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Research article

Analysis of genetic diversity among pomegranates cultivated in Diyala, Iraq using RAPD-PCR method

Zainab M. Hamid[1]*, Silva A. Yakoub Zokian[1]

ABSTRACT

This study attempts to identify genetic diversity among four genotypes of pomegranates cultivated in Diyala city, Iraq and find the genetic polymorphism among them by using DNA markers generated by polymerase chain reaction (PCR). Total genomic DNA of genotypes studied was extracted from dry young leaves by using cetyltrimethyl ammonium bromide (CTAB) procedure. Molecular analysis was performed by using twelve random markers in random amplified polymorphic DNA (RAPD-PCR) technique. The analyses based on five primers OPH – 08, OPH – 13, OPH – 18, RI – 3 and RI – 16, which used in amplification and polymorphisim for the four cultivated genotypes studied. The genetic polymorphisms value of each primer was determined and ranged between 31 to 100%; primers OPBA–3 and OPBB–4 produced the highest percent of genetic polymorphism compared with primer OPH–13. The genetic diversity and the isolation of the four cultivated genotypes of pomegranates confirmed obviously by RAPD-PCR technique.

Keywords: Genotypes, Pomegranates, RAPD-PCR technique.

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INTRODUCTION

Pomegranate belongs to the order Myrtales and most likely originated from Saxifragales [1]. The Lythraceae family is likely to be an initial form, which initiated the Sonneratiaceae and Punicaceae families [2]. However, the genus Punica, described for the first time in 1753 by Linnaeus, had tropical ancestors close to Lythraceae and Sonneratiaceae [3]. The genus Punica belongs to the family Punicaceae and consists of two species: *Punica granatum* L. and P. protopunica Balf. However, some authors classified the ornamental dwarf pomegranate (P. nana L.) as a distinct species [4]. P. protopunica is endemic to the Socotra Island, Yemen [5].

The pomegranate probably originated in Iran [6,7] and from there it divided according to other regions like Mediterranean countries, India, China, Pakistan and Afg-

*Correspondence: zainabmahmoodbotanymaster91@yahoo.com
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq
Full list of author information is available at the end of the article

hanistan, possibly through ancient trade routes. It is one of the oldest known edible fruits [8]. The wild types of central Asia vary widely in fruit size, sweetness, ripening time, juiciness and the proportions of seeds [9]. Molecular marker techniques based on PCR, such as random amplified polymorphic DNA (RAPD) is powerful tools for plant breeding and genetic analysis, so the RAPD technique provides a convenient and rapid assessment of diversity in the genetic composition of the related individuals and has been employed in a large number of plants for the determination and assessment of genetic diversity [10,11]. Pomegranate contains exceptionally high amounts of polysaccharides, secondary metabolites and polyphenols including mainly ellagitannin, gallotannin, and anthocyanin which interfere with DNA isolation procedure. In pomegranate, several DNA extraction methods have been successfully used for plant [12,13]. However, these methods are not useful for RAPD analysis. Dyiala city is quite rich in cultivating pomegranate and genetic study is very little in this area. Therefore, in this study, we try to find a rapid, simple and inexpensive protocol for isolation of high quality DNA from pomegranate of Dyiala city and can be used for RAPD analysis to identify cultivated genotypes.

MATERIALS AND METHODS

Plant material

Four samples of young leaves of adult trees collected from different geographic populations of Dyiala city (**Table 1**). The leaf samples were washed three times in sterile distilled water, frozen in liquid nitrogen and stored at −80°C for further study.

Table 1. List of pomegranate genotypes used in the study.

Sample name	Location
Sweet Iraqi pomegranate	Diyala, Iraq
Blue sour Iraqi pomegranate.	Diyala, Iraq
Blue curvature Iraqi pomegranate.	Diyala, Iraq
Shahrabani Iraqi pomegranate.	Diyala, Iraq

DNA extraction

DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) procedure described by Doyle and Doyle [14] with some modifications. Briefly, Young leaves (25 mg) were ground to a fine powder in liquid nitrogen and mixed with 500 µl of extraction buffer (3% CTAB; 100 mMTris–HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.3 M NaCl; 4% PVP (poly vinyl pyrrolidone) [15]; 2% β-mercaptoethanol). Samples were incubated at 65°C for 20 min, subsequently, washed with an equal volume of chloroform/isoamylalcohol (24:1), and centrifuged at 12000 rpm for 10 min. The supernatant was mixed with 200 µl potassium acetate (koAc) and 500 µl of cold isopropanol carefully and cooled for 15 min and the DNA was pelleted by centrifugation at 12000 rpm for 15 min. Pellets of DNA were then washed with 70% ethanol twice, dried and dissolved in 200 µl of TE buffer (10 m MTris–HCl, pH 8.0, 1 mM EDTA, pH 8.0) and extracted DNA was stored at −20°C for later use. Purity and concentration measured using the standard method [16]. The yield of the DNA extracted from the pomegranate leaf tissue was in range of (44.5 - 1247.7) µg per gram of pomegranate leaf tissue with purity of (1.6 - 1.97).

PCR amplification with RAPD primers

The RAPD primers were procured from Alpha-DNA (Canada) (**Table 2**). On the basis of the screening, primers resulting in discrete well-separated bands on agarose gels were selected for amplification. All RAPD reactions were carried out in 25 ml volumes and contained 4 µl of template DNA, 1 µl of RAPD primer, 12.5µl Master Mix, and 6.5 Nuclease-free water (Promega, USA). The amplification reactions were carried out using MyCycler (BioRad, USA), which was programmed to include pre-denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 44°C for 1:30 min, and extension at 72 °C for 2 min. The final cycle allowed an additional 10 min of extension at 72 °C and hold on 4 °C.

Agarose gel electrophoresis

The PCR products were electrophoresed on 1.5% agarose gel using 1×TBE buffer and 1 ml of 10 mg/ml ethidium bromide at constant voltage of 100 V/cm for 90 min. After electrophoresis the gel was visualized and archived using UV transilluminator major science (USA). The patterns were photographed and stored as digital pictures in gel documentation system.

RESULTS and DISCUSSION

RAPD-PCR technique used to reveal DNA polymorphism in DNA of the studied genotypes of pomegranate in order to classify types cultivated in Dyiala- Iraq by using DNA markers. The primers were classified into two groups according to results obtained. The first group, that gave results in term of amplification and polymorphism, included OPH – 08, OPH – 13, OPH – 18, RI – 3, RI – 16. The second group included OP – 04, OPA– 9, OPA – 19, OPAH– 17, OPB – 11, OPBA– 3, OPBB – 4 primers gave amplification and polymorphism of the genomic DNA for some species, while no amplification was detected with other species. A total of 121 useful bands were scored from the amplification products with the twelve primers of DNA from five pomegranate genotypes (**Table 2**).The number of amplification generated by each primer varied from 2 (OPB–11) to 15 (RI–16) and ranged in size from 115bp (OPH–18) to 2079 bp (OPAH–17) (**Fig. 1**).

In general, sufficient polymorphism existed to allow distinction between the genotypes tested with, polymorphism ranged from 31 to 100 %, primers OPBA–3 and

Table 2. Distinct characteristic of random primers included in the study: Primer's name, Sequence, total number of bands, number of polymorphic bands and percentage of polymorphism in pomegranate genotypes studied.

NO.	Primer name	Sequence	Total No. of bands	Total No. of main bands	Number of unique bands	Number of polymorphic bands	Polymorphism %
1	OPA – 04	AATCGGGCTG	**33**	**14**	**2**	**12**	**86**
2	OPA – 9	GGGTAACGCC	**21**	**9**	**1**	**8**	**89**
3	OPA – 19	CAAACGTCGG	**21**	**8**	**1**	**7**	**88**
4	OPAH – 17	CAGTGGGGAG	**27**	**11**	**2**	**9**	**82**
5	OPB – 11	GTAGACCCGT	**2**	**2**	**2**	------	------
6	OPBA – 3	GTGCGAGAAC	**26**	**9**	------	**9**	**100**
7	OPBB – 4	ACCAGGTCAC	**29**	**10**	------	**10**	**100**
8	OPH – 08	GAAACACCCC	**20**	**9**	**2**	**7**	**78**
9	OPH – 13	GACGCCACAC	**37**	**13**	**3**	**4**	**31**
10	OPH – 18	GAATCGGCCA	**36**	**12**	**2**	**6**	**50**
11	RI – 3	GTCCGTGAAC	**21**	**9**	**2**	**6**	**67**
12	RI – 16	GTCGCCGTCA	**34**	**15**	**5**	**8**	**53**
Total			**307**	**121**	**22**	**86**	

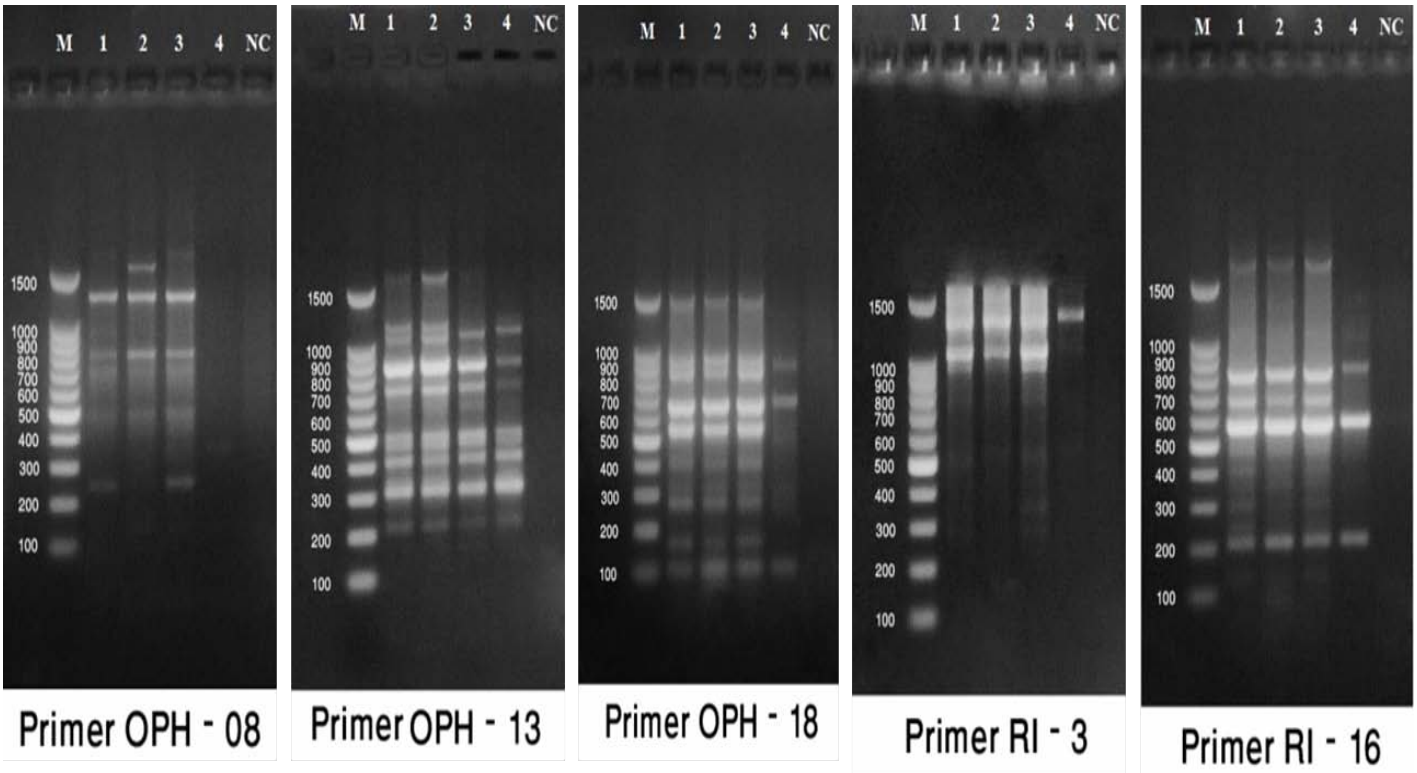

Fig 1. Agarose gel electrophoresis of RAPD-PCR reaction for random primers OPH – 08, OPH – 13, OPH – 18, RI – 3 and RI – 16 for DNA samples of Pomegranate genotypes. Bands were fractionated by electrophoresis on a 1.5% agarose gel (90 min, 100 V/cm, 1X tris-borate buffer) and visualized under UV light by ethidium bromide staining. M, 100 Kb ladder; NC, negative control. Pomegranate genotypes.: 1.Sweet Iraqi p., 2. Blue sour Iraqi p., 3. Blue curvature Iraqi p., 4.Shahrabani Iraqi.

OPBB–4 produced the highest percent of polymorphism compared with primer OPH – 13. Of these 121 PCR products generated, 10.74 % (13 bands) were monomorphic across all genotypes and there are 22 unique bands (**Table 3,4,5,6,7**).

Table 3. The polymorphic, monomorphic and unique bands with their molecular weight for primer OPH–08 to different pomegranate genotypes. 1, Presence of band; 0, absence of band (red); unique bands (green); 1. Sweet Iraqi p., 2. Blue sour Iraqi p., 3. Blue curvature Iraqi p., 4.Shahrabani Iraqi pomegranate.

Number	Band molecular weight in bp	Pomegranate four genotypes			
		1	2	3	4
1	1819	1	0	1	0
2	1675	0	1	0	0
3	1387	1	1	1	0
4	881	1	1	1	0
5	747	1	1	1	0
6	521	1	1	1	0
7	461	0	1	1	0
8	365	0	0	0	1
9	258	1	0	1	0
Total number of band		6	6	7	1

Table 4. The polymorphic, monomorphic and unique bands with their molecular weight for primer OPH–13 to different pomegranate genotypes. 1, Presence of band; 0, absence of band (red); unique bands (green); polymorphic bands (blue), manomorphic band. 1. Sweet Iraqi p., 2. Blue sour Iraqi p., 3. Blue curvature Iraqi p., 4.Shahrabani Iraqi pomegranate.

Number	Band molecular weight in bp	Pomegranate four genotypes			
		1	2	3	4
1	1692	1	1	0	0
2	1337	0	0	0	1
3	1237	1	1	0	1
4	1182	0	0	1	0
5	1142	1	1	0	0
6	982	0	0	0	1
7	915	1	1	1	0
8	798	1	1	1	1
9	685	1	1	1	1
10	537	1	1	1	1
11	440	1	1	1	1
12	336	1	1	1	1
13	236	1	1	1	1
Total number of band		10	10	8	9

Many bands appeared in most of the genotypes and were absent in only a few genotypes. The remaining 86 bands (71.07% of the total products scored) were polymorphic among the studied genotypes; the method of calculation percentage of polymorphism was done by dividing the number of polymorphic bands upon total number of bands then multiplying by 100, this was a relatively high level of the percentage of polymorphic bands obtained by random primers compared to reports of other RAPD studies in pomegranate which were 47% [17] and 53% [18], while this percentage was less comparatively to other pomegranate studies at 93.72% [19]. Other studies like in Euphorbia or in Rice the percentage of polymorphism were (54%) [20] and (87%) [21], respectively. A total of 86 (71.07%) polymorphic bands were observed, ranging from 4 (OPH – 13) to 12 (OPA–04) bands with an average of (7.8) polymorphic bands per primer across all the four pomegranate genotypes. This average was less to that observed in other pomegranate study using RAPD markers with south Tunisian pomegranate genotypes, This report observed that the average number of polymorphic bands per primer were 9.1 [22]. The average number of polymorphic bands was relatively higher than earlier reports, with an average of 6 and 3.8 polymorphic bands per primer [13,16].

Table 5. The polymorphic, monomorphic and unique bands with their molecular weight for primer OPH–18 to different pomegranate genotypes. 1, Presence of band; 0, absence of band (red); unique bands (green); polymorphic bands (blue), manomorphic band. 1. Sweet Iraqi p., 2. Blue sour Iraqi p., 3. Blue curvature Iraqi p., 4.Shahrabani Iraqi pomegranate.

Number	Band molecular weight in bp	Pomegranate four genotypes			
		1	2	3	4
1	1518	1	1	1	0
2	963	1	1	1	1
3	2075	1	1	1	0
4	723	0	0	0	1
5	673	1	1	1	0
6	565	1	1	1	0
7	518	0	0	0	1
8	420	1	1	1	0
9	336	1	1	1	1
10	285	1	1	1	1
11	175	1	1	1	0
12	115	1	1	1	1
Total number of band		10	10	10	6

Table 6. The polymorphic, monomorphic and unique bands with their molecular weight for primer RI – 3 to different pomegranate genotypes. 1, Presence of band; 0, absence of band (red); unique bands (green); polymorphic bands (blue), manomorphic band. 1. Sweet Iraqi p., 2. Blue sour Iraqi p., 3. Blue curvature Iraqi p., 4.Shahrabani Iraqi pomegranate.

Number	Band molecular weight in bp	Pomegranate four genotypes			
		1	2	3	4
1	1565	1	1	1	0
2	1438	0	0	0	1
3	1158	0	0	1	1
4	1352	1	1	1	0
5	993	1	0	1	0
6	810	1	1	1	0
7	553	1	1	1	1
8	362	0	0	1	0
9	289	1	0	1	0
Total number of band		6	4	8	3

The examination of electrophoresis gels and analysis of DNA banding patterns confirmed that the Sweet Iraqi pomegranate, Blue sour Iraqi pomegranate and Blue curvature Iraqi pomegranate genotypes had high

degree of similarity in the DNA pattern with most of primers compared with other genotypes, but there were clear differences among them especially in unique bands. The RAPD assay generated specific products in all of the genotypes studied. These may be used as DNA fingerprints for identification. 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 [23].

Table 7. The polymorphic, monomorphic and unique bands with their molecular weight for primer RI–16 to different pomegranate genotypes. 1, Presence of band; 0, absence of band (red); unique bands (green); polymorphic bands (blue). 1. Sweet Iraqi p., 2. Blue sour Iraqi p., 3. Blue curvature Iraqi p., 4.Shahrabani Iraqi pomegranate.

Number	Band molecular weight in bp	Pomegranate four genotypes			
		1	2	3	4
1	1716	1	1	1	0
2	1465	1	0	0	0
3	1360	0	1	0	0
4	1165	0	0	0	1
5	918	0	0	0	1
6	847	1	1	1	0
7	712	1	1	1	0
8	599	1	1	1	1
9	487	1	1	1	0
10	428	1	1	1	0
11	392	0	1	1	0
12	318	1	0	1	0
13	251	1	0	0	0
14	222	1	1	1	1
15	140	1	0	1	0
Total number of band		11	9	10	4

Also, these results confirm the isolation of the fife cultivated genotypes of pomegranate from each other clearly; and this corresponds to their morphological features. This method may be applied in isolation of similar genotypes that could not be isolated by using other morphological characteristics.

Many studies showed RAPD PCR efficiency for identification of cultivars and for determination of the genetic relationships among varieties like Zokian and El-Banna [20,24]. The present molecular-systematic study can be applied to diagnosis the rest of genotypes of pomegranate cultivated in Iraq.

Conflict of interest

The authors declare that they have no conflict of interests.

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Author affiliation:
1. *Department of Biology, Collage of Science, University of Baghdad, Baghdad, Iraq.*