنف (*C. hyemalis*) الذي تم جمعه من منطقة عجلون و خمس عينات من الصنف (*C. moabiticus*) الذي تم جمعه من منطقة المفرق. استُخدمت أربع تركيبات مختلفة من البادئات الانتقائية (AFLP) لتوليد المعلمات و تحليل الارتباط الجيني. وقد تم إنتاج ما مجموعه 869 معلماً من جميع العينات (منها 93.09% كانت متعددة الاشكال) وتم تحليلها باستخدام التقنية المناسبة بالحاسوب (NTSYS-PC software). إن درجات التشابه الوراثي تم التعبير عنها برسم بياني يحتوي مجموعتين رئيسيتين. تم حساب عدد العلامات الوراثية المتعددة الاشكال التي تم فحصها ببادئة منفردة لكل صنف تم جمعه. المجموعة الأولى تألفت من جميع العينات التي تنتمي للصنف (*C. hyemalis*) بمعامل تشابه وراثي مقداره 61.5%. المجموعة الثانية تألفت من الصنف (*C. moabiticus*) بمعامل تشابه بلغ 83%. هذه النتائج تشير إلى أن طريقة (AFLP) طريقة فعالة في تحليل التباين الجيني لنبات الزعفران.

الكلمات الدالة: مضاعفات الحامض النووي غير المتماثلة، الاختلافات الوراثية، الزعفران، شمال الأردن.

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تاريخ استلام البحث 2009/7/20 وتاريخ قبوله 2010/6/14.