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Authentication of *Euphorbia peplus* L. Family Euphorbiaceae Growing in Egypt Using Finger Printing

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Abstract

RAPD-PCR was performed using six random primers to identify the genetic diversity among six plant samples belong to two genera (*Euphorbia* and *Ricinus*). The dendrogram, based on genetic distance, depict the relationship among the investigated plant samples, separate clearly the six samples. The closest relationship was observed between *E. geniculata* and *E. aphylla*; and *E. pulcherrima* and *E. peplus*, while this relationship was quite separated between these four samples and the other two samples *E. cactus* and *R. communis*. Fragments generated by the six primers show a polymorphism ratio of 88.9%. Bands 3500 and 750 bp generated by primer OP-Z13, and also bands 2000, 1500, 1400, 1200, 1000, 720 and 550 bp generated by primer OP-A09 existing only in the plant samples of *E. geniculata* and *E. aphylla*, which suggest that these bands can be used as a positive molecular marker to identify these plant samples. Bands 2500, 1720, 1650, 1300, 950 and 250 bp generated by primer OP-A09, and band 1200 bp generated by primer OP-A20 and band 350 bp generated by primer OP-Z19 and band 250 bp generated by primer OP-Z17 were common in all plant samples of family *Euphorbiaceae*. Moreover, band 430 bp generated by primer OP-Z17 was characterized for *Ricinus communis* and absent in other plants of genus *Euphorbia*. Also, band 2700 bp generated by primer OP-A20 and band 210 bp generated by primer OP-Z19 existing only in *Euphorbia peplus*. This study highlights the usefulness of RAPD assay for determining genetic variation in different plant genera and for estimating genetic distances between different plant samples. Moreover, knowledge of genetic distance among genera and species, and genetic diversity/structure within genera could be useful for conservation of genetic resources. Data presented here are the first report in Egypt of genetic variation inside genera *Euphorbia* and *Ricinus* described at the molecular level. We consider this work as a first step in molecular characterization of genera *Euphorbia* and *Ricinus*, thus, it is recommended to extend the panel of samples and primers in the future.

***Keywords**: Fingerprinting, RAPD-PCR, Genetic marker, Euphorbia, Ricinus.*

Introduction

Authentication of medicinal plants is a critical issue (Core, 1962; Lawrence, 1968 and Kirticar and Basu, 1975). Macroscopic and microscopic studies can be used, as they are rapid and inexpensive identification techniques. Chemical analysis is the best method for the detection of contaminants and could be an excellent method for plant identification (Jassbi, 2006; Ria *et al.,* 2009 and Uchida *et al.,* 2010). Molecular biology offers an assortment of techniques that can be very useful for authentication of medicinal plants,

DNA base-pair sequences guides the production of proteins and enzymes. These in turn decide features such as leaf shape and flower color, as well as direct synthesis of a wide range of phytochemicals in plants. DNA fingerprint can distinguish plants from different families, genera and species. DNA fingerprinting refer to the use of techniques based polymerase chain reaction (PCR), a system for the amplification of DNA, reveal the specific profile for a particular organism which is a unique as a fingerprint (Powledge, 1995 and Rosso and Philippe, 2001). Medicinal plants have always an important place in the therapeutic system. The use of natural products in the treatment of various diseases has played an important role in medical therapy for many years and plants of the genus *Euphorbia* are known to possess considerable medicinal and economic importance components (Tian *et al.,* 2010 and Chaabi *et al.,* 2007). Euphorbiaceae is one of the largest families of higher plants comprising about 283 genera and 7500 species (Core, 1962; Lawrence, 1968; Tackholm, 1974; Kirticar and Basu, 1975; Watt and Breyer-Brandwijk, 1962; Boulos, 1980 and Benson, 1957) that are further characterized by the frequent occurrence of milky sap. Genus *Euphorbia* is the one of the largest six genera of flowering plants with approximately 1600 species (Boulos, 1980 and Chopra, 1973). Economically, important products including foods, drugs and rubber (Watt and Breyer-Brandwijk, 1962 and Boulos, 1980) are obtained from certain *Euphorbia* species. Reviewing the available literature, genetic diversity of genera *Euphorbia* and *Ricinus* growing in Egypt has not been sufficiently studied. This provoked us to carry out this study to provide information at the molecular level about the authentication and genetic diversity of these genera, employing RAPD-PCR molecular markers.

Materials and Methods

Six plant samples belong to tow genera, *Euphorbia* (1= *E. geniculate*, 2= *E. pulcherrima*, 3= *E. peplus*, 4= *E. aphylla* and 5= *E. cactus*) and *Ricinus* (6= *R. communis*) collected from Assiut University Campus, were subjected to Random Amplified Polymorphic DNA (RAPD) technique using six decamer random primers, obtained from Operon Technologies Inc.: USA.

Extraction and Purification of Genomic DNA: Total genomic DNA was extracted using the Qiagen DNeasy (Qiagen Santa Clara, CA). This was performed following the manufacturer's instructions. DNA concentration was determined by diluting the DNA (1:5) in dH_2O. The DNA samples were electrophoresed in 1% agarose gel against 10µg of a DNA size marker. This marker covers a range of concentration between 95ng to 11ng. Samples concentration was adjusted at 25 ng/µl using TE buffer pH 8.0.

PCR conditions for RAPD analysis:

PCR for RAPD analysis was done using 25 ng genomic DNA of six plant samples. The PCR mixture and amplification conditions were prepared according to Williams *et al.,* (1990) with minor modifications. A set of six primers RAPD (OP-A09, OP-A20, OP-B03, OP-Z13, OP-Z17 and OP-Z19) were used. The amplifi-

cation reaction was carried out in 25 µl reaction volume. The contents of PCR mixture are shown in Table 1. PCR amplification cycles were performed in a Perkin-Elmer/GeneAmp® PCR system as follows: Pre-Denaturation (one cycle) at 94 °C for 5 min, followed by 40 cycles of: Denaturation step at 94 °C for 1 min, Annealing step at 36 °C for 1 min, and an elongation step at 72 °C for 1.5 min, then final extension at 72 °C for 7 min.

Table 1. contents of PCR mixture for RAPD analysis

0.2 mM dNTPs	2.5 µl
1X reaction buffer + $MgCl_2$	2.5 µl
1 µM primer	3.0 µl
25 ng template DNA	1.0 µl
1 units Taq (super thermal)	1.0 µl
H_2O	15.0 µl
Total volume	25.0 µl

The amplification products were revolved by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 95 volts. PCR products were visualized on UV Transilluminator and photographed. RAPD-PCR profiles were analyzed using Gene profiler 3.1 software. The banding profiles were scored (using 1kb DNA Ladder RTU, promega) and the presence or absence of each size class was scored as (+) or (-), respectively. The scored bands were analyzed by unweighted pair-group method based on arithmetic mean (UPGMA) to estimate similarity, genetic distances and reconstruct the dendogram.

Results

Genetic diversity and relationship between six species belong to two genera (*Euphorbia* and *Ricinus*) were studied using Random Amplified Polymorphic DNA (RAPD) technique. Six oligodecamers arbitrarily primers used in the present investigation to generate RAPD profiles from the six plant samples. All primers were amplified successfully on the genomic DNA from taken samples yielding distinct RAPD patterns.

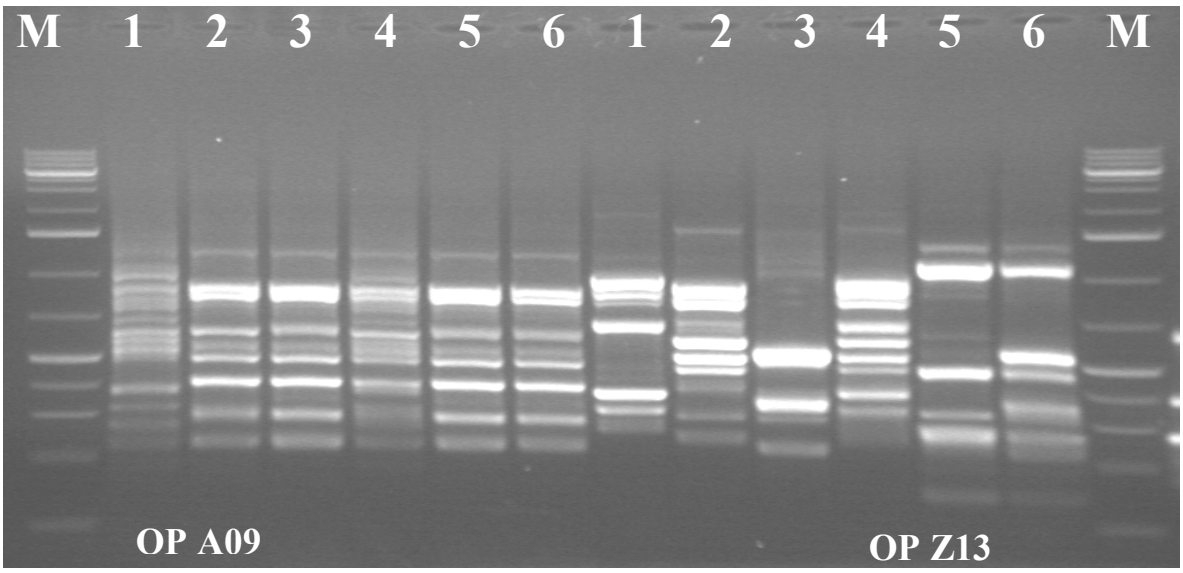

Figure 1. Agarose-gel electrophoresis of RAPD products generated by primer OP-A09 and OP-Z13 in the examined samples, M = DNA marker.

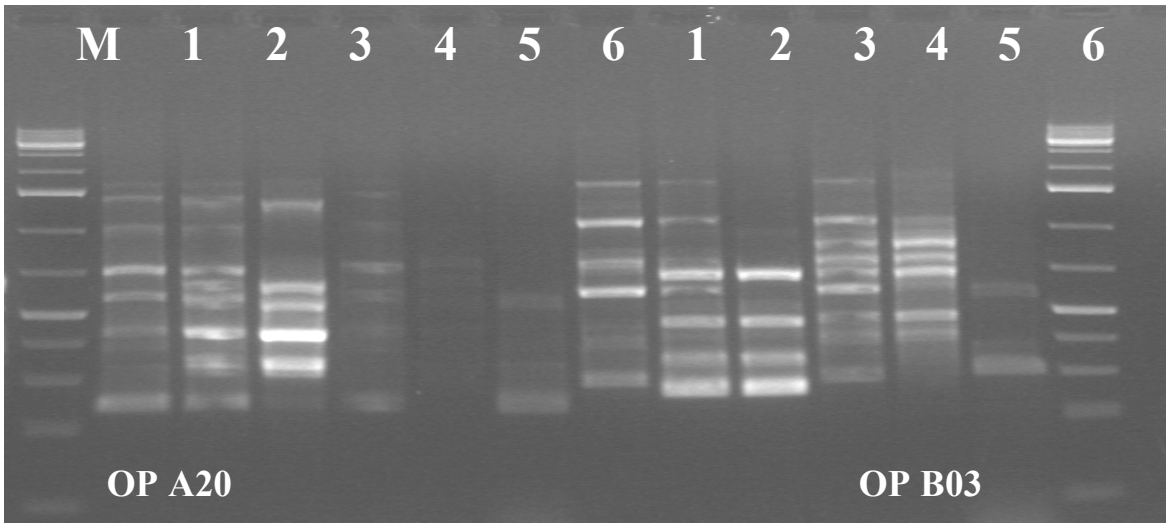

Figure 2: Agarose-gel electrophoresis of RAPD products generated by primer OP-A20 and OP-B03 in the examined samples, M = DNA marker.

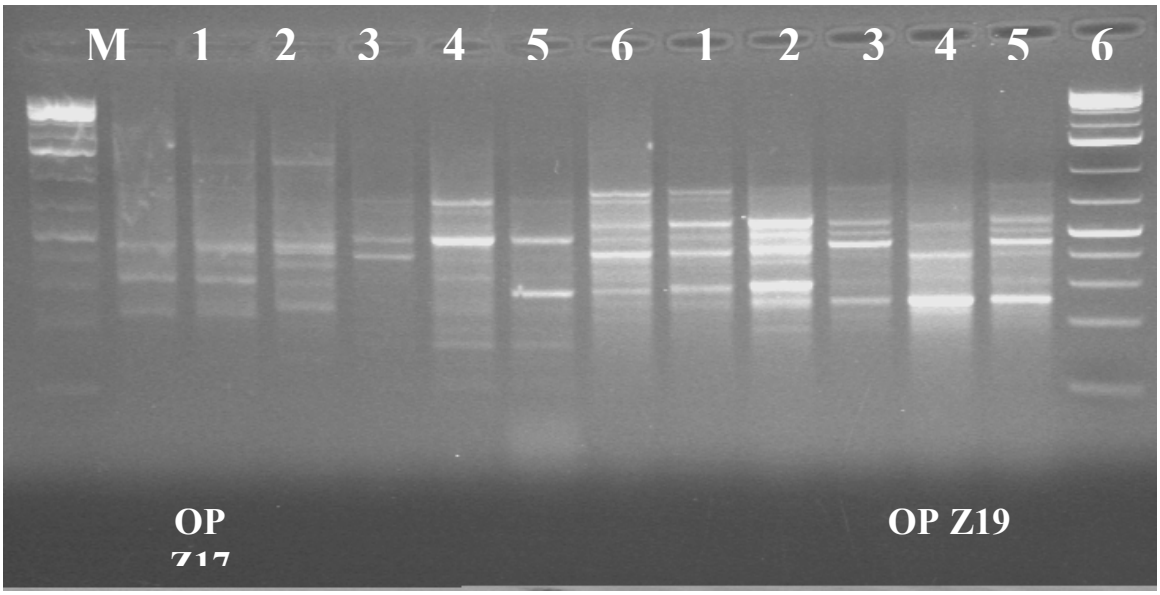

Figure 3. Agarose-gel electrophoresis of RAPD products generated by primer OP-Z17 and OP-Z19 in the examined samples, M = DNA marker.

Table 2. Summary of all fragments generated by the assay of the six primers, and their molecular size (bp) in all six samples of *Euphorbia* and *Ricinus* where (+) means presence and (-) means absence.

Primer	M.W.	Samples						Primer	M.W.	Samples					
code	(bp)	1	2	3	4	5	6	code	(bp)	1	2	3	4	5	6
OPZ13	3500	+	-	-	+	-	-	OPA09	950	+	+	+	+	+	+
OPA20	3200	+	+	+	+	-	-	OPZ17	900	+	+	+	-	-	-
OPB03	3200	+	+	-	+	-	-	OPA20	850	+	+	+	-	-	-
OPZ13	3000	-	+	-	+	-	-	OPZ19	850	+	+	+	+	-	+
OPZ17	3000	+	+	+	-	-	-	OPA09	750	-	+	+	+	+	+
OPA20	2800	+	+	-	+	-	-	OPA20	750	+	-	-	-	-	
OPZ13	2800	-	-	-	-	+	+	OPB03	750	+	+	+	+	+	-
OPA20	2700	-	-	+	-	-	-	OPZ13	750	+	-	-	+	-	-
OPA09	2500	+	+	+	+	+	+	OPZ17	750	-	-	-	+	-	-
OPZ13	2200	-	-	-	-	+	+	OPA09	720	+	-	-	+	-	-
OPB03	2100	+	+	-	+	+	-	OPZ17	700	-	+	+	-	+	+
OPA09	2000	+	-	-	+	-	-	OPZ19	700	+	+	+	-	+	+
OPA20	2000	+	+	-	+	-	-	OPB03	650	+	-	-	+	+	+
OPA09	1720	+	+	+	+	+	+	OPZ13	650	+	-	+	+	+	+
OPB03	1720	-	-	-	+	+	-	OPA20	580	+	+	+	-	-	+
OPZ13	1720	+	+	-	+	-	-	OPA09	550	+	-	-	+	-	-
OPZ19	1720	+	+	+	+	-	+	OPZ19	550	+	-	-	-	+	+
OPA09	1650	+	+	+	+	+	+	OPZ17	530	-	-	-	-	+	-
OPZ13	1620	+	+	-	+	-	-	OPA09	500	-	+	+	-	+	+
OPB03	1600	+	+	-	+	+	-	OPA20	500	-	+	+	-	-	-
OPA09	1500	+	-	-	+	-	-	OPB03	500	-	+	+	-	-	+
OPA20	1500	+	+	-	+	+	-	OPZ13	500	-	+	+	-	+	+
OPB03	1500	-	+	+	+	+	-	OPZ17	500	+	+	+	-	-	-
OPZ13	1500	+	+	-	+	-	-	OPZ19	470	+	+	+	-	-	-
OPZ17	1500	-	-	-	+	+	+	OPB03	450	+	-	-	+	-	+
OPZ19	1500	+	+	-	-	-	-	OPZ17	430	-	-	-	-	-	+
OPA09	1400	+	-	-	+	-	-	OPA09	400	+	-	-	-	-	-
OPA09	1300	+	+	+	+	+	+	OPZ13	400	+	-	-	-	+	+
OPA20	1300	-	+	+	-	-	-	OPZ13	350	-	+	-	-	+	+
OPZ13	1300	-	+	-	+	-	-	OPZ19	350	+	+	+	+	+	+
OPA09	1200	+	-	-	+	-	-	OPA20	320	+	+	+	+	-	+
OPA20	1200	+	+	+	+	+	+	OPB03	300	-	+	+	-	-	-
OPB03	1200	+	+	-	+	+	+	OPZ17	300	-	-	+	-	-	-
OPZ19	1200	+	+	+	+	+	+	OPA09	250	+	+	+	+	+	+
OPA09	1100	-	+	+	-	+	+	OPZ13	250	-	-	+	-	-	+
OPZ13	1100	-	+	+	+	-	+	OPZ17	250	+	+	-	-	+	+
OPA09	1000	+	-	-	+	-	-	OPZ19	210	-	-	+	-	-	-
OPB03	1000	-	+	+	+	+	-	OPZ17	200	-	-	-	-	+	+
OPZ17	1000	-	-	-	+	+	+	OPZ13,17	150	-	-	-	-	+	+
OPZ19	1000	+	+	+	+	-	+	**Total**	**81**	**50**	**50**	**39**	**48**	**38**	**40**
OPZ13	980	-	+	-	+	+	+								

The amplified fragments per primer varied between 10 (OP-Z19) and 17 (OP-A9 and OP-Z13), with an average 13.5 bands per primer. These fragments have a size ranged from 3500 to 150 base pairs (bp). A total number of 81 bands were amplified, with only 9 bands being monomorphic (11.1%) and the other 72 bands were polymorphic with a polymorphism ratio of 88.9%. Primer OP-A09 reacted with six plant samples generating 17 fragments ranged in size from 2500 to 250 bp (Figure 1). The

number of fragments generated by this primer varied among samples where the highest number was 14 observed in samples number 1 and 4, while the lowest number was 9 in samples number 2, 3, 5 and 6. Fragment of size 400 bp was generated only from sample 1 and absent in all other samples. Meanwhile, fragment of size 750 bp was absent in sample number 1 only and present in the other samples. Primer OP-Z13 (Figure 1) generated 17 fragments ranged in size from 3500 to 150 bp. The lowest number of fragments generated by this primer was 4 observed in sample number 3, while the highest number was 10 in samples 4 and 6. Fragment of size 650 bp was absent in sample number 2 only and present in the other samples. Primer OP-A20 reacted with the samples generating 12 fragments having sizes ranged from 3200 and 320 bp. The number of fragments generated by this primer varied among samples from two in sample number 5, to ten in sample number 2. Fragments of size 2700 and 750 bp were generated only with samples 3 and 1, respectively and absent in all other samples. Meanwhile, fragment of size 320 bp was absent in sample number 5 only and present in the other samples. The results of RAPD analysis obtained by primer OP-B03 are illustrated in Figure 2 and Table 2. Primer OP-B03 produced 12 fragments ranged in size from 3200 to 300 bp. The lowest number of fragments generated by this primer was four observed in sample number 6, while the highest number was ten in sample number 4. Fragments of size 1200 and 750 bp were absent only in samples number 3 and 6, respectively and present in the other samples. Primer OP-Z17 (Figure 3) generated 13 fragments ranged in size from 3000 to 150 bp. The number of fragments generated by this primer varied among samples from three in sample number 4 to seven in samples number 5 and 6. The fragments with size 750 and 530 bp were present only with samples number 4 and 5, respectively and absent in all other samples. Also, fragments with size 430 and 300 bp were present only with samples number 6 and 3, respectively and absent in all other samples. Primer OP-Z19 (Figure 3 and Table 2) generated 10 fragments ranged in size from 1720 to 210 bp. The lowest number of fragments generated by this primer was four observed in sample number 5, while the highest number was nine in sample number 1. The fragment with size 210 bp was present only with sample number 3 and absent in all other samples. Meanwhile, fragments of size 1720, 1000 and 850 bp were absent in sample number 5 only and present in all other samples. Also, fragment of size 700 bp was absent in sample number 4 only and present in all other samples.

Genetic distances and cluster analysis based on RAPD results:

Pairwise genetic similarity (GS) was estimated for the six combined primers (Table 3). The results showed that the highest percentages of similarity were 76.9 % (between samples 5 and 6) and 76.3 % (between samples 1 and 2). A dendogram, indicating genetic relationships between the six plant samples of *Euphorbia* and *Ricinus*, was generated by cluster analysis (UPGMA) with the RAPD

data (Figure 4). Plant samples were classified into two main clusters. The first cluster contains samples number 5 (*E. cactus*) and 6 (*R. communis*). The second cluster divides into two sub-clusters, one of them contains samples number 1 (*E. geniculate*) and number 4 (*E. aphylla*). The second sub-cluster contains samples number 2 (*E. pulcherrima*) and number 3 (*E. peplus*).

Table 3. The similarity matrix in percentage, among the six examined plant samples based on RAPD banding pattern analysis and Jaccard's Coefficient.

Samples	1	2	3	4	5	6
1	100					
2	76.3	100				
3	67.3	66	100			
4	50.6	46.5	75.9	100		
5	49.4	50	58.4	61.5	100	
6	46	53.5	57.5	50	76.9	100

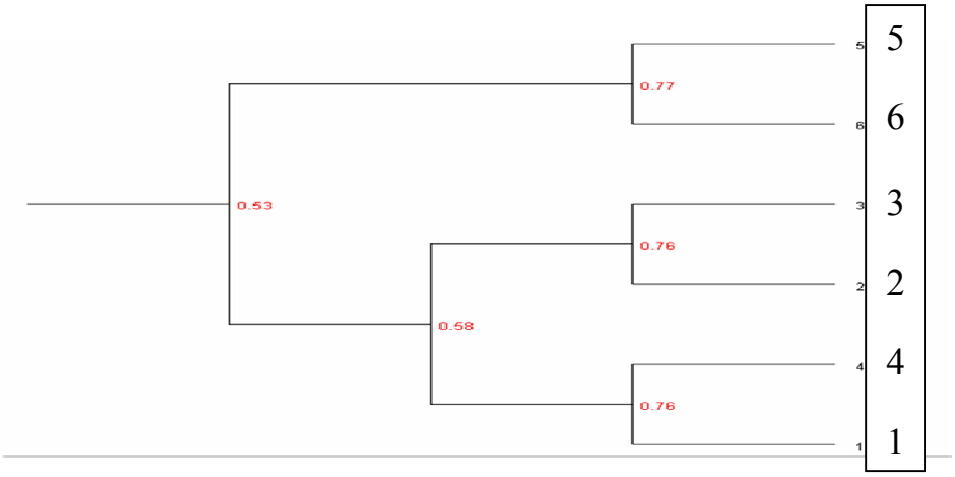

Figure 4. UPGMA dendrogram of the six plant samples of *Euphorbia* and *Ricinus* based on values of genetic distances calculated from all.

Discussion

Random amplified polymorphic DNA (RAPD) was proved to be a powerful tool in different genetic analyses (Williams *et al.,* 1990). This approach detects DNA polymorphisms based on amplification using single DNA fragments. It is specific, quick and do not require previous DNA sequence information (Williams *et al*., 1990; Park *et al*., 2000 and Ragot and Hoisington, 1993). RAPD-PCR was performed using six random primers to identify the genetic diversity among six plant samples belong to two genera (*Euphorbia* and *Ricinus*). After estimation of genetic similarity and establishment of dendrogram, subjects are classified into two distinct groups with low

bootstrap value (53%). The first group contains two subjects, *Euphorbia cactus* and *Ricinus communis*, with significant bootstrap value (77%). The second group compromises two distinct clusters (subcluster) with low bootstrap value (58%). The first cluster is contains two subjects, *Euphorbia geniculata* and *Euphorbia aphylla*, supported by high bootstrap value (76%). The second cluster is contains also two subjects, *Euphorbia pulcherrima* and *Euphorbia peplus*, with significant bootstrap value (76%). Complete analysis of the resulted bands reveals interesting findings (Table 4). Fragments generated by primers show a polymorphism ratio of 88.9%. Bands 3500 and 750 bp generated by primer OP-Z13, and also bands 2000, 1500, 1400, 1200, 1000, 720 and 550 bp generated by primer OP-A09 existing only in the plant samples *E. geniculata* and *E. aphylla*, which suggest that these bands generated by these primers can be used as a positive molecular marker to identify these plant samples and the active constituents of these plants are closely related. In addition, bands 2800, 2200 and 150 bp generated by primer OP-Z17 existing only with plant samples *E. cactus* and *R. communis* which suggest that these bands can be used as a positive molecular marker to identify these plant samples and support our finding of dendogram (Fig. 4). Also, band 1300 bp generated by primer OP-A20 and band 300 bp generated by primer OP-B03 existing only in the plant samples *E. pulcherrima* and *E. peplus* which suggest that these bands can be used as a positive molecular marker to identify these plant samples and support our finding of dendogram (Fig. 4). Bands 2500, 1720, 1650, 1300, 950 and 250 bp generated by primer OP-A09, and band 1200 bp generated by primer OP-A20 and band 350 bp generated by primer OP-Z19 and band 250 bp generated by primer OP-Z17 were common in all plant samples of family *Euphorbiaceae*. Moreover, band 430 bp generated by primer OP-Z17 was characterized for *Ricinus communis* and absent in other five plants of genus *Euphorbia*. Also, band 2700 bp generated by primer OP-A20 and band 210 bp generated by primer OP-Z19 existing only in *Euphorbia peplus*. The dendrogram, based on genetic distance, depict the relationship among the investigated plant samples, separate clearly the six samples. The closest relationship was observed between *E. geniculata* and *E. aphylla*; and *E. pulcherrima* and *E. peplus*, while this relationship was quite separated between these four samples and the other two samples *E. cactus* and *R. communis*.

Table 4. Summary of the molecular marker (Fingerprinting) produced by different primers used with species belong to *Euphorbia* and *Ricinus*.

Plant species	Primer code	Band size in bp	Type of marker
E. geniculata	OP-A20	750	Positive
	OP-A09	750	Negative
		400	Positive
E. pulcherrima	OP-Z13	650	Negative
E. peplus	OP-A20	2700	Positive
	OP-B03	1200	Negative
	OP-Z17	300	Positive
	OP-Z19	210	Positive
E. aphylla	OP-Z17	750	Positive
	OP-Z19	700	Negative
E. cactus	OP-Z19	1720	Negative
		1000	Negative
		850	Negative
	OP-Z17	530	Positive
	OP-A20	320	Negative
R. communis	OP-B03	750	Negative
	OP-Z17	430	Positive

This study highlights the usefulness of RAPD assay for determining genetic variation in different plant genera and for estimating genetic distances between different plant samples. Moreover, knowledge of genetic distance among genera and species, and genetic diversity/structure within genera could be useful for conservation of genetic resources. Data presented here are the first report in Egypt of genetic variation inside genera *Euphorbia* and *Ricinus* described at the molecular level. We consider this work as a first step in molecular characterization of genera *Euphorbia* and *Ricinus*, thus, it is recommended to extend the panel of samples and primers in the future.

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توثيق نسب نبات الافوربيا بيبلس ل. التابع للعائلة اللبنية الذي ينمو في مصر باستخدام البصمة الوراثية

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الملخص

توثيق نسب النباتات الطبية يعد من الموضوعات الهامة. والنباتات التابعة للعائلة اللبنية ، والتي لها استخدامات طبية كثيرة ، والتي تنمو في مصر لم تلقي دراسة وافية. لذلك تم عمل البصمة الوراثية باستخدام عدد ستة باديئات بتقنية الـ PAPD-PCR لتحديد الاختلافات الوراثية لستة عينات نباتية تتبع اثنين من الاجناس هما الافوربيا والريسيناس. اظهرت النتائج ان البصمة الوراثية لنبات الافوربيا بيبلس تشبه كثيرا البصمة الوراثية لنبات الافوربيا بلكيريما كما ظهر ذلك من شجرة النسب. ايضا يوجد تقارب وراثي بين الافوربيا جينكيولاتا والافوربيا افيلا. بينما ظهر ان العلاقة الوراثية بين تلك النباتات وكلا من الافوربيا كاكتس والريسيناس كوميونس اكثر تباعدا. الشظايا الناتجة بالبادئات اظهرت نسبة 88.9% لتعدد الاشكال. والحزم الوراثية 3500 و 750 والناتجة بالباديئ OP-Z13 وكذلك الحزم 2000 و 1500 و 1400 و 1200 و 1000 و 720 و 550 والناتجة بالباديئ OP-A09 تنشاء فقط في نباتات الافوربيا جينكيولاتا والافوربيا افيلا ويمكن استخدامها كواسمات وراثية لتحديد تلك النباتات. ايضا الحزمة 2700 الناشيئة بالباديئ OP-A20 وكذلك الحزمة 210 الناشيئة بالباديئ OP-Z19 توجد فقط في نبات الافوربيا بيبلس ويمكن استخدامها كواسمات وراثية موجبة لتحديد تلك النباتات. علاوة علي ذلك الحزم 2500 و 1720 و 1650 و 1300 و 950 و 250 الناتجة بالباديئ OP-A09 والحزمة 1200 الناتجة بالباديئ OP-A20 والحزمة 350 الناتجة بالباديئ OP-Z19 والحزمة 250 الناتجة بالباديئ OP-Z17 كانت موجودة بصورة عامة في كل نباتات العائلة ايوفوربيسيا. علي الجانب الاخر الحزمة 430 الناتجة بالباديئ OP-Z17 كانت مميزة لنبات الريسيناس كوميونس ولاتوجد في جنس الايفوربيا.

هذه الدراسة تلقي الضوء علي فائدة تقنية الـ RAPD لتحديد التباين الوراثي للاجناس النباتية. علاوة علي ان توافر المعلومات عن التباعد الوراثي للاجناس والانواع قد يكون ذو فائدة للمحافظة علي المصادر الوراثية. النتائج المذكورة في هذا البحث تعد الاولي في مصر لتحديد التباين الوراثي لاجناس الافوربيا والريسيناس علي المستوي الجزيئي.