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REGULAR ARTICLE

Genotyping and identification of six date palm (*Phoenix dactylifera* L.) cultivars of the Gaza Strip by random amplification of polymorphic DNA

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Abstract

Date palm (*Phoenix dactylifera* L.) is one of the major fruit crops in the Gaza Strip. The main cultivars are Hayani, Bentaisha, Barhi, Zahedi, Ameri and Halawy. Because of the difficulty associated with morphological identification of date palm cultivars, development of cultivar specific genetic markers have been extensively studied. The purpose of this study was to optimize and apply a reliable molecular marker protocol for genotyping and identification of these cultivars. Random amplification of polymorphic DNA (RAPD) was used to study the genetic diversity among the six cultivars using 42 primers. Genetic similarity matrices were constructed for the six cultivars using the Nei and Li formula and clustered with the UPGMA to determine the relationships between the six cultivars. Nine primers were informative and reproducible, and gave a number of polymorphic bands (on average, 7.2 bands per primer). The overall level of polymorphism was 49%. Genetic similarity among the six cultivars ranged from 76.3 to 93.5%. Some primers gave reproducible cultivar-specific bands that may be reliable to identify that cultivar. Our study introduces for the first time a molecular marker approach, capable of distinguishing and studying the genetic diversity among the six date palm cultivars in the Gaza Strip.

Key words: Date palm, Cultivars, Gaza Strip, Molecular identification, PCR, *Phoenix dactylifera*, RAPD

Introduction

Date palm (*Phoenix dactylifera* L.), has been economically, spiritually and ornamentally associated with eastern Mediterranean life for at least the last 6,000 years (Popenoe, 1913; Barreveld, 1993; El Hadrami and Al-Khayri, 2012). The date palm can tolerate long periods of drought, and thus is widely grown and its fruits consumed in the dry regions of the Middle East, North Africa and the Indian subcontinent. The most important date-producing countries are Egypt, Saudi Arabia, Iran, United Arab Emirates, Algeria, Iraq, Pakistan, Oman, Tunisia and Libya (FAO, 2013). Date palm reportedly has more than 3,000 varieties around the world (Zaid, 2002). The overall production of dates has increased in the last 10 years worldwide to reach 7.5 million metric tons in 2011 (FAO, 2013). In the Palestinian territories, there has been an increase in date production to 4,688 metric tons in 2011 (FAO, 2013).

In the Gaza Strip, most of the date palms are planted in the coastal area and in many cases the plantations utilize mixed farming systems. The main date palm cultivars in the Gaza Strip are Hayani and to a lesser extent Bentaisha. In 1999 other cvs. such as Barhi, Zahedi, Ameri and Halawy were introduced (Banna, 2007).

Date palm cultivars are very difficult to distinguish by morphology. They are distinguished by the characters of the fruits, which are produced only after 4-5 years (Sedra et al., 1998). Therefore, molecular techniques may be a more reliable approach for cultivar discrimination. Genotyping using DNA-based markers has been used in the assessment of genetic diversity in plant species for different purposes (Agarwal et al., 2008; Appleby et al., 2009). It provides useful information regarding genetic diversity and the relationship between cultivars, while unaffected by environmental factors and the developmental stage of the plant. Among the various kinds of genotyping techniques characterized so far, restriction fragment length polymorphism (RFLP) was the first and until recently the most commonly used for estimation of the genetic

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diversity in plant species (Barnes, 1991). However, it is a time-consuming and labor-intensive assay**.** Polymerase chain reaction (PCR)-based techniques, which include random amplification of polymorphic DNA (RAPD), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) are playing an increasingly important role in DNA fingerprinting and pedigree analysis (Al-Khalifah and Askari, 2003; Agarwal et al., 2008; Al-Khalifah et al., 2012; Khanam et al., 2012; Zhao et al., 2013).

The arbitrarily designed RAPD short primers (usually 10-mer) may be used to reproducibly amplify segments of genomic DNA from a wide variety of species (Powell et al., 1995). Polymorphism among the amplification products is frequently detected and is useful as a genetic marker. RAPD assay may in some instances detect single base changes in genomic DNA (Atienzar et al., 2002)**.** However**,** distinguishable RAPD fingerprints among different varieties are obtainable only if suitable primers are used and PCR conditions are optimized (Ramos et al., 2008). On the other hand, the RAPD technique is relatively cheap, easily performed and interpreted, and possesses high discriminatory power and typing capacity (Buckingham, 2012).

So far, the discrimination between date palm cultivars, based on morphology, has created obstacles in proper date palm agriculture, and that may lead to serious problems in terms of time and cost. Therefore, in this study, we have optimized a molecular method capable of distinguishing among the six different Gaza Strip date palm cultivars using RAPD technique. The genetic diversity between these cultivars was also studied.

Table 1. A summary of the morphological characteristics of six date palm cultivars present in the Gaza Strip.

Fruit Charact-eristics	Date palm cultivar name					
	Hayani	Bentaisha	Halawy	Zahedi	Barhi	Ameri
Morphology						
Color	Dark red	Dark red	Yellow	Yellow	Yellow	Reddish yellow
Shape	Oblong, 4-7cm	Globular, 2-3 cm	Oblong, small to medium size	Ovate, medium in size	Ovate, 3-4 cm	Oblong, 4-5 cm
Weight	12-20 g	15-20 g	7-10 g	7-10 g	15-20 g	15-23 g
Consistency	High moisture content, (about 72%)	A high moisture content (about 89%)	Low-moderate moisture content	Dry at consumption	High moisture (about 75%)	Moderate moisture (about 62%) and high fiber content
Other features	The main cultivar in Gaza strip. Collected either red or black (rutab).	Less common cultivar than Hayani. Collected either red or black (rutab). Sweeter taste than Hayani.	Distinctive rich flavor and extremely sweet taste. Consumed fresh fruit at the Khalal stage.	Palm is fast growing and heavy bearing. Sugary fruits. Consumed as dry dates (tamar).	High quality and heavy yield. Excellent flavor with little astringency at Khalal stage. Marketed and consumed only as fresh fruit on strands at the Khalal stage.	Eaten dried.

Materials and Methods

Plant material source and preparation

Young leaves were collected from 9-year-old well characterized female date palm trees. Four samples, each from a different tree, were collected from each cultivar (Ameri, Halawy, Zahedi, Barhi, Bentaisha and Hayani). The tested date palms were selected, following consultation with the Palestinian Agricultural relief Committee (PARC). The PARC imported the four yellow cvs. (Halawy, Zahedi, Barhi and Ameri) in 1999 and distributed them to local farmers. Therefore, they provided information about the location of cultivars, age of the palm and evidence of fruit production. Leaf samples were collected from three areas of the Gaza Strip (Rafah, Deir al-Balah and Az-Zawayda). Table 1 summarizes some of the fruit morphological characteristics of the six cultivars. washed with 500 µl 70% ethanol. The pellet was dissolved in 100µl TE buffer and kept on ice for one hour, then mixed gently.

DNA extraction

DNA was extracted from 100 mg new tender white to pale yellow leaves to increase the DNA yield, and to avoid high levels of endogenous phenolics, polysaccharides, or other substances present in green leaves that may interfere with DNA extraction. In order to assure the cultivar type, 9-year-old trees were selected provided that they had already borne fruit.

The leaves were frozen and ground under liquid nitrogen to a fine powder using a mortar and pestle. The liquid nitrogen was allowed to evaporate and the powder was either stored at -70 ºC or immediately subjected to DNA extraction procedures using two methods. The first method involved the commercially purchased DNeasy plant mini kit (Qiagen, Germany), according to the manufacturer recommendations. The second protocol followed a previously published manual protocol (Dellaporta et al., 1983) with reduced plant material weight (100 mg instead of 1 g) using 10 ml falcon tubes and Sorvall centrifuge (Thermo Scientific). Ground plant tissue was lysed for 10 min at 65ºC with 1.5 ml extraction buffer (100 mM Tris-HCl pH 8; 50 mM EDTA pH 8; 500 mM NaCl; 10 mM 2-mercaptoethanole) and 100 µl 20% SDS. The lysate was incubated with 500 µl of 5 M potassium acetate for 20 min on ice followed by centrifugation at 25000 xg, 4ºC for 20 min. The supernatant was poured through a miracloth filter to a new tube containing 1 ml isopropanol and 1 ml 5 M ammonium acetate and incubated at -20ºC for 20 min. The nucleic acid was pelleted by centrifuge for 15 min. at 20000 xg; the supernatant was gently discarded and the pellet was dried for 5 min. The pellet was resuspended in 700 µl TE buffer (50 mM Tris 10 mM EDTA, pH 8) and contaminating RNA was digested with 4µl of 100 mg/ml RNase A for 1 hour at 37ºC. Insoluble debris was pelleted with 75 µl of 3 M sodium acetate and centrifugation for 15 min 25000 xg. The supernatant was then transferred to a clean tube containing 500 µl isopropanol and incubated at room temperature for 5 min. The DNA was pelleted at 20000 xg for 15 min, and the pellet

After purification, the DNA concentration was spectrophotometrically determined at absorbance of 260 nm, and its integrity was determined with 1% agarose mini-gel electrophoresis at 7 V/cm.

RAPD-PCR

A list of primers analyzed in the study is provided in Table 2 (Operon Technologies Inc., USA). The primers included 42 sequences selected from previously published reports based on reproducibility (Corniquel and Mercier, 1994; Aitchitt et al., 1998; Mokhtar et al., 1998; Ouenzar et al., 1998; Sedra et al., 1998; Mokhtar et al., 2000; Soliman et al., 2003; Saker et al., 2006; Eissa et al., 2009). The PCR reactions were performed in a final volume of 25 µl containing 30 ng total genomic DNA, 200 µM of each dNTP, 50 pmole primer and 1.25 units of Taq DNA polymerase (Promega,USA). The PCR cycling parameters consisted of an initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 35ºC for 1 min and extension at 72ºC for 2 min and a final delay phase at 72°C for 5 min. The quality of PCR was assessed by running a water negative control in each experiment. The amplified PCR fragments were separated on 1.4% agarose gel in 1x TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3 at 25°C), stained with ethidium bromide (0.5 µg/ml) and visualized by UV trans-illumination.

Data analysis

The results of RAPD were scored based upon the absence (-) or presence (+) of bands and the number of bands per primer. The scored data was transformed into numerical values based on the similarity and genetic distance. For each primer the scoring system was followed on the basis of the number of bands in a single cultivar and absent in the others; as well as the number of common bands in all cultivars.

Table 2. Sequences of 42 10-mer random primers analyzed with RAPD technique.

Primer No.	*Sequence 5' to 3'	Primer No.	Sequence 5' to 3'
1	CAGGCCCTTC	22	CTGCTGGGAC
2	AGGGGTCTTG	23	GTAGACCCGT
3	GTGATCGCAG	24	CCTTGACGCA
4	CAATCGCCGT	25	TTCCCCCGCT
5	TCGGCGATAG	26	TCCGCTCTGG
6	CAGCACCCAC	27	GGAGGGTGTT
7	TCTGTGCTGG	28	TTTGCCCGGA
8	TTCCGAACCC	29	GTGTGCCCCA
9	AGCCAGCGAA	30	CACCGTATCC
10	AGGTGACCGT	31	GAGAGCCAAC
11	CAAACGTCGG	32	CCCAAGGTCC
12	GTTGCGATCC	33	AAGACCCCTC
13	GTTTCGCTCC	34	GTTGGTGGCT
14	TGATCCCTGG	35	GGGAACGTGT
15	CATCCCCCTG	36	CTCAGTCGCA
16	GGACTGGAGT	37	ACATGCCGTG
17	TGCGCCCTTC	38	AGGCCCCTGT
18	TGCTCTGCCC	39	ATGCCCCTGT
19	GGTGACGCAG	40	AAAGCTGCGG
20	GTCCACACGG	41	GGGTTGTTGG
21	TGGGGGACTC	42	GTGGGTGTTG

* The primers sequences were obtained from (Corniquel and Mercier, 1994; Aitchitt et al., 1998; Mokhtar et al., 1998; Ouenzar et al., 1998; Sedra et al., 1998; Mokhtar et al., 2000; Soliman et al., 2003; Saker et al., 2006; Eissa et al., 2009).

The NL coefficient calculations were made to determine the similarity value between two cultivars using a single primer (Nei and Li 1979) using the following formula: NL = 2a/(b + c)); where, (a) number of similar bands in both cultivars, (b) total number of bands in the first cultivar and (c) total number of bands in the second cultivar. The similarity values for each primer were used for construction of a binary matrix for the six cultivars. The average of similarity values between each two cultivars using all primers was calculated and included in a single binary matrix. This matrix was also used for construction of a dendrogram according to UPGMA method (Michener and Sokal, 1957) using the online dendrogram construction utility, DendroUPGMA (http:// genomes.urv.cat/UPGMA) (Garcia-Vallvé et al., 1999).

Results and Discussion

Validation of DNA extraction and RAPD protocols

The quality and quantities of DNA may be important for the success of highly sensitive RAPD application. Extraction of the plant DNA using commercially ready-made reagent recipes is usually more convenient than methods with manual preparations, because it saves time, requires minimal preparation of buffers and reagents, and is less prone to error. However, commercial kits are more expensive. In this work a manual protocol for DNA extraction was successfully adapted with few modifications (Dellaporta et al., 1983).

The scale and purity of DNA extracted by the "Dellaporta protocol" and the commercial kit was satisfactory (concentration: 35-125 ng/µl and 25-65 ng/µl respectively). No degradation of DNA was observed (Figure 1), although the purity of manually extracted DNA was slightly reduced (260/280 OD ratio = 1.5 compared to 1.6-2 in case of the commercial kit). This was not critical for the quality of PCR reactions and did not compromise the number and quality of bands obtained in this study. Therefore we used the manual protocol in the rest of the study.

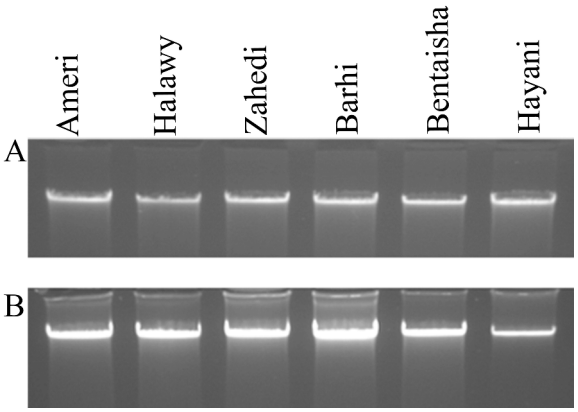

Figure 1. Agarose gel electrophoresis of total DNA. A representative 1% agarose gel showing DNA extracted from the six cultivars using the DNeasy plant mini kit (A) and the "Dellaporta protocol" (B).

For PCR reaction optimization, the GoTaq DNA polymerase kit (separate Taq DNA polymerase, buffer, MgCl2 and dNTPS) was used, rather than a ready-made master mix, which allowed adjustment of reactants amounts and concentration. The results of this work showed that the optimum PCR reaction composition for RAPD test are 1.25U Taq DNA polymerase, 200 µM each dNTPs, 1.5 mM $MgCl_2$, 50 pm of each primer and 30ng DNA.

The results showed that RAPD is sensitive to changes in the PCR kit components. This agrees with many previous works which discussed the RAPD reproducibility and sensitivity when changing the PCR reaction components (Sharma et al., 1995; Skorić et al., 2012). Therefore, the optimal RAPD conditions (concentration of the primer, MgCL2, Taq DNA polymerase and dNTPs;

the amount of DNA and amplification conditions) were maintained for fingerprinting and identification of date palm in the study.

RAPD fingerprinting of date palm cultivars

The RAPD principle is based on amplifying fragments of the target DNA with unknown sequences. Therefore, a large number of short primers with randomly selected sequences are used to find a few suitable ones. Forty-two random primers were examined in our study, 9 primers showed a number of polymorphic and reproducible bands in all six date palm cultivars (Table 3). Primers that generated either none or weak amplification patterns were discontinued. The reproducibility of the primers with identifiable polymorphic bands was confirmed with another three different samples of each date palm cultivar, collected from different regions of Gaza Strip. The 9 reproducible primers gave a total of 65 bands, including 32 polymorphic and 33 monomorphic bands (49% polymorphism). In comparison, the level of polymorphism obtained by similar studies was 58.5% (Haider et al., 2012) and 66% (Sedra et al., 1998). The number of bands ranged from 3 to 8 for each cultivar (on average 7.2 bands per primer), and the size of bands was 280-2000 bp.

The bands were recorded as present (+) or absent (-), and the genetic diversity of the six date palm cultivars was calculated using the similarity coefficient, and the UPGMA method was used to construct a dendrogram (Table 4 and Figure 2). The similarity values obtained from the nine primers ranged from 76.3 to 93.5% with a mean of 84.9% (Table 5). The highest similarity value (93.5%) was between Bentaisha and Hayani cvs.; both have red fruits while the other four cultivars have yellow fruits (Table 1), while the lowest similarity value (76.3%) was recorded between cvs. Hayani and Zahedi. The Ameri and Hayani cvs., which have similar fruit shape and size, although slightly different in color, also showed high similarity (91.7%). Barhi and Halawy cvs., which have similar sweet and least acidic fruits (Pundir and Porwal, 1998), have also demonstrated a high similarity value (91.8%).

The agarose gel of some primers gave reproducible cultivar-specific bands (Figure 3). Primers 6 and 33 repeatedly gave a band of about 1200 bp and 1000 bp, respectively, with Zahedi. Therefore, these primers might be reliable to identify the Zahedi cv. Primers 7 and 26 gave a band of at about 450 bp and 700 bp, respectively, with the four yellow fruit cultivars, which was absent in the two red fruit cvs. Hayani and Bentaisha. Thus they may be useful for distinguishing these two groups according to the fruit color. Similarly, primer 39 gave a specific band at about 630 bp with Ameri cv.

The RAPD primer No. 6 gave the highest polymorphism among the six date palm cultivars (9 polymorphic out of 11 bands). However, a single RAPD primer usually fails to distinguish among the six cultivars alone. Therefore, a combination of more than one primer must be used to identify the six cultivars.

Table 3. Polymorphism of nine RAPD primers applied on six date palm cultivars.

Primer ID	No. of polymorphic bands*	No. of monomorphic bands	Total No. of bands per primer	Polymorphism %
6	9	2	11	81.8
7	3	3	6	50
15	3	3	6	50
16	3	3	6	50
26	4	5	9	44.4
29	4	4	8	50
33	1	5	6	20
35	1	3	4	25
39	4	5	9	44.4
Total	32	33	65	49 %

* Polymorphic bands are those present only in a fraction of cultivars. Monomorphic bands are those present in all cultivars.

Table 4. RAPD profile for the six date palm cultivars obtained by nine primers.

Primer ID	Band size (bp)	Date palm cultivars					
		Ameri	Halawy	Zahedi	Barhi	Bentaisha	Hayani
6	2000	-	-	-	-	-	+
	1700	-	-	-	-	+	+
	1600	-	-	-	+	-	-
	1200	-	-	+	-	-	-
	830	+	-	+	+	+	+
	780	+	+	+	+	+	+
	720	+	-	-	-	+	+
	600	+	-	-	-	+	+
	570	-	+	+	-	-	-
	380	-	+	+	+	-	-
	350	+	+	+	+	+	+
7	1500	+	+	+	+	+	+
	820	+	+	+	+	+	+
	600	+	+	+	+	-	+
	490	+	+	+	+	-	-
	380	+	+	+	+	-	+
	320	+	+	+	+	+	+
15	2000	+	+	+	+	+	+
	1000	+	+	+	+	+	+
	750	-	+	-	+	+	+
	730	+	-	+	-	-	-
	600	-	+	-	+	-	-
	520	+	+	+	+	+	+
16	1400	-	+	+	+	+	-
	1100	+	-	-	-	-	+
	700	-	+	+	-	-	-
	540	+	+	+	+	+	+
	450	+	+	+	+	+	+
	320	+	+	+	+	+	+
26	1050	-	+	-	-	-	-
	950	+	+	+	+	+	+
	900	+	+	-	-	+	+
	800	+	+	+	+	+	+
	770	+	+	+	+	+	+
	700	+	+	+	+	-	-
	630	+	+	+	+	+	+
	480	+	-	+	-	+	+
	390	+	+	+	+	+	+
29	1600	-	+	-	+	-	-
	1200	+	+	+	+	+	+
	1100	-	-	+	-	+	+
	1050	+	+	+	+	+	+
	950	+	+	+	-	+	+
	750	+	+	+	+	+	+
	460	+	+	+	+	+	+
	280	+	-	-	-	-	+
33	1450	+	+	+	+	+	+
	1000	-	-	+	-	-	-
	750	+	+	+	+	+	+
	700	+	+	+	+	+	+
	450	+	+	+	+	+	+
	300	+	+	+	+	+	+
35	1050	+	+	+	+	+	+
	680	+	+	+	+	+	+
	500	+	+	+	+	+	+
	300	-	+	-	+	-	-

Table 4. Contd..

Primer ID	Band size (bp)	Date palm cultivars					
		Ameri	Halawy	Zahedi	Barhi	Bentaisha	Hayani
39	1450	+	+	+	+	+	+
	1300	+	+	+	+	+	+
	1300	+	+	+	+	+	+
	700	-	+	-	+	-	-
	650	-	-	+	+	-	-
	630	+	-	-	-	-	-
	580	+	+	-	+	+	+
	450	+	+	+	+	+	+
	300	+	+	+	+	+	+

The size of amplification fragments was estimated by comparison to 100 bp ladder as a

(+) indicates the presence of an amplified DNA fragment and (–) absence of that fragment.

Table 5. Similarity matrix based on the Nei and Li coefficients of the six date palm cultivars obtained from 9 RAPD markers.

Cultivar	Ameri	Halawy	Zahedi	Barhi	Bentaisha	Hayani
Ameri	1	0.816	0.845	0.917	0.870	0.917
Halawy		1	0.848	0.918	0.808	0.796
Zahedi			1	0.845	0.860	0.763
Barhi				1	0.804	0.792
Bentaisha					1	0.935
Hayani						1

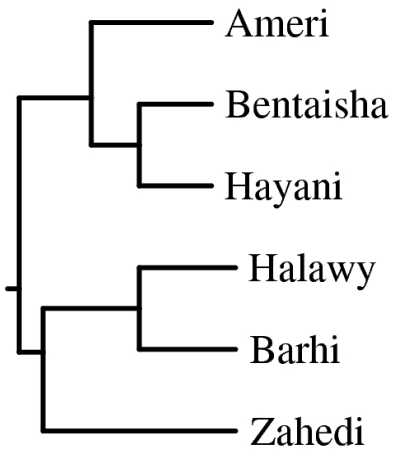

Figure 2. Phylogenetic dendrogram of six date palm cultivars using nine RAPD primers. The dendrogram was constructed according to the UPGMA method, using the online dendrogram construction utility, DendroUPGMA. Each arm of the dendrogram corresponds to the scaled genetic distance.

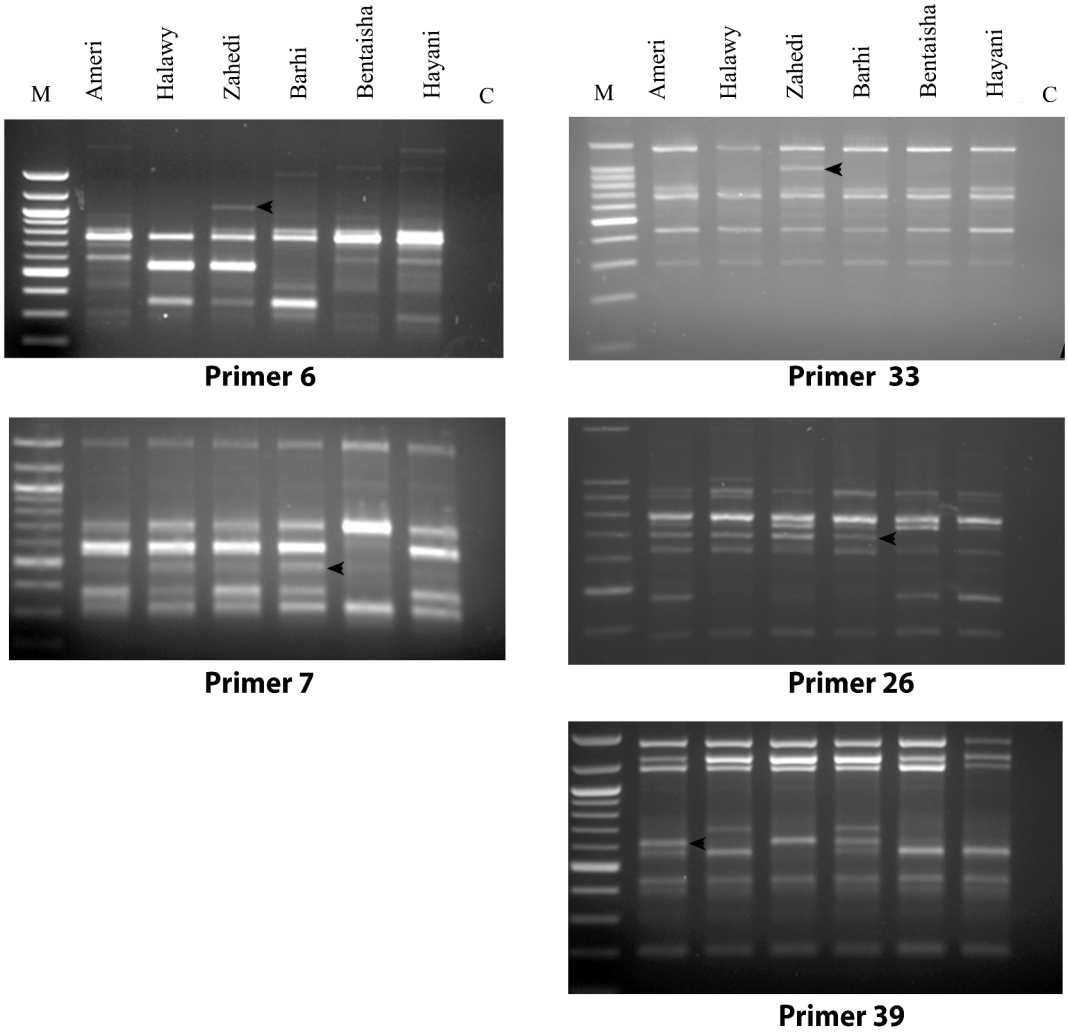

Figure 3. Agarose gel electrophoresis of RAPD experiments showing amplification with primers that gave specific marker bands with certain cultivars. These bands are indicated by black arrows. The name of cultivar is indicated above each lane and the primer number is indicated below each plate. **M** is a 100 bp DNA ladder and **C** is a negative PCR-control. The text and Table 4 give approximate size of the bands.

Conclusion

This is the first study to address genetic characterization of six date palm cultivars of the Gaza Strip. Among them, two cvs. (Hayani and Bentaisha) have been grown for a very long time while the other four were imported from other Palestinian areas in 1999 (Ameri, Halawy, Zahedi and Barhi). A RAPD protocol was optimized and found capable of differentiating all the six cultivars using 9 primers, which may be applied at an early stage of the tree growth, thus eliminating the need to wait for the fruit stage for cultivar identification.

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