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RAPD-PCR analysis of some species of *Euphorbia* grown in University of Baghdad Campus in Jadiriyah

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This study attempts to identify species of *Euphorbia* (*Euphorbia peplus*, *Euphorbia helioscopia*, *Euphorbia granulata* and *Euphorbia hirta*) grown in University of Baghdad Campus in Jadiriyah and determine the genetic polymorphism among them by using DNA markers generated by polymerase chain reaction (PCR). Total genomic DNA of species studied was extracted from dry seeds by using commercial kit. Molecular analysis was performed by using nine random markers in random amplified polymorphic DNA (RAPD-PCR) technique. RAPD-PCR analyses based on three primers A13, C05 and D20 gave results in term of amplification and polymorphisim for the four species studied. The genetic polymorphisms value of each primer was determined and ranged between 47 to 84%; primer A13 produced the highest percent of genetic polymorphism compared with primer C05. RAPD-PCR technique confirmed the isolation of the four species of *Euphorbia* obviously.

Key words: *Euphorbia* spp., random markers in random amplified polymorphic DNA (RAPD-PCR), monomorphic, polymorphic, random primers.

INTRODUCTION

The genus *Euphorbia* spp. is one of the largest and most complex genera of flowering plants. High morphological plasticity and diversity of this genus make taxonomical studies attractive for botanists. Also, this species have shown their own economic value and hence contribute to the floristic wealth of tropical and subtropical countries of the world as well as reputed for the production of valuable secondary metabolites like alkaloids, flavonoids and terpenes in nature. There are about 44 species of *Euphorbia* in Iraqi flora (Radcliff-Smith, 1980), and more than four species in University of Baghdad Campus in Jadiriyah (Zokian, 2011). Early proponents of molecular systemic claimed that molecular data were more likely to reflect the true phylogeny than morphological data, ostensibly because they reflected gene-level changes, which were thought to be less subject to convergence and parallelism than were morphological traits.

In many cases, molecular data supported the morphology of groups that were recognized on morphological grounds. More importantly, molecular data often allowed syste-matizes to choose among competing hypotheses of relationships (Judd et al., 1999). Genetic identification can be performed by examining morphological or phonotypical characteristics but such characteristics are affected by environmental conditions. However, DNA based techniques allow scanning the genome directly without being environmental affected. Today, genetic variations between species can be revealed in short time

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Table 1. The names of the random primers used in the study and their equences (Ahmadikhah and Alvi, 2009).

Number	Primer's name	Sequence 5'------ 3'
1	A07	GAAACGGGTG
2	A08	GTGACGTAGG
3	A13	CAGCACCCAC
4	C05	GATGACCGCC
5	D20	ACCCGGTCAC
6	P06	TCGGCGGTTC
7	P07	CTGCATCGTG
8	R02	GTCCTCGTGT
9	R03	ACGGTTCCAC

and easily, and the population can be examined rapidly through random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) (Sesli and Yegenoglu, 2010).

In this study, there was an attempt to identify the four species of *Euphorbia* (*Euphorbia peplus*, *Euphorbia helioscopia*, *Euphorbia granulata* and *Euphorbia hirta*) distributed at University of Baghdad Campus by using the technique of RAPD- PCR.

MATERIALS AND METHODS

Molecular weight markers

The DNA markers (Bench top PCR markers 50 to 1000 bp and 1 kb DNA ladder 250 to 10000 bp) were prepared according to the manufacturer instructions.

DNA extraction from dry seeds of *Euphorbia*

The DNA was extracted from dry seeds by using commercial kit; High Pure GMO sample preparation kit provided by Roche - Germany.

Estimation of DNA concentration by spectrophotometer

5 µl of each sample were added to 495 µl of distilled water (DW) and mixed well to determine the DNA concentration and its purity by using the spectrophotometer. A spectrophotometer was used to measure the optical density (OD) at wave length of 260 and 280 nm. An OD of 1 corresponds to approximately 50 µg/ml for double stranded DNA (Maniatis et al., 1982). The concentration of DNA was calculated according to the formula:

DNA concentration (µg/ml) = O.D 260 nm × 50 × dilution factor

The spectrophotometer was used also to estimate the DNA purity ratio according to this formula:

DNA purity ratio = O.D 260 nm / O.D 280 nm

This ratio was used to detect nucleic acid contamination in protein preparations. DNA quality can be also assessed by simply analyzing the DNA by agarose gel electrophoresis (Maniatis et al., 1982).

Agarose gel electrophoresis

Agarose gels in different concentrations were used (0.8% for extracted DNA, and 1.2% for visual checking to separate DNA fragments of RAPD product). Gels were run horizontally in 0.5X Tris-borate-EDTA (TBE) buffer. Electrophoresis buffer was added to cover the gel and run for 2 h at 5 V/cm. Agarose gels were stained with ethidium bromide 0.5 µg/ml for 20 to 30 min. DNA bands were visualized by UV transilluminator at 365 nm wavelength (Maniatis et al., 1982). A gel documentation system was used to document the observed bands.

RAPD-PCR analysis of genomic DNA of *Euphorbia* species

Randomly primers

Nine random sequence decamer primers were used, synthesis by Alpha DNA-Canada from different series (A, C, D, P and R) in a lyophilized form and were dissolved in sterile distilled water to give a final concentration of 10 pmol/µl as recommended by provider. The primers used and their sequences are listed in Table 1.

Go Taq®Green master mix (2X)

Go *Taq*®Green master mix is a ready to use mixture that contains *Taq* DNA polymerase, $MgCl_2$, pure deoxynucleotides (dNTPs), reaction buffer and two dyes (blue and yellow) that allow monitoring of progress during electrophoresis, with concentration 2X. Go *Taq*®Green master mix was provided by Promega-USA. Amplification was performed on ice in aseptic conditions in laminar air flow using 0.2 ml tight cap Eppendorf tubes. A negative control reaction in each PCR experiment was set up containing all components of the reaction without template DNA so that any contaminating DNA present in the reaction would be amplified and detected on agarose gel.

Protocol of RAPD-PCR

PCR was performed with a protocol which includes the following:

PCR mix

About 12.5 µl of the PCR ready mix (Go *Taq*®Green Master Mix) was added when the final reaction volume was 25 µl to obtain a final concentration 1X as recommended by provider and sterile distilled water was used to achieve a total volume of 25 µl after added each of primers and DNA template.

Amplification reaction

Amplification of random fragments of genomic DNA was preformed with the following master amplification reaction. RAPD-PCR master mix (final reaction volume = 25 µl).

PCR program

The amplification program was run as follows: Initial denaturartion at 94°C for 5 min with a total number of 45 cycles. Denaturation at 94°C for 1 min, annealing at 36°C for 1 min extension at 72°C for 2 min and final extension at 72°C for 10 min. Approximately, 20 µl of PCR amplified products were separated by electrophoresis in 1.2% agarose gels (2 h, 5 V/cm, 0.5 X Tris-borate buffer). Gels stained with ethidium bromide, PCR products were visualized by UV transilluminator and then were imaged by gel documentation system (Hashemi et al., 2009). The amplified products usually consist of 1 to 10 discrete bands and may increase to 15 bands, the molecular weight of RAPD-PCR products estimated by comparing with the marker 1 kb DNA ladder 250 to 10,000 bp.

RESULTS AND DISCUSSION

DNA extraction from dry seeds of *Euphorbia* species

The extraction of genomic DNA from dry seeds of *Euphorbia* spp. using commercial kit produced good quality and high purity of intact DNA to use in the RAPD-PCR analysis. The DNA yield of *E. peplus*, *E. helioscopia*, *E. granulata* and *E. hirta* were 11.5, 18.5, 9.5 and 14.0 µg per mg, respectively, of dry seeds powder, while the purity of the extracted DNA were 1.3, 1.6, 1.3 and 1.4, respectively. The integrity of the extracted DNA was checked by agarose gel 0.8%.

Molecular biological studies of plants, such as the PCR techniques, require pure DNA (Kang and Yang, 2004; Ahmadikhah and Alvi, 2009). One of the advantages of the PCR techniques is the rapid DNA analysis of many plant samples using small quantities of DNA. The DNA samples extracted from seeds were very stable and could be stored at 4 to -80°C for a long time without degradation; therefore, it could be used in further studies (Ahmadikhah and Alvi, 2009).

RAPD-PCR analysis

RAPD-PCR technique was used to reveal DNA polymorphism in DNA of the studied *Euphorbia* spp. in order to search for the sources of differences that could be used as a DNA marker represent the *Euphorbia* spp. The primers used in this study were selected randomly. Nine primers had been tested with same DNA samples under optimum conditions. The primers were classified into three groups according to results obtained. The first group gave no amplified products and this group includes (P07). Similar results were reported in different studies and a number of random primers were scored as non amplification producing primers (Al-Judy, 2004; Sujatha et al., 2005; Younan, 2010; Sesli and Yegenoglu, 2010). The second group that gave results in terms of amplification and polymorphism include A13, C05 and D20. The third group which includes A08, A07, R02, R03 and P06 primers gave amplification and polymorphism of the genomic DNA for some species, while no ampli-fication was detected with other species. The reasons of failure of these primers to amplify genomic DNA may be absence of suitable priming site for these primers on template DNA (Devos and Gale, 1992). The analysis of PCR amplified DNA fragments rely on several bases including the absence or presence of bands, and diffe-rences in molecular weight.

Levels of polymorphisms were gene-rated in this study among the four species of *Euphorbia* and also some primers generate unique bands that could be used as a DNA marker to distinguish between the local species of *Euphorbia*. In some instances, the reasons behind DNA polymorphism among samples may be due to a single base changes in genomic DNA.

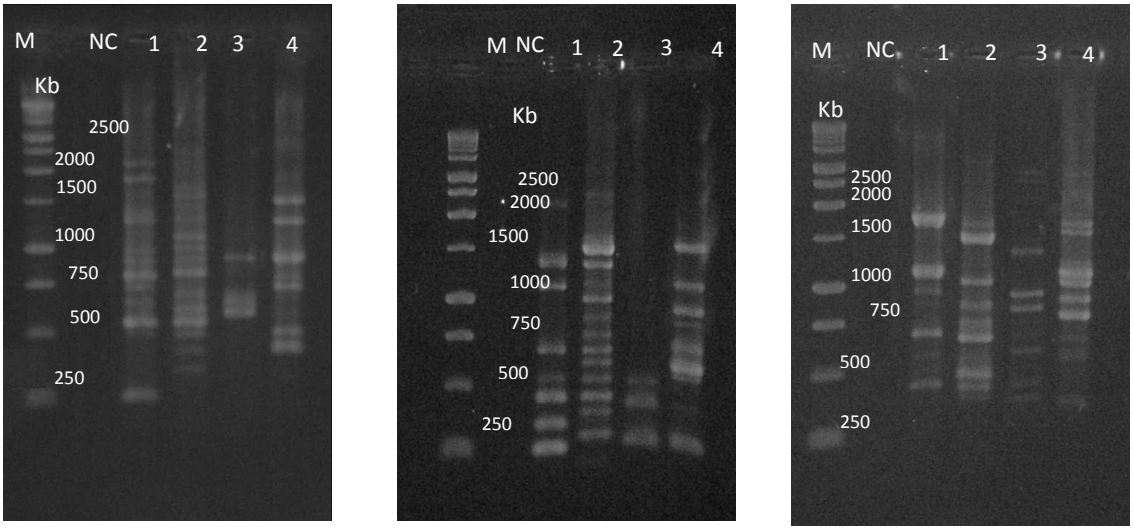
Other sources of polymorphisms may include deletions of a priming site, insertions that render priming sites too distant to support amplification, or insertions that change the size of a DNA segment without preventing its ampli-fication (Williams et al., 1990). Furthermore, it had been reported that single nucleotide changes in a primer sequence caused a complete change in the pattern of amplified DNA segments.

Primer A13

PCR results of primer A13 amplified genomic DNA of species studied of *Euphorbia* showed 33 bands as a total number of bands, distributed into 19 main bands that were polymorphic bands. The range of bands between 3 to 12 bands, *E. granulata* produced the lower number of bands only 3 bands, while *E. helioscopia* produced the highest number of bands (12 bands) as shown in Figure 1. Primer A13 generated eight unique bands, the second, third, tenth and nineteenth bands with molecular weight of about 2712, 2402, 1283 and 527 bp respectively, which distinguished *E. peplus*. The other unique bands were the first, fourth, eighth and eighteenth bands with molecular weight of about 2955, 2213, 1500 and 618 bp, respectively, which differentiated *E. helioscopia* from other species of *Euphorbia* as shown in Table 2.

Primer C05

Genomic DNA of *Euphorbia* spp. was amplified by using primer C05 and the results included a total number of 30 bands distributed into 17 main bands. Out of the 30 bands, 8 were polymorphic, ranging in molecular weight of 439 to 1569 bp, and one band were monomorphic with molecular weight of about of 690 bp (Table 3). The present monomorphic band in result of PCR reaction



Primer A13 **Primer C05** **Primer D20**

Figure 1. Agarose gel electrophoresis of RAPD-PCR reaction for random primers A13, C05 and D20 for DNA samples of Euphorbia species. Bands were fractionated by electrophoresis on a 1.2% agarose gel (2 h, 5 V/cm, 0.5X tris-borate buffer) and visualized under UV light by ethidium bromide staining. M, 1 Kb ladder; NC, negative control. Euphorbia spp.: 1, E. peplus; 2, E. helioscopia; 3, E. granulate; 4, *E. hirta*.

Table 2. The polymorphic, monomorphic and unique bands with their molecular weight for primer A13 to different *Euphorbia* species.

Number	Band molecular weight in bp	Euphorbia four spp			
		1	2	3	4
1	2955	0	1	0	0
2	2712	1	0	0	0
3	2402	1	0	0	0
4	2213	0	1	0	0
5	2630	0	1	0	1
6	1810	1	0	0	1
7	16450	1	1	0	0
8	1500	0	1	0	0
9	1400	1	0	1	1
10	1283	1	0	0	0
11	1090	1	1	0	0
12	970	1	0	0	1
13	865	1	1	1	0
14	814	0	1	1	0
15	775	1	1	0	0
16	750	0	1	0	1
17	690	0	1	0	1
18	618	0	1	0	0
19	527	1	0	0	0
Total number of band		11	12	3	6

1, Presence of bands; 0, absence of bands; , unique bands; , polymorphic bands. *Euphorbia* spp: 1, *E. peplus*; 2, *E. helioscopia;* 3, *E. granulate*; 4. *E. hirt.*

Table 3. The polymorphic, monomorphic and unique bands with their molecular weight for primer C05 to different *Euphorbia* species.

Number	Band molecular weight in bp	Euphorbia four spp			
		1	2	3	4
1	1569	1	1	0	1
2	1321	0	0	0	1
3	1225	1	0	0	0
4	1169	0	0	1	0
5	1090	1	0	0	1
6	1031	0	1	0	1
7	960	1	0	1	0
8	910	0	1	0	1
9	871	0	0	1	0
10	825	0	0	0	1
11	560	0	1	1	0
12	690	1	1	1	1
13	605	0	0	1	1
14	492	0	1	0	0
15	439	1	1	1	0
16	401	0	0	1	0
17	376	0	0	0	1
Total number of band		9	8	7	6

1, Presence of band; 0, absence of band; , unique bands; , monomorphic bands; , polymorphic bands. 1, *E. peplus*; 2, *E. helioscopia;* 3, *E. granulate*; 4, *E. hirt.*

means there was share DNA fragment in genomic of all four species of *Euphorbia.* Primer C05 produced bands in range 6 to 9 bands. *E. peplus* produced the lowest number of bands (6 bands), while *E. hirta* had the highest number of bands that produced 9 bands as shown in Figure 1. Primer C05 generated eight unique bands as shown in Table 3. *E. peplus* had one unique band which was the third band with molecular weight of about 1225 bp, as well as *E. helioscopia* distinguished by one unique band, the fourteenth band with molecular weight 492 bp, while *E. granulata* was differentiated by three unique bands, the fourth, ninth and sixteenth bands, a molecular weight of about 1169, 871 and 401 bp, respectively. Also *E. hirta* distinguished by three unique bands, the second, tenth and seventeenth bands, a molecular weight of approximately 1321, 825 and 376 bp, respectively.

Primer D20

The results of PCR reaction of primer D20 that reacted with genomic DNA of the species of *Euphorbia* showed 31 bands as a total number of bands, distributed into 16 main bands, of which 21 bands were polymorphic, the range of their molecular weight were between 241 to 2476 bp (Table 4), and there were one monomorphic band with molecular weight of about 431 bp. *E. helioscopia* produced the highest number of bands (13 bands) compared with *E. granulata* that produced three bands. This primer generated six unique bands (Table 4). The first, sixth, thirteenth bands with molecular weight of approximately 2476, 980 and 391 bp, respectively, distinguished *E. helioscopia*. The other unique bands were the second, fourteenth and sixteenth bands with molecular weight of about 2240, 339 and 250 bp, respectively, that distinguished *E. peplus* as in Figure 1.

Primers A08, A07, R02, R03 and P06

PCR results of primer A08 showed nine bands as a total number of bands for the two species *E. helioscopia* and *E. peplus*. All bands were unique, the molecular weight of these bands ranged from (500 to 2000) bp. This primer produced amplification products with range of five bands of the first species and 4 bands of the second. The genomic DNA of the two species of *E. granulata* and *E. helioscopia* were amplified by using the primers A07, R02, R03 and P06, while the genomic DNA of *E. peplus* and *E. hirta* had no amplification. The results of primer

Table 4. The polymorphic, monomorphic and unique bands with their molecular weight for primer D20 to different *Euphorbia* species.

Number	Band molecular weight in bp	Euphorbia four spp			
		1	2	3	4
1	2476	0	1	0	0
2	2240	1	0	0	0
3	1500	0	1	0	1
4	1300	1	1	0	0
5	1100	1	1	0	0
6	980	0	1	0	0
7	880	0	1	0	1
8	790	0	1	0	1
9	678	1	1	0	1
10	590	0	1	0	1
11	520	1	1	1	0
12	431	1	1	1	1
13	391	0	1	0	0
14	339	1	0	0	0
15	275	0	1	1	1
16	250	1	0	0	0
Total number of bands		7	3	13	8

1, Presence of band; 0, absence of band; , unique bands; , monomorphic bands; , polymorphic bands. *Euphorbia* spp: 1, *E. peplus*, 2. *E. helioscopia;* 3, *E. granulate*; 4. *E. hirt.*

A07 were eight bands as a total number of bands, the molecular weight of these bands ranged between 500 to 2100 bp. This primer produced amplification products with range of three bands for *E. granulata* and five bands for *E. helioscopia*. The genomic DNA of these two species was amplified by using primer R02; the results that appeared were seven bands as a total number of bands, molecular weight of bands were ranged between 545 to 1400 bp. This primer gave amplification products with range of four bands for *E. granulata* and three bands for *E. helioscopia*. While the results of primer R03 included total number of bands for the two spp., which were 12 bands, molecular weight of bands ranged between 350 to 2200 bp. This primer gave amplification products with range of nine bands for *E. granulata* and three bands for *E. helioscopia.* The primer P06 amplified genomic DNA of *E. granulata* and *E. helioscopia*. The total number were two bands, the molecular weight of the first band was about 2400 bp for *E. granulata* and the second band was about 1000 bp for *E. helioscopia*.

RAPD-PCR analyses were based on the second group of primers (A13, C05 and D20) because it gave results in terms of amplification and polymorphism for the species of *Euphorbia* as shown in Table 5. These primers produced a total of 52 main bands across the four species (Table 6). Of these 52 PCR products generated 3.85% (two bands) were monomorphic across the studied species. The remaining 50 bands (96.2% of the total products scored) were polymorphic among the species studied. This means that there is high difference among the genotypes of the species of *Euphorbia*. A total of 50 (96.2%) polymorphic bands were observed ranging from eight to 11 bands. The primer A13 gave the highest number of polymorphic bands (11), while the minimum number of polymorphic bands (8) by using C05 primer. The average number of polymorphic bands per primer among the species was 9.3. Polymorphism of each primer was calculated as the percentage of polymorphic bands to the number of total main bands produced by the designated primer. The obtained high polymorphism rate indicates a high genetic diversity. The number of bands generated by each primer varied, A13 generated maximum number of bands (33) while R02 amplified minimum number of bands (2). The variation in the number of bands amplified by different primers influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome (Kernodle et al., 1993). Visual examination of electrophoresis gels and analysis of banding patterns confirmed that *E. peplus* and *E. helioscopia* had high degree of similarity in the pattern of DNA with most of primers compared with other species, but there were

Table 5. The species of *Euphorbia* and the primers that appeared the unique bands, the number and molecular weight of these bands.

Species name	Primer	Molecular weight of unique bands (bp)	Unique bands number
E. peplus	A13	2712	2nd
		2402	3rd
		1283	10th
	C05	527	19th
		1225	3rd
	D20	2240	2nd
		339	14th
		241	16th
E. helioscopia	A13	2955	1st
		2213	4th
		1500	8th
	C05	618	18th
		492	14th
	D20	2476	1st
		980	6th
		391	13th
E. granulata	C05	1169	4th
		871	9th
		401	16th
E.hirta	C05	1321	2nd
		825	10th
		376	17th

Table 6. Distinct characteristic of random primers included in the study: Primer's name, total number of bands, number of polymorphic bands and percentage of polymorphism in species of *Euphorbia.*

Number	Primer	Total number of main bands	Number of polymorphic bands	Polymorphism %
1	A13	19	11	84
2	C05	17	8	47
3	D20	16	9	56
Total		52	28	-

clear differences among them especially in terms of unique bands. While *E. hirta* and *E. granulata* had less similarity pattern of DNA. The RAPD assay generated specific products in all of the species studied. These may be used as DNA fingerprints for species identification. It would be of immense use for the establishment of proprietary rights and the determination of species purity.

On the other hand, RAPD markers had been useful as the first step to produce a genetic map in plants with unknown, much or less known genetic series (Sesli and Yegenoglu, 2010). On the other hand, these results confirm the isolation of the four species of *Euphorbia* from each other obviously; as well as distinction of *E. peplus* and *E. helioscopia* from the other two species *E. hirta* and *E. granulata*, and this corresponds to the morphological features of these species. These results may be applied in isolation of similar species that could not be isolated by using other qualities and characteristic features. Tian et al. (1997) showed RAPD PCR were efficient for identification of poinsettia cultivars and for determination of the genetic relationships among cultivars. Chowda-Reddy et al. (2012) showed RAPD-PCR to examine molecular variability and to select individuals with different fingerprints, and relation to recent

changes in the epidemiology of tomato leaf curl disease in South India of *Bemisia tabaci* adults from various host-plant species. Genetic variations were tested in normal and fasciated stems of *Euphorbia lactea* using RAPD-PCR fingerprints. PCR yielded different polymorphic banding patterns that were unique to each primer and distinguishable over all samples and indicated that occurrence of fasciation in *E. lactea* is an epigenetic mutation of tissues (El-Banna et al., 2013). This is one of the goals of this molecularystematic study which can be applied in such methods in the diagnosis of the rest of *Euphorbia* species existing in Iraq.

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