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A preliminary Study on Genetic Diversity Among Different Genotypes of Manfalouty Pomegranate Cultivar
B- Molecular Genetics Identification Using RAPD Markers

Safia A. Abou-Taleb[*], T. K. El-Bolok[**] and Hala N. Abdallah[*]
[*]*Olive & Semi-Arid Zone Fruits Deptartment, Horticulture Research Institute, Agricultural Research Centre, Cairo, and* [**]*Olive & Semi-Arid Zone Fruits Deptartment, Horticulture Research Institute, Agricultural Research Centre, Souhag. Egypt.*

Genetic variability of Manfalouty pomegranate (*Punica granatum L.*), the most distributed commercial cultivar in Egypt, was investigated at a private farm located in El-Badari – Assuit governorate, Egypt. A preliminary surveys for 5 years in the field among Manfalouty cultivar was done and resulted in the selection of four promising pomegranate genotypes. Those genotypes proved to have desired characteristics and were given different names by local farmers i.e. (1) Abo- Shelh, (2) Almekhtat, (3) Abo-Shoka, and (4) Genah – Aldabor. The selected genotypes were characterized with molecular genetics identification using RAPD-PCR analysis. Polymorphism was detected among the selected genotypes by RAPD primers using five arbitrary primers for the four selected genotypes in comparison with Manfalouty cultivar. The percentage of polymorphism revealed by different primers ranged from 50% to 87.5% with an average of 64.44%. The UPGMA analysis of genetic distance of the four selected pomegranate genotypes and Manfalouty cultivar showed that, the highest value of similarity index was (0.86) occurred between Manfalouty cultivar and genotype (no.2) while the lowest value of similarity index was (0.67) occurred between the cultivars genotype (no.2) and genotype (no.4). From this study, we confirmed that diversity among the fruit characteristics by indication of genetic relatedness, while molecular tools are valuable to study such similarities.

Generally, it be can concluded from this study that, RAPD markers were able to distinguish the genetic diversity among the different pomegranate genotypes.

Pomegranate is considered an excellent tree for growing in arid zones for its resistance to drought conditions. It is now widely cultivated in Mediterranean, in tropical and subtropical areas. Some parts of the Mediterranean area are also

considered as native of pomegranate. Many cultivars/varieties are listed and collections are already established in different countries, but interchange of plant material is still not frequent.

The description of pomegranate germplasm is based mainly on pomological and agronomic criteria and genetic studies are rare (Mars, 2000). Morphological evaluation especially fruit characteristics, juice parameters and soluble solids/acidity ratio which are stable across environments, are important traits for discriminating the cultivars and also to discriminate to some degree the homonymy; but they are not reliable markers for identification of synonymous genotypes (Durgaç *et al*., 2008). DNA markers are independent from environmental interactions and show high level of polymorphism therefore, they are considered as useful tools for determining genetic relationships and diversity. Recently, different molecular markers have been used to characterize and discriminate different pomegranate cultivars or the polyclonal varieties (Sheidai & Noormohammadi 2005 and Zamani *et al*., 2007). Due to the long history of Egyptian pomegranate cultivation and the related vegetative propagation, several cases of homonymy and synonymy can be observed among this germplasm. Moreover, it is very important for using a sensitive and credible molecular technique to detect the DNA variation and identify the pomegranate germplasm, by helping breeders and nurserymen with the selection and propagation of a cultivar (Nemati *et al*., 2012). A wide range of molecular markers have been used to assess genetic diversity of pomegranate cultivars as well as wild genotypes from different parts of the world. Random amplified polymorphic DNA (RAPD) markers have provided reliable and highly polymorphic information to discriminate pomegranate cultivars (Durgaç *et al*., 2008 and Ercisli *et al*., 2011). These molecular markers provide an opportunity for direct comparison and identification of different genetic material independent of any influences (Bautista *et al*., 2003 and Zhao & Pan, 2004). Collecting and evaluating more pomegranate populations for various characteristics may help in selecting new desirable types (Verma *et al*., 2010). Studying the genetic diversity as well as cultivar identification by using various molecular markers including RAPD (Random Amplified Polymorphic DNA) has been performed in several plant species (Bautista *et al*., 2003 and Zhao & Pan, 2004). These molecular markers provide an opportunity for direct comparison and identification of different genetic material independent of any influences. This study was aimed to identify molecular diversity of four promising genotypes among Manfalouty pomegranate cultivar.

Material and Methods

Plant materials

Four promising pomegranate genotypes of Manfalouty cultivar (the highest cultivated area and commercial cultivar in Egypt), were selected for flowering, yield and molecular studies after field preliminary surveys for 5 years. They have desired characteristics and were named by the local farmers differently:

Genotype no. (1): Abo - Shelh
Genotype no. (2): Almekhtat
Genotype no. (3): Abo-Shoka
Genotype no. (4): Genah – Aldabor

The selected trees were of the same age (15 years – old), grown at a private farm located in El-Badari – Assuit governorate, Egypt. The soil texture is heavy clay and flood irrigation system was applied from Nile. The trees were planted at 6 x 6 m. and subjected to the same standard cultivation practices (according to the Ministry of Agriculture and reclamination lands recommendations).

RAPD-PCR analysis

DNA Extraction

Young and freshly excised tissue were collected separately for each parent and used for DNA extraction was performed as described by (Dellaporta *et al*., 1983). About 0.1 g (fresh weight) of plant tissues was grounded to fine powder in liquid N_2 in a mortar. Before the tissue thawed, 1 ml extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA and 0.5 M NaCl) and 0.2 ml 20% SDS were added. The mixture was incubated at 65 ^{o}C in water bath for 20 minutes. Then 1 ml of phenol, chloroform and isoamyl alcohol (25: 24: 1) was added. Centrifugation was performed at 10,000 rpm for 10 minutes. The supernatants of each sample were transferred separately to new tubes, and then 1 ml of chloroform and isoamyl (24: 1) was added. Centrifugation was performed at 10,000 rpm for 10 minutes. The supernatants of each sample were transferred separately to a new tube, then 1 ml of iso-propanol was added and kept overnight in a freezer. Centrifugation was performed at 10,000 rpm for 10 minutes. The resulted pellets containing DNA were washed in 1 ml ethanol (70%). Centrifugation was performed at 10,000 rpm for 2 minutes. The DNA pellets were re-suspended in 200 µl of TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) buffer. DNA was quantities by quantitatively determined and gel electrophoresis.

Polymerase Chain Reaction (PCR)

In order to obtain clear reproducible amplification products, different preliminary experiments were carried out in which a number of factors were optimized. These factors included PCR temperature cycle profile and concentration of each of the template DNA, primer, $MgCl_2$ and Taq polymerase. A total of twenty-one random DNA oligonucleotide primers were independently used according to (Williams *et al*., 1990) in the PCR reaction.

The eppendorf master cycler PCR amplification was performed in a 25 µl reaction volume containing the following: 2.5 µl of dNTPs (2.5 mM), 1.5µl of Mg Cl_2 (25 mM), 2.5 µl of 10x buffer, 2.0 µl of primer (2.5 µM), 2.0 µl of template DNA (50 ng/µl), 0.3 µl of *promega Taq* polymerase (5 U/µl) and 14.7 µl of sterile ddH_2O. The reaction mixtures were overlaid with a drop of light mineral oil per sample. Amplification was carried out in Techni TC-512 PCR System. The reaction was subjected to one cycle at 95 ºC for 5 minutes, followed by 35 cycles at 94 ºC for 30

seconds, 37 ºC for 30 seconds, and 72 ºC for 30 seconds, then a final cycle of 72 ºC for 5 minutes. PCR products were run at 100 V for one hour on 1.5 % agarose gels to detect polymorphism between selected parents of squash under study. After electrophoresis, the RAPD patterns were visualized with UV transilluminator. RAPD markers were scored from the gels as DNA fragments present or absent in all lanes. PCR amplification was performed using five random 10 mer arbitrary primers synthesized by (Operon biotechnologies, Inc. Germany) OPA 10, OPA 13, OPGO4, OPG 15 and OPM01.The amplified DNA bands generated by each primer were counted and their molecular sizes were compared with those of the DNA markers. The bands scored from DNA profiles generated by each primer were pooled together. Then the presence or absence of each DNA band was treated as a binary character in a data matrix (coded 1 and 0, respectively) to calculate genetic similarity and to construct dendrogram tree among the studied four pomegranate genotypes and Manfalouty cv. Calculation was achieved using Dice similarity coefficients (Dice, 1945) as implemented in the computer program SPSS-10.

Results and Discussion

RAPD-PCR analysis

Polymorphism detected by RAPD primers.

RAPD amplification from all DNA samples of different pomegranate genotypes collected from Assuit governorate. Data of the amplified fragments using those five arbitrary primers for the four selected plants in comparison with Manfalouty cultivar resulted in multiple banding profiles with all selected primers (Fig. 1)**.** The total number of amplified fragments among tested primers ranged from 4 to 16 fragments. 3` anchored OP-A10 primer amplified the highest number of fragments (16 bands); while primer OP-C04 produced the lowest number of fragment (4 bands). The average number of fragments/primer was 9, and the size of these fragments ranged from 155 – 1386 bp. Four of the five selected primers produced polymorphic band (Table, 1). The number polymorphic fragments ranged from 4 to 16 fragments resulting in an average of polymorphism/ primer of (5.6). Primers OP-A10 revealed the highest number of polymorphic fragments (14); however the lowest number of polymorphic fragments was detected by primer OP-C15. The percentage of polymorphism revealed by different primers ranged between 0 % to 87.5 % with an average of 64.44%.

TABLE 1. Pomegranate accessions (collected from Assuit governorate) characterized by total amplified fragments, polymorphic fragments and polymorphism % for each primer.

Primer Names	Total Amplified Fragments	Monomorphic Fragments	Polymorphic Fragments	Polymorphism %
OP-A10	16	2	14	87.5
OP-A13	8	3	5	62.5
OP-C04	4	4	-	0
OP-C15	8	4	4	50.00
OP-M01	9	3	6	66.67
Total	45	16	29	64.44
Average	9	3.2	5.8	

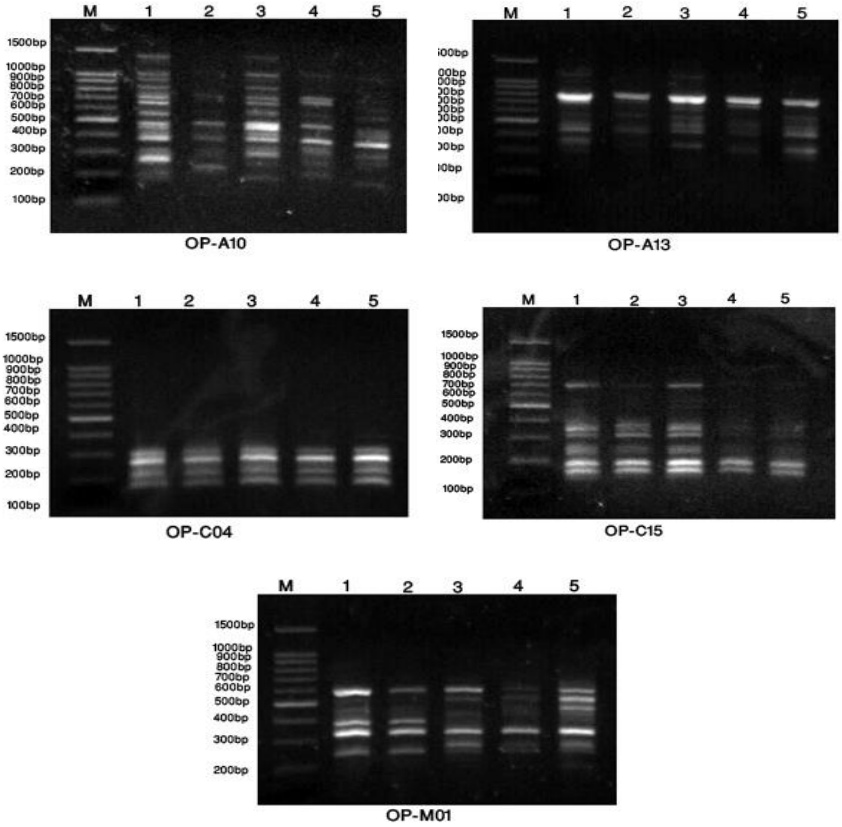

Fig. 1. RAPD profiles of five pomegranate genotypes amplified with five primers for each analysis.

Cluster analysis

The UPGMA cluster analysis of genetic distance among the five selected pomegranate cultivars is shown in Fig 2. The similarly matrix (Table 2), showed that the highest similarly index (0.86) was recorded between pomegranate genotype (Manfalouty cultivar) and pomegranate genotype (no.2) while the lowest similarly index was detected (0.67) between pomegranate genotype (no. 2) and pomegranate genotype (no. 4). A dendrogram for the genetic relationship among the four selected genotypes and Manfalouty cultivar was carried out as in Fig. 2 which separated the five selected pomegranate genotypes into two major groups. The first group included genotype (no. 4) only, while the second group divided into two sub main groups, the first sub main group included only genotype (no. 1) and the second included the other pomegranate genotypes.

TABLE 2. Similarity coefficient among Manfalouty and four pomegranate genotypes based on five RARPs markers.

	M	1	2	3	4
M	1				
1	0.78	1			
2	0.86	0.75	1		
3	0.77	0.75	0.81	1	
4	0.66	0.69	0.67	0.76	1

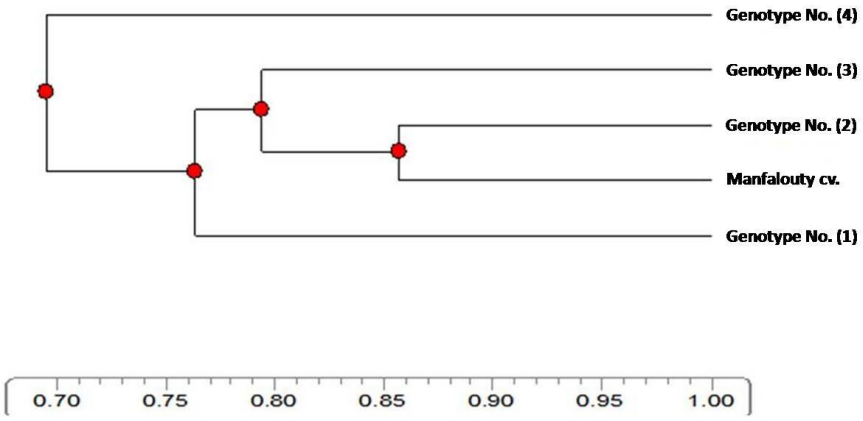

Fig. 2. Dendrogram for the Manfalouty Cultivar and 4 pomegranate genotype accessions (collected from AssuitAssuit governorate) constructed from the RAPD generated data using UPGMA method and similarity matrices computed according to Dice's similarity coefficient.

Genotype identification by Unique RAPD markers.

Unique markers are defined as bands which are present or absent, that specifically identify a cultivar from the others. These bands could be used for genotype identification, discrimination and labeling purposes (Table 3). The RAPD markers generating primers and the positive and/or negative markers approximate size are shown in Table 3. There was a reproducible association between the number of informative bands and the proportion of pomegranate cultivars that could be differentiated through unique markers. Three primers (OP-A10, M-01and C-15) were able to generate unique markers (positive and/or negative) that could differentiate pomegranate genotypes while, two primers (OP-A13 and OP-CO4) were failed to differentiate any genotypes.

TABLE 3. Pomegranate accessions (collected from Assuit governorate) characterized by unique positive and/or negative RAPDs markers, marker size and total number of RAPD marker identifying each genotype.

Cultivars	Primer	Unique positive		Unique negative		Total
		Size in bp	Total	Size in bp	Total	
Genotype Manfalouty	OP-A10	943	1	-	-	1
Genotype 1	OP-M01	----	---	538	1	2
				456	1	
Genotype 2	C-15	529	1	-	-	2
		466	1			
Genotype 4	OP-A10	358	1	689	1	7
				645	1	
		155	1	432	1	
	OP-M01	653	1	-	-	
		211	1	-	-	

The number of generated unique markers ranged from 1 to 7 markers. The maximum number of unique markers was identified with the genotype (no.4) by 7 markers. On the other hand, genotype (Manfalouty), genotype (no.1) and genotype (no.2) were characterized by one and two unique markers, respectively, while the genotype (no.3) can't characterized by unique markers.

The previous results are in harmony with Sarkhosh *et al*. (2006) who studied RAPD markers to determine the diversity level among 24 Iranian pomegranate genotypes. One hundred decamer random primers were used for PCR reactions, among which 16 showed reliable polymorphic patterns. These primers produced 178 bands, of which 102 were polymorphic. Cluster analysis of the genotypes was performed based on data from polymorphic RAPD bands, using Jaccard's similarity coefficient and UPGMA clustering method. The highest and lowest similarities detected between genotypes were 1.00 and 0.00, respectively. At a similarity of 60%, the genotypes were divided into four sub-clusters. Cophenetic correlation coefficient between similarity matrix and cophenetic matrix of dendrogram was relatively high (r = 0.9) showing the goodness of fit of the dendrogram. RAPD markers showed to be a useful tool for studying the genetic diversity of pomegranate. On the other hand, Hasnaoui *et al*. (2010) investigated the genetic diversity among Tunisian pomegranate cultivars using universal primers, the random amplified polymorphic DNA (RAPD) method was used to generate banding profiles from a set of twelve cultivars. Data was then computed with appropriate programs to construct a dendrogram illustrating the relationships between the studied cultivars. The present data proved the efficiency of the designed method to examine the DNA polymorphism in this crop since the tested primers are characterized by a collective resolving power of 12.83. In addition, the cluster analysis has exhibited a parsimonious tree

branching independent from the geographic origin of the cultivars. In spite of the relatively low number of primers and cultivars, RAPD constitutes an appropriate procedure to assess the genetic diversity and to survey the phylogenetic relationships in this crop. The results are also in harmony with Sheidai *et al*., (2008) where RAPD markers variations were studied in ten pomegranate cultivars. Forty RAPD primers were used producing 2050 bands in total. Two hundred and fifteen bands were polymorphic (about 10.50%) and 1835 bands were monomorphic (80.50%). Primers H02 and R01 produced the highest number of polymorphic bands (13 bands, 0.63%), while primer R15 produced no polymorphism.

It can be generally concluded from RAPD analysis and UPGMA analysis of the studied pomegranate genotypes that there was genetic differentiation among them, however there was no correlation between morphological and molecular analyses. The reasons for low correlation between morphological and molecular dendrograms may be due to the effects environmental conditions on morphological characters. It is known that the genes controlling morphological characters studied are different from those illustrated by RAPD analysis, moreover in RAPD, repetitive non- coding sequences might be present too. These differences were also reported in previous studies performed in pomegranate cultivars (Sarkhosh *et al*., 2006, Zamani *et al*., 2007 and Durgac *et al*., 2008). It is known that some mutations and genetic changes that are easily recognizable not detectable by application of molecular markers (Garcia *et al*., 2002 and Talebi Baddaf *et al*., 2003).

This study provides a scientific basis for the future selection and management of pomegranate germplasm as well as indicated the level of polymorphism among pomegranate and these genotypes could be used in future breeding programs.

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دراسة أولية عن التنوع الجينى بين أنماط وراثية مختلفة من صنف الرمان المنفلوطى

ب- التحديد الوراث الجزيئي باستخدام علامات RAPD

صفية عبد المنعم أبو طالب*، طارق خلف أحمد البلك و هالة نجيب محمد عبد الله***

*قسم بحوث الزيتون وفاكهة المناطق شبه الجافة - معهد بحوث البساتين - مركز البحوث الزراعية - القاهرة و ** قسم بحوث الزيتون وفاكهة المناطق شبه الجافة - معهد بحوث البساتين - مركز البحوث الزراعية – سوهاج - مصر.

أجريت دراسة الاختلافات الوراثية فى صنف الرمان المنفلوطى الأكثر انتشارا على المستوى التجاري فى مصر والمنزرع فى مزرعة خاصة بالبدارى - محافظة أسيوط – مصر بعد عملية مسح أولى لمدة خمس سنوات فى الحقل.
تم انتخاب أربعة أنماط وراثية واعدة لصنف الرمان المنفلوطى حيث أنها أثبتت أن لها صفات مرغوبة وأطلق عليها المزارعون أسماء مختلفة (١- ابو شلح ، ٢- المخطط ، ٣-أبو شوكة ، ٤- جناح الدبور).

تم تحديد التباين الوراثي عن طريق تحليل RAPD باستخدام خمس بوادئ لمقارنة الأربع أنماط وراثية المختارة بالصنف المنفلوطى. تراوحت النسبة المئوية للتباين الموضحة بواسطة البوادىء المختلفة بين ٥٠٪ - ٨٧,٥٪ ومتوسط ٦٤,٤٤٪ بالنسبة للتحاليل الخاصة بشجرة UPGMA لقياس القرابة الوراثية للأربعة أنماط المختارة للرمان والصنف المنفلوطى أوضحت أن القيمة الأعلى لمؤشر التشابة وهو (٠,٨٦) بين الصنف منفلوطى والنمط الوراثى رقم (٢) بينما القيمة الأدنى لمؤشر التشابة وهو (٠,٦٧) بين النمط الوراثى رقم (٢) والنمط الوراثى رقم (٤). هذه الدراسة تؤكد ان التنوع بين خصائص الثمار هو وراثي عن طريق تحديد القرابة الوراثية ، وأن دراسة الوراثة الجزئية تؤكد وجود التنوع الوراثي الحقيقي.
يمكن أن نستنتج من هذه الدراسة أن دلالات RAPD كانت قادرة على التمييز بين التنوع الجيني بين الأنماط الوراثية المختلفة للرمان.