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MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF FIVE EGYPTIAN POMEGRANATE CULTIVARS

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Abstract: Random amplified polymorphic DNA (RAPD) analysis was performed on five local cultivars of pomegranate (*Punica granatum*), which vary in fruit splitting resistance, grown in the farm of Faculty of Agriculture, Assiut University. The cultivars investigated were Manfalouty, Nab-Elgamal, Wardy, Araby and Higazy. Five selected primers, namely OPC-7, OPA-3, OPE-7, OPI-2and OPO-12, produced 52 DNA fragments in total, and among which 29 fragments (55.76 %) were reproducible polymorphic amplified products while, 23 DNA fragments (44.23 %) were conserved among the five cultivars. The degree of polymorphism detected made possible the identification of each cultivar by combining the RAPD banding patterns of the five primers. The amplification products obtained with the five primers were used to identify cultivar- specific markers and to estimate the genetic relationships among pomegranate genotypes. Nineteen cultivar-specific markers were generated. The largest number of cultivar-specific markers was generated for OPC-7 primer (6 markers). Five specific genetic markers of the fruit splitting resistant cultivars (Araby and Higazy) were identified. A dendrogram was performed using the UPGMA cluster analysis to indicate the genetic relationships among the cultivars. The similarity values among genotypes ranged between 0.675 and 0.889 with an average of 0.782. The results of this study clearly indicate the utility of RAPD markers for the detection of genetic variation in pomegranate and suggest that RAPD markers have good potential for identifying pomegranate cultivars.

Key words: Pomegranate, *Punica granatum*, RAPD-PCR, dendrogram

Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruit. Its history dates to very ancient times. It is widely distributed in the tropical and subtropical regions of the world, even if its importance in the world-wide trade is still very limited. In recent years the area of this crop has increased, mainly because its medical properties, its high adaptability to versatile conditions especially stress conditions (Haggag and El-Shemy, 1987). The fruit and trees of pomegranate can be utilized in various ways and is

expected to become an important raw material for industrial use (Onur, 1988).

Almost all pomegranate cultivars grown throughout the world are of local types selected by unknown persons and maintained by vegetative propagations. The grown local material may be considered as the pomegranate primary genetic pool (Mars, 1996). Despite the large number of local varieties, in almost all countries, varietal differences and some physiological disorders exert a major impact on fruit quality and consequently marketability. Fruit splitting, as one of the physiological disorders, is a common problem with pomegranate in the hot and dry climate of Upper Egypt .However, the differences among the cultivars with respect to fruit splitting seems to be significant. Little knowledge is known about genetic diversity and polymorphism of the local cultivars. Classical approaches for identification and analysis of genetic variability in pomegranate cultivars are based on morphological, physiological and agronomic traits (Mars and Sayadi, 1992; Al Kahtani, 1992). However; these traits have limitations since they are influenced by environment and the need for extensive observation of mature plants. Moreover, the morphological and agronomic traits are limited and do not cover the entire genome which limits their use in assessing genetic diversity.

Although, there are many molecular techniques available for plant scientists to characterize genetic recourses and cultivars (Staub *et al.,* 1996), RAPD markers proved to be effective for identification and discrimination of cultivars in various plant species such as date palm (Trifi *et al*., 2000) mulberry (Orham *et al*.,2007) and olive (Ganino *et al*.,2007). Recently, RAPD markers were used to determine the genetic diversity level among Iranian pomegranate genotypes (Sarkhosh *et.al.* 2006) and to examine the genetic relationships among pomegranate cultivars of southern Anatolia Region of Turkey (Ercisli *et.al*; 2007).

Pomegranate is considered an important fruit in Upper Egypt region, particularly in Assiut. Although pomegranate is generally grown in Assiut as a low input culture, genetic studies are rare or lacking entirely because pomegr-anate has not been subject of scientific investigation..

Very little is known of the extent of genetic diversity of pomegranate cultivars in Egypt. Therefore, the present work was designed specially to shed light on this aspect. The study was conducted on five local pomegranate cultivars, which are dominate in Assiut region**,** varied in fruit splitting resistance and morphologically well identified throughout phenotypic characteri-zation. The objectives of the present

investigation were to (1) gather data on molecular evaluation of such cultivars (2) study the genetic relationships among the cultivars and (3) identify specific genetic markers of the fruit splitting resistant cultivars.

Materials and Methods

Plant materials:

The present study was carried out during 2008 growing season on 35 years old pomegranate trees grown in the farm of faculty of Agriculture, Assiut University, Assiut, Egypt. The study was conducted on five local pomegranate cultivars dominate in Assiut region, namely: Hegazy, Arabi, Manfalouty, Wardy and Nab El-Gamal. The cultivars varied in cracked fruit percentage as shown in Table (1).

Table (1): Cracked fruit percentage in five pomegranate cultivars.

Cultivars	Cracked fruit %
Hegazy	0.75
Arabi	2.50
Manfalouty	12.33
Wardy	14.68
Nab El-Gamal	18.68

The cracked percentages of the five cultivars were recorded by Dr. El-Agamy, Department of Horticulture, Faculty of Agriculture , Assiut University, Assiut, Egypt, in the (Egypt-USA project, Improvement of pomegranate for export in Egypt MUCIA, 2004).

Molecular analysis

Young leaves were collected from 5 trees for each cultivar and immediately frozen in liquid nitrogen and stored at -80°c. DNA was extracted from the leaf samples following the protocol for minipreps by using CTAB (Dellaportta, *et.al;* 1983). For RAPD analysis, the method described by Williams *et al.* (1990) was used to optimize the RAPD conditions.

Twenty different primers among the stocks available in our laboratory were tested (obtained from Operon technologies, Almeda, CA, USA) on a sample of the cultivars. Primers that produce reproducible, polymorphic bands were used to amplify the rest of the cultivars. Five 10-mer primers which were found to be polymorphic were used to generate

the RAPD markers. Amplification reactions were done in a thermal cycle (Prekin Elmer co.) The reaction mixtures were 50 µl volume consisting of 10 x PCR buffer, $MgCl_2$ 50 mM dNTP's (2 mM), primer (5µl) template DNA (10 ng/µl) Taq DNA polymerase (5units). The amplification reaction program consisted of an initial denaturation step at 94°C for 1 min and 45 cycles of 1 min at 94°C. 1 min at 36°C, 2 min at 72°C with a final extension time 7 min at 72°C. PCR products were stored at 4°C before analysis. The amplification products were separated by electrophoresis in 1.5% agarose gels in 1 x TBE buffer for 3h at 70 volts and photographed under UV light using DC 120 Digital cameras (Kodak, Rochester, N.Y.). A 1 kb DNA ladder was used as the molecular standard in order to confirm the appropriate RAPD markers.

Statistical analysis:

RAPD data were recorded as (1) for the presence of a band and (0) for its absence by using the software package MVSP (Multi-Variant Statistical Package) and pairwaise comparison between cultivars tested were used to calculate the Nei and Li's coefficient (Nei and Li, 1979) of genetic similarity matrix. Hierarchical cluster analysis to produce a dendrogram was performed using unweighted pair-group with arithmetic average (UPGMA).

Results

Variability study with RAPD markers:

Preliminary experiments were carried out to determine primers which produced good and reproducible polymorphic bands. Five random primers out of 20 primers evaluated were selected according to polymorphism and reproducibility. The details of the primers producing polymorphic bands are presented in Table 2.

These five primers produced 52 DNA fragment in total, among them 23 monomorphic and 29 fragments were polymorphic in one or more genotypes (Table 2).The total number of bands per primer ranged from 8 (OPO-12) to 16 (OPC-7) with an average 10.4 bands per primer. The highest number of polymorphisms was obtained with primer (OPC-7). The average number of polymorphic bands per primer was 5.4. The frequency of polymorphic bands varied from 0.25 (OPO-12) to 0.813 (OPC-7) with an average of 0.558. Amplified DNA products ranged in length from 196bp (OPI-7) to 2519bp (OPE-7).

Cultivars identification

The variability found allowed identification of cultivars in various independent ways: unique RAPD markers, unique banding patterns and combination of the banding pattern provided by different primers (Fig. 1).

In the present study we have found 19 unique markers (presence or absence of one band as compared to the rest of the cultivars) that allow the identification of four cultivars (table 3). Nine out of such markers were specific for Wardy cultivar. Five specific genetic markers of the fruit splitting resistant cultivars were identified, two markers were specific for Araby cultivar and three markers were specific for Higazy cultivar. The largest number of cultivar- specific markers was generated for OPC-7 primer (6 markers). Primers differed in their ability to differentiate, depending on the number of polymorphic bands and their frequencies. Using a combination of the five primers included in this study allowed identification of all pomegranate cultivars studied.

Genetic relationships among pomegranate cultivars:

The amplification products obtained with the five primers were analyzed to estimate the genetic relationships between pomegranate genotypes (Table 4).

The similarity values among genotypes ranged between 0.675 and 0.889 with an average of 0.782. The greatest similarity value (0.889) was obtained between the cultivars Araby and Nab-Elgamal and the least similarity value (0.675) was obtained between the cultivars Manfalouty and Hegazy (Table 4). The dendrogram resulting from the UPGMA cluster analysis is shown in Figure (2).

Discussions

The success of any genetic conservation or breeding program dependent on understanding the amount and distribution of genetic variation present in the genetic pool. The identification and characterization of pomegranate cultivars based on morphological traits alone is difficult and not necessarily accurate. The diversity of pomegranate cultivars requires precise method of discrimination for cultivars identification.

The results of this study clearly indicate the utility of RAPD markers for the detection of genetic variation in pomegranate. The results of RAPD analysis obtained showed a considerable amount of genetic variation present in the five cultivars investigated, although individual primer differed in the amount of variation they detected.

The high percentage of polymorphism among the cultivars tested suggested the existence of high genetic polymorphism in these pomegranate cultivars. Similarly high polymorphisms were reported among 24 Iranian pomegranate genotypes (Sarkhosh *et al.,* 2006) as well as among cultivars of southern Anatolia Region of Turkey (Ercisl *et al.,* 2007) and among popular cultivars from the Eastern Mediterranean region of Turkey (Durgac *et al.,* 2008). Such high degree of RAPD

polymorphism observed in the present material (the 5 primers generated one or more polymorphic markers, and frequency of polymorphic marker was as high as 55.8%) suggests that RAPD can efficiently be used in cultivar identification of pomegranate, as observed already in crops like orange (Shaaban *et al,* 2006), date palm (Trifi *et al.,* 2000) mulberry (Orhan *et al.,* 2007) and olive (Ganino *et al.,* 2007). Therefore, pomegranate breeding programs based on selection and hybridization will benefit from the data and the patterns reported in this study. In addition, the large number of cultivar- specific markers (19 markers) and the unique banding patterns generated in the present work suggest that RAPD markers have good potential for identifying pomegranate cultivars, and would be especially useful in identifying young trees that have not yet begun to fruit.

Moreover, the high similarity values among genotypes, which ranged between 0.675 and 0.889, developed in this study provide breeders of pomegranate in Egypt with a starting point for increasing the genetic diversity in their crosses.

Table(2): Details of five selected 10-mer primers and corresponding numbers of RAPD DNA markers (polymorphic and monomorphic).

Primer	Sequence	Band size(Bp)	No. of amplified bands	Monomorphic bands		Polymorphic bands	
				No	%	No	%
OPY-7	GTCCCGACGA	231 -2449	16	3	18.75	13	81.25
OPA-3	AGTCAGCCAC	388 -1826	10	4	40.00	6	60.00
OPE-7	AGATGCAGCC	271 -2519	8	3	37.50	5	62.50
OPI-2	GGAGGAGAGG	196 -1504	10	7	70.00	3	30.00
OPC-18	TGGGGGACTC	328 -937	8	6	75.00	2	25.00
Total			52	23	75.00	29	55.77

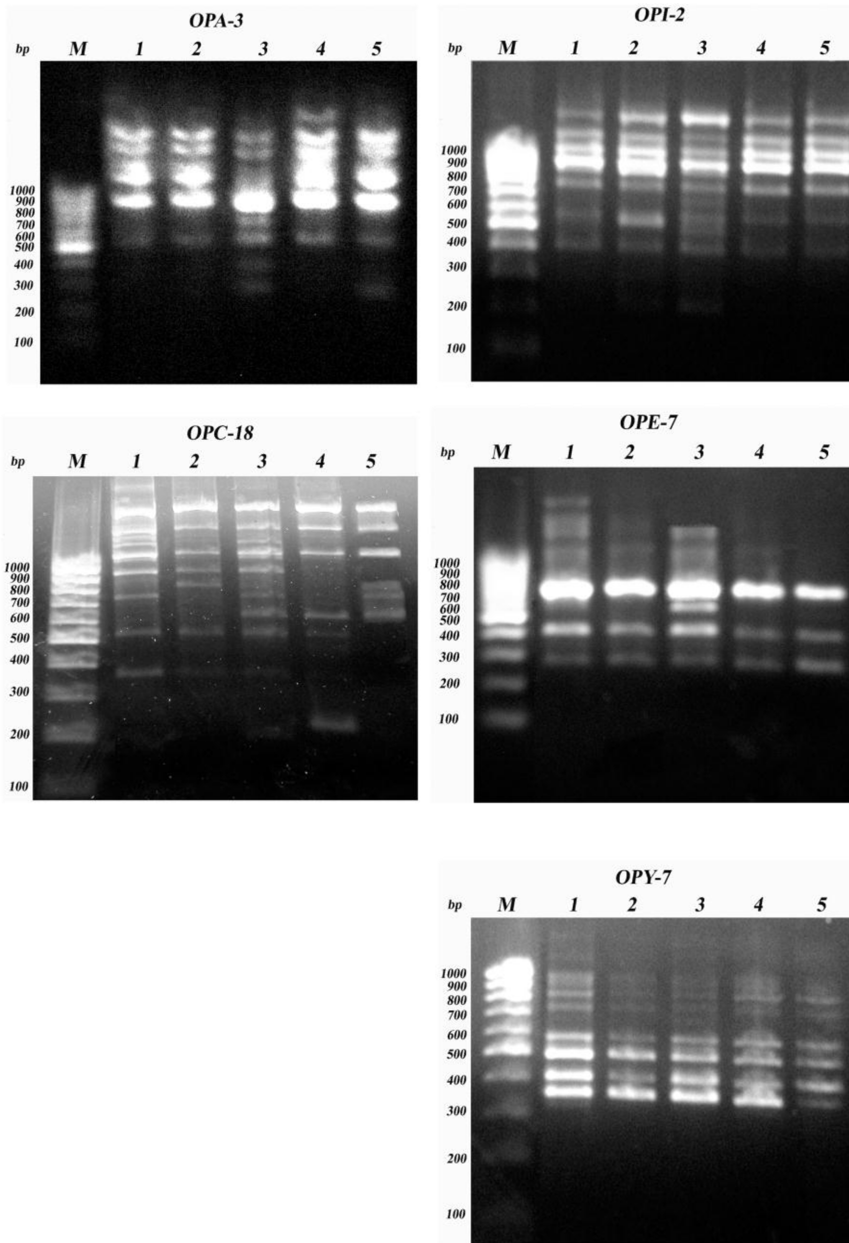

Fig.(1): Agarose gel electrophoresis of RAPD profiles in 5 pomegranate genotypes (1-5) generated by five RAPD primers. Where (1): Manfalouty , (2): Nab-El-Gamal, (3): Wardy, (4): Araby and (5): Higazy**.**

Table (3): DNA specific markers in five pomegranate cultivars; based on RAPD analysis data

Primer / Cultivars	OPC-7		OPA-3		OPE-7		OPI2		OPY-12		Total	
	+	-	+	-	+	-	+	-	+	-	+	-
Manfalouty	2479 1545 1328				2519 2041						5	
Nab-El-Gamal												
Wardy			802 482	1398 1215	618		614 462 196		628		7	2
Araby	231		1828								2	
Higazy		1456 546								937		3
Total	4	2	3	2	3		3		1	1	14	5

Table(4): Genetic similarity values calculated from the total DNA fragments amplified from the five pomegranate cultivars using five random primers.

cultivars	1	2	3	4	5
1- Manfalouty	--				
2- Nab-El-Gamal	0.845	--			
3- Wardy	0.79	0.784	--		
4- Araby	0.8	0.889	0.74	--	
5- Higazy	0.765	0.852	0.732	0.867	--

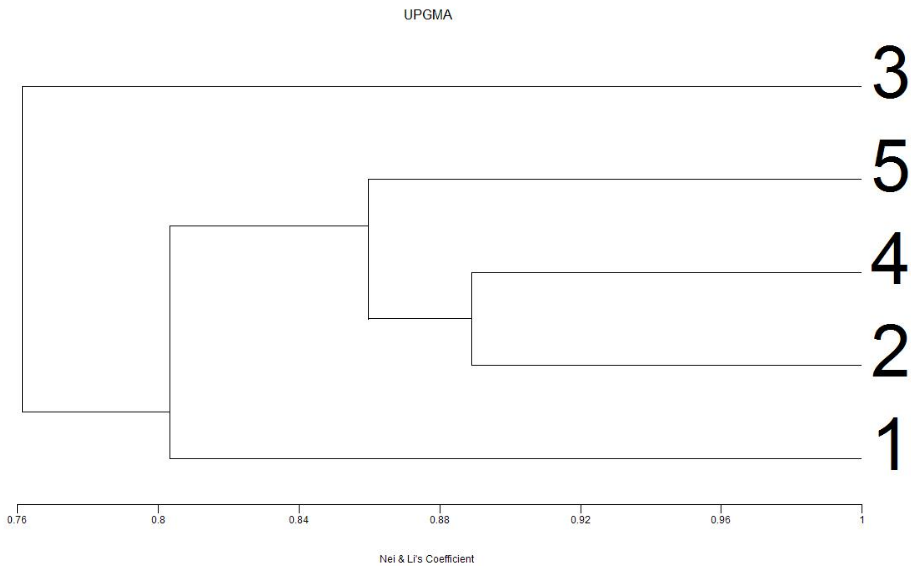

Fig.(2): Dendrogram demonstrating the relationship among the five pomegranate genotypes based on data recorded from polymorphism of RAPD markers. Where (1): Manfalouty , (2): Nab-El-Gamal, (3): Wardy, (4): Araby and (5): Higazy**.**

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تحديد الخصائص الجزيئية والتمييز بين خمسة أصناف مصرية من الرمان

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تم استخدام تحليل RAPD-PCR على خمسة أصناف محلية من الرمان هى (المنفلوطى وناب الجمل والوردى والعربى والحجازى) تختلف فى مقاومتها لصفة تشقق الثمار، ونامية فى مزرعة كلية الزراعة جامعة أسيوط. وقد تم استخدام خمسة بادئات Primers لدراسة هذه الصفة هى:

. (OPC-7, OPA-3, OPE-7, OPI-2 and OPO-12)

تفاعلت الخمسة بادئات المستخدمة مع الخمسة أصناف من الرمان فأنتجت اجمالى ٥٢ شظية DNA . أظهرت ٢٣ شظية من هذه الشظايا تماثلا فى كل أصناف الرمان المختبرة بنسبة ٤٤,٢٣ % بينما ٢٩ شظية كانت متعددة الصور polymorphic فى الأصناف المدروسة ونسبتها كانت ٥٥,٧٦ %. ودرجة تعدد الصور المتحصل عليها أوضحت أنه من الممكن التمييز بين كل صنف من الرمان بإستخدام هذه البادئات الخمسة. كما اظهرت نتائج تحليل RAPD-PCR باستخدام الخمسة بادئات امكانية تحديد واسمات جزيئية (markers) خاصة بأصناف الرمان الخمسة وكذلك لتحديد القرابة النباتية بين أصناف الرمان.

كان العدد الإجمالى للواسمات الجزيئية المتخصصة ١٩ شظية DNA . وأظهر البادئ OPC-7 أكبر عدد من الواسمات الجزيئية المتخصصة (٦ واسمات). كما أظهر التحليل أن هناك خمسة واسمات جزيئية متخصصة خاصة بصفة مقاومة تشقق الثمار وذلك فى الصنفين العربى والحجازى المقاومين للتشقق.

تم عمل التحليل العنقودى Dendrogram UPGMA cluster analysis لتوضيح التماثل الوراثى بين أصناف الرمان. وتراوحت درجة التماثل الوراثى بين الأصناف من ٠,٦٧٥ إلى ٠,٨٨٩ بمتوسط ٠,٧٨٢ . وأوضحت نتائج هذه الدراسة مدى أهمية استخدام الواسمات الجزيئية الناتجة من تحليل الـ RAPD لتعيين الإختلافات الوراثية الموجودة فى أصناف الرمان كما أنها طريقة فعالة ومفيدة للتمييز بين أصناف الرمان المختلفة يمكن استخدامها لتساعد مربى الرمان فى الاسراع من عملية الانتخاب.