The Journal of Animal & Plant Sciences, 27(4): 2017, Page: 1287-1294
ISSN: 1018-7081

MOLECULAR IDENTIFICATION OF SOME SEEDLING OF DATE PALM *(Phoenix dactylifera L.)* MALES' TREES

S. A. Seif El-Yazal[1], H. F. Alharby[2], M. A. Seif El-Yazal[3], G. M. Hassan[4] and M. M. Rady[3]

[1]Department of Horticulture, [3]Department of Botany, [4]Department of Genetics, Faculty of Agriculture, Fayoum University, 63514 Fayoum, Egypt
[2]Department of Biological Sciences, Faculty of Science, King Abdulaziz University, 21589 Jeddah, Saudi Arabia

Corresponding author E-mail: mmr02@fayoum.edu.eg; mrady2050@gmail.com

ABSTRACT

The present investigation was conducted during the season of 2014 using five seedlings of date palm males selected from the evaluation of fifty male seedlings of date palm grown in Tamiya district, Fayoum Governorate, Egypt. To facilitate the selection and identification of good male pollinators for further using in breeding programs, the selection was carried out for promising males to be used to pollinate the females of date palm. The selected five male seedlings of date palm were analyzed using RAPD marker. Among a total of 77 bands obtained, 75 bands were polymorphic and the other 2 bands were monomorphic. Amplification size ranged between 280 and 2000 bp. UPGMA based cluster diagram showed that the all five different genotypes were grouped into two major clusters. Polymorphism percentage (96.4%) was indicated a high genetic diversity among the date palm studied.

Keywords: Date palm males, Genetic diversity, RAPD markers

INTRODUCTION

Date palm (*Phoenix dactylifera L.*) is one of the oldest cultivated trees worldwide. In Egypt, date palm has been discovered since prehistoric times and can be grown well in both Nile Valley and desert districts. It could also be grown under unfavorable environmental condition in most countries all over the world. Date palm tree is considered one of the most important fruits where it is widely distributed in different areas in Egypt from the Mediterranean coast up to Aswan. Many investigators have had studied the effect of pollens obtained from different cultivars of date palm males on fruit set, yield and fruit physical and chemical properties (i.e., fruit weight, flesh weight, fruit length and diameter, and tannins content) of several cultivars of date palm females (Metwaly *et al.*, 2009; Maryam *et al.*, 2016). The direct influence of the male parent on the development of the date fruit is precise and definite, and varies with the particular male used to fertilize the female flowers. Each male exerts approximately the same effect on fruit of all varieties in different years. Therefore, it is important to select and identify superior male in a term of fertilization. Development of suitable genetic markers may allow the researchers to estimate the genetic diversity which will ultimately help in the genetic conservation of date palm. The progress of any genetic reservation or breeding program is dependent on understanding the amount and distribution of the genetic variation presented in the genetic pool (Jubrael *et al.*, 2005). Many investigators tended to estimate the genetic diversity in date palm (Younis *et al.*, 2008; Ponnuswami *et al.*, 2008). Data based on molecular markers such as RFLPs and RAPDs have been used to characterize the date palm genotypes. The DNA fingerprinting techniques have the advantage that the DNA content of a cell is independent of environmental conditions, organ specificity and growth stage (Ainsworth *et al.*, 1996). To understand and analyze the genetic relationships and genetic diversity among and within date palm varieties, random amplified polymorphic DNA (RAPD) marker has been used widely and efficiently to characterize many date palm varieties (Khanam *et al.*, 2012). Estimating the genetic distance assists in studying genetic diversity, a trait that is important for parent selection associated with genetic mapping and for marker-assisted selection in breeding programs (Lapitan *et al.*, 2007; Trethowan and Kazi, 2008).

Therefore, in this study we have attempted to identify sex-specific DNA markers for the selective five date palm males using RAPD-PCR to facilitate the selection and identification of good male pollinators for further using in breeding programs to increase the yield and improve the physical and chemical characters of fruits. Consequently, these males could be used as promising pollinators for date palm females after being subjected to propagation programs, including tissue cultural programs.

MATERIALS AND METHODS

Plant materials: This study was conducted during the successive season of 2014 on five male seedlings of date palm coded by numbers of 2, 10, 29, 40 and 46. The five date palm males were select according to the evaluation results of previously studies (Mohamed, 2004; Moustafa *et al.*, 2006). Based on these evaluation results, fifty seedlings of palm males were evaluated recognized as date superior pollinators. They were about 20 years old and grown in a clayey loam soil at Tamiya district, Fayoum Governorate, Egypt to select the suitable and most promising ones to be used in pollinating the female date palms.

DNA preparation: Young and fresh leaf samples were collected separately from each of the five date palm males. The selected leaf samples were firstly cleaned carefully with distilled water to remove the waxy layer, and were then saved in ice box and were quickly transported to laboratory. One gram of each leaf sample was cut into small pieces and grinded into fine powder in liquid nitrogen. Extraction of DNA was performed using DNeasy Plant Maxi kit protocol (QIAGEN).

DNA qualitative and quantitative determination: The quantity of DNA was measured by recording the A260nm in a 1 cm quartz cuvette in a spectrophotometer and by applying the following equation:

DNA concentration (ng μl^{-1}) = AB 260 nm × 50 × dilution factor ……(1)

The DNA fragments were determined visually on Agarose gel, by comparison of their staining intensities with those of known quantities of DNA marker of a similar size. The quality of extracted DNA was performed using 0.8% Agarose gel. An UV Trans illuminator was used to visualize the DNA bands.

PCR conditions and electrophoresis: An initial screening of nine RAPD decamer was performed to test their readability and amplification profiles for polymorphism. After this screening procedure, five RAPD were selected (Table 1). The PCR analysis was performed in 25 µl volume containing 2.5 mM MgCl, 0.2 mM dNTPs, 20 mM primer, 1 µl genomic DNA and 1 U Taq DNA polymerase (Bioron, Germany). All reactions were performed in the thermal cycler 2720 (Applied Biosystems, USA). The RAPD program was performed as 1 cycle of 94ºC for 5 min and 40 cycles of 94ºC for 1 min, 36ºC for 1 min and 72ºC for 2 min, then, a final extension step 72ºC for 10 min. To visualize the PCR products, 15 µl of each reaction was loaded on 1.8% agarose gel. The gel was run at 90 V for about 2 h and visualized with UV transilluminator and photographed. The PCR reaction that generated high level of polymorphism was repeated twice in order to verify the reproducibility of scored polymorphic bands. This procedure allowed only those bands present in all replicated experiments to be scored as markers. Amplicon sizes were estimated using 100-bp DNA standards (Bioron, Germany).

Amplified polymorphic DNA (RAPD analysis): The RAPD-PCR fingerprinting patterns were carried out with Computer-assisted analysis using RAPD software package, version 1.4 (Armstrong *et al.*, 1994). Similarity of the band profiles was performed on the basis of the Jaccard similarity coefficient and UPGMA-based grouping in the NTSYS software (version 2.02), presence of a band was defined as 1 and absence of the band was defined as 0. The Jaccard similarity coefficient for five random primers in the tested genotypes of date palm was calculated. In addition, date palm genotypes were clustered using Un weighted Pair Group Method with Arithmetic Mean (UPGMA) (Jaccard, 1908).

RESULTS AND DISCUSSION

Extraction of DNA: Genomic DNA was successfully extracted and observed to have sharp band. Using the DNeasy plant maxi kit method, the clear band and purity of DNA was obtained. Extraction DNA from samples is the first step for all molecular marker type. DNA can be extract either from fresh, lyophilized, preserved or dried samples but to have good quality DNA fresh material is recommended (Semagn et al., 2006). There are difficulties to get plant DNA free from contaminating proteins and polysaccharides. The kit was used as for proteins remover. Most of the plant cells had very tough cell wall and make used vigorous method to breaking the cell. The excessive force makes the degradation very high molecular weight molecules thought the shearing. The force make also can produce DNase and made low quality of DNA (Croy et al., 1993).

RAPD profiles analysis: Out of nine selective RAPD universal primers tested, only five gave clear amplification. A total of 77 scorable bands were generated from those primers with average polymorphic bands were obtained with 15.4 bands per primer (Table 2). The number of amplified fragments per cultivar varied from 8 bands for the primer OPA16 showing the lowest primer efficiency to 20 bands for the primer OPA20 showing the highest primer efficiency. The primers OPA11, OPA15 and OPA20 amplified only polymorphic bands (100% polymorphism). While the primers OPA09 and OPA16 amplified 95% and 87% polymorphism, respectively (Table 2 and Fig. 1).

The results of this study showed clear and high yields of amplified DNA fragments. RAPD method appears to be a powerful technique for the analysis of genetic diversity of data palm. RAPD data showed high polymorphism (96.4%) among the five cultivars. this results are disagree with the results obtained by Hussein

et al. (2005) and Adawy *et al*. (2004) reported low RAPD polymorphism an Egyptian date palm cultivars (25.2% and 18.9%, respectively). Moghaieb *et al*. (2010) reported relatively high RAPD polymorphism (60.2 %). Morphological markers for identifying the cultivars are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith and Smith, 1989). In contrast, DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationships studies (Reddy *et al.*, 2002). Similarly, quantity and purity of extracted genomic DNA plays crucial role for analysis of molecular diversity and optimization of different parameters for PCR. The absence of amplification for some samples could be due to DNA quality. According to Nguyen (2005) RNA and other contaminants would not have been easily detected when performing gel electrophoresis. Ratios above 2.0 correspond to RNA contamination, while ratios below 1.6 suggest protein contamination (Sinha *et al*., 2001). Beside that genomic DNA concentration is an important factor in whether the reaction is productive or not. The PCR cycle conditions can be one of the factors, like at certain temperatures, the primers might not bind to the template DNA (Caetano-Anollés, 1998). Other than DNA, this could result from imprecise pipetting or unintentional overlooking when doing master mix for PCR reaction. Another factor for unsuccessful PCR product might be due to the primers not finding complementary bases on the template DNA (Williams, 1999).

Genetic distances and cluster analysis: Among the five date palm males studied with nine primers, only five primers were used to assess genetic relationships. These primers were identified as OPA09, OPA11, OPA15, OPA16 and OPA20. A total of 75 reproducible polymorphic and 2 monomorphic bands were identified and scored as RAPD markers. The genetic distance was calculated for the 75 RAPD polymorphic fragments of the 5 cultivars (Tables 3). The date palm male T_2 was highly divergent from date palm male T_4 and T_5 with distances 0.59. The date palm male T_3 is closely related to date palm male T_1, T_2 and T_4 with distances 0.49. Jaccard's similarity coefficient matrix was used to generate a dendrogram using Unpaired Group with Mathematical Average (UPGMA) clustering method using NTSYS and later it was bootstrapped to confirm the grouping (Fig. 2). According to RAPD analyses that revealed by 72 polymorphic band, UPGMA ordered the date palm cultivars into two main clusters irrespective of their origin at similarity level of 0.49 (Fig. 2). The first one consisted of the male T_3 and the second consisted of two sub-clusters, the first one consisted of one group of T_2 and T_4 and the second sub-cluster consisted of two sub-clusters, the first contained the male T_1 while the second sub-cluster consisted of the last male T_5 (Fig. 2). At the polymorphism level, a high level of polymorphism was generated utilizing the 5 RAPD primers (Table 3 and Fig. 2). The highest number of amplicons was generated from primer OPA20 (20 amplicons), while the lowest was generated from primer OPA16 (8 amplicons). All RAPD primers used in the present study allowed for enough distinction among the five palm cultivars. Overall comparison among cultivars across the five primers revealed the power of RAPD in distinguishing among the palm cultivars grown in Fayoum Governorate. A low number of RAPD amplicons per primer was sufficient to produce useful fingerprints for palm cultivar discrimination.

The RAPD-based phylogenetic analyses have been conducted using neighbor-joining and/or UPGMA with Jaccard and raw-character-difference distances (Pharmawati *et al.*, 2004; Simmons and Webb, 2006). The RAPD markers have been extensively used in investigations aiming to reveal the level and apportionment of genetic diversity, genetic relationships among species or subspecies, and sex identification. Obtaining a marker linked to a gene or genomic region through RAPD analysis depends to a larger extent on chance, because random sequences are used as PCR primers. The RAPD markers have been successively used for phylogenetic studies in many plant species (Xuemei *et al.*, 2012), and the RAPD analysis could be used for an effective identification and DNA fingerprinting of date palm (Abdulla and Gamal, 2010; Jan *et al.*, 2011). The genetic similarity matrices were estimated for the date palm cultivars and used to develop dendrogram revealing the genetic relationships. Moreover, the polymorphism detected and its reproducibility suggests that RAPD markers are reliable for identification of date palm cultivars (Eissa *et al.*, 2009). Our results are supported by Sonboli *et al.* (2011) and Xuemei *et al.* (2012) that the RAPD marker system reveals high levels of polymorphism among species indicating its effectiveness for evaluating the intra specific genetic diversity in the genus.

Table 1. Nucleotide sequence of ten 10 mer primers used for the random amplification of polymorphism DNA.

S/N	Primer name	Primer sequence	GC%
1	OPA20	GTTGCGATCC	60
2	OPA16	AGCCAGCGAA	60
3	OPA15	TTCCGAACCC	60
4	OPA11	CAATCGCCGT	60
5	OPA09	GGGTAACGCC	70

Table 2. Random primers showing polymorphism among five seedling of date palm (*Phoenix dactylifera* L.).

Primer	No. of samples	Total band obtained	Polymorphic band	Monomorphic band	polymorphic%
OPA09	5	19	18	1	95
OPA11	5	17	17	0	100
OPA15	5	13	13	0	100
OPA16	5	8	7	1	87
OPA20	5	20	20	0	100
Total		77	75	2	482
Average		15.4	15	4	96.4

Table 3. Banding patterns and molecular weight of RAPD-PCR fragments detected in the five seedling of date palm using five OPA primers.

primer	Band No.	MW(bp)	Date palm				
			T_1	T_2	T_3	T_4	T_5
OPA09	1	1500	1	0	1	1	0
	2	1300	0	0	1	0	1
	3	1200	0	1	1	1	0
	4	1150	1	0	0	0	1
	5	1000	0	1	0	0	1
	6	950	0	0	1	1	1
	7	900	1	1	0	0	1
	8	850	0	1	0	0	0
	9	800	0	0	1	0	0
	10	700	0	0	0	1	0
	11	650	1	1	0	1	1
	12	550	0	0	1	0	0
	13	500	1	1	0	1	1
	14	450	1	1	1	1	1
	15	300	0	0	0	0	1
	16	280	0	0	0	0	1
OPA11	1	1400	1	0	0	1	0
	2	1200	1	0	0	1	0
	3	750	0	1	0	1	0
	4	700	1	1	1	0	0
	5	680	0	0	0	1	1
	6	650	1	1	0	1	1
	7	600	1	1	0	1	1
	8	500	0	1	0	1	0
	9	450	0	1	0	0	0
	10	400	1	0	1	0	0
	11	300	1	0	0	0	0
OPA15	1	1500	1	0	0	0	0
	2	950	0	1	1	1	0

	3	900	1	0	1	0	0
	4	700	1	1	1	1	1
	5	500	0	1	1	1	1
	6	450	1	1	1	0	1
	7	320	1	1	0	1	0
	1	950	0	1	0	0	0
	2	650	1	1	1	1	1
	3	500	1	1	1	0	0
OPA16	4	450	0	1	0	1	0
	5	400	0	1	0	0	1
	6	350	0	1	0	0	0
	7	280	1	0	0	0	1
	1	2000	1	0	0	0	0
	2	1500	0	0	1	1	0
	3	1200	1	1	0	0	1
	4	1000	0	0	1	0	0
	5	950	1	1	1	1	0
OPA20	6	800	0	1	1	0	1
	7	700	1	1	1	1	1
	8	600	1	1	0	1	1
	9	500	1	0	0	1	1
	10	300	1	0	0	1	0

Table 4. Similarity coefficient percentages among five date palm males based on RAPD analysis.

Date palm	T_1	T_2	T_3	T_4	T_5
T_1	100				
T_2	56.90	100			
T_3	49.00	49.00	100		
T_4	54.95	59.00	49.00	100	
T_5	56.90	54.95	49.00	54.95	100

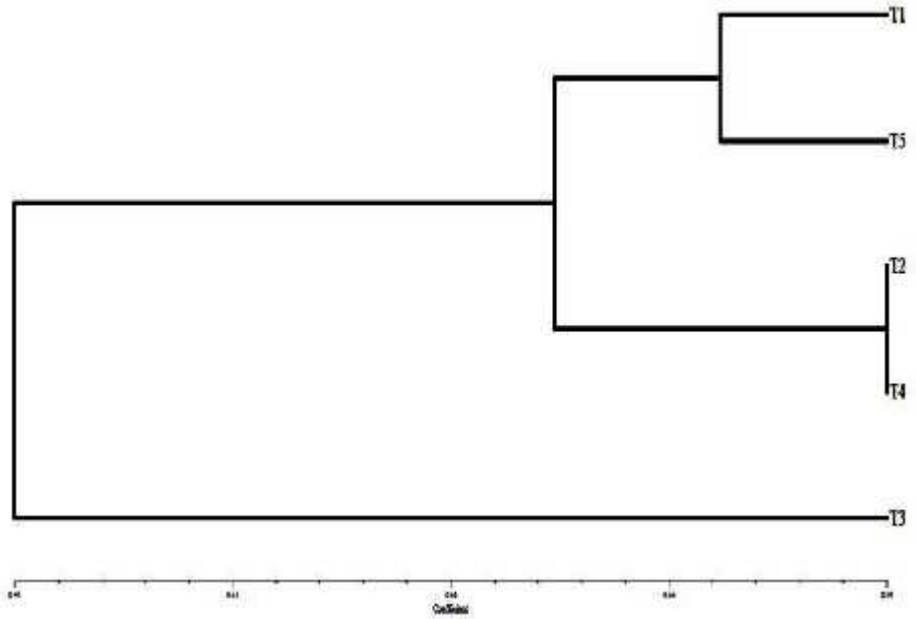

Fig. 1. RAPD Profiles of the five seedling of date palm amplified with RAPD primers OPA09, OPA11, OPA15, OPA16 and OPA20. M: Molecular marker 1kbDNA Ladder, lanes from 1 to 5 Represents: Date palm No., respectively.

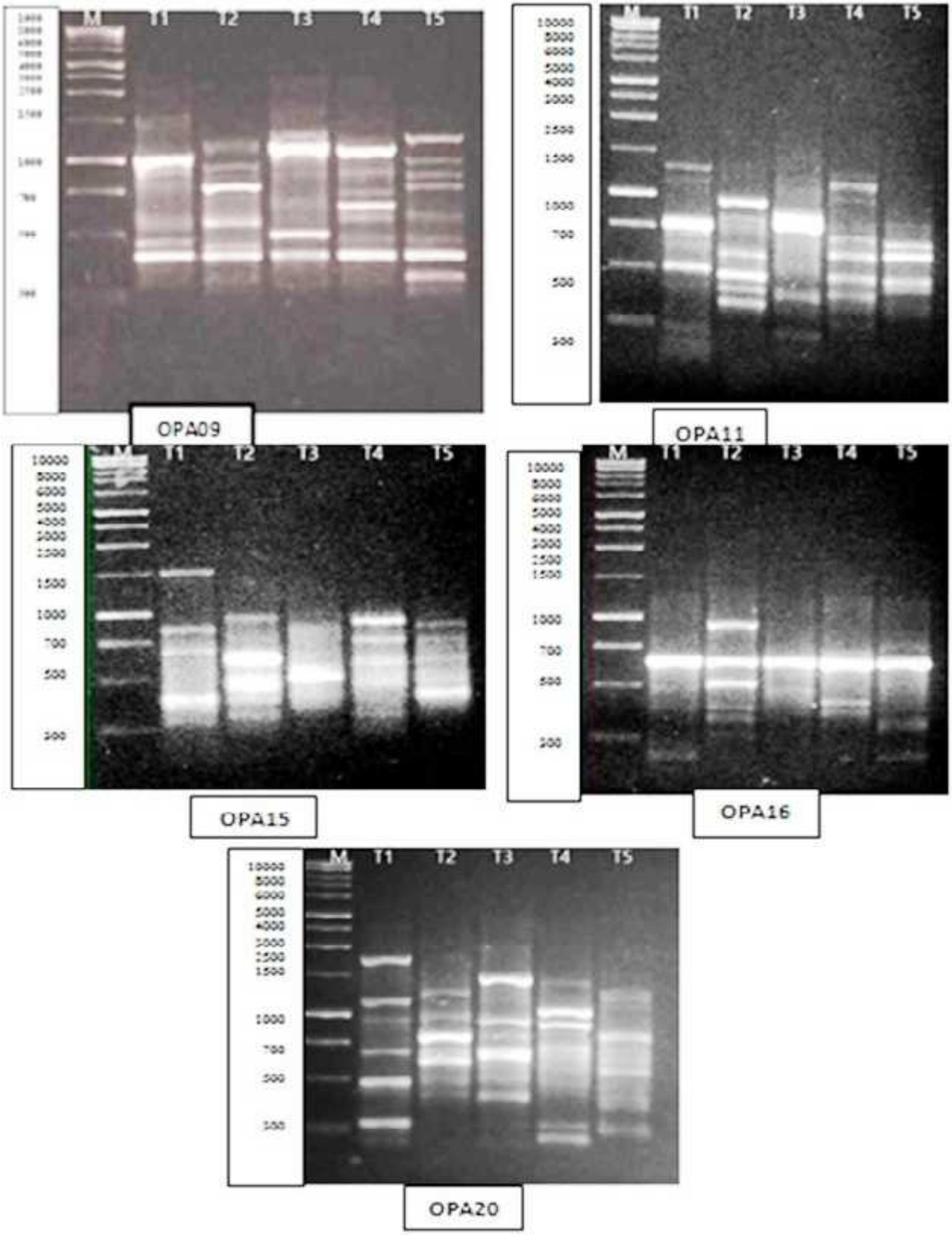

Fig. 2. Dendrogram of the five seedling of date palm amplified with RAPD primers OPA09, OPA11, OPA15, OPA16 and OPA20. M: Molecular marker 1kbDNA Ladder, lanes from 1 to 5 represents: Date palm No., respectively.

Conclusion: The RAPD-PCR is a useful and an effective tool for estimating the genetic variability and the genetic relationships among closely related genotypes of date palm. This study has provided an efficient procedure that can be used routinely to identify date palm cultivars, and can be used to study the genetic diversity of ornamental date palms that are difficult to classify according to their morphological traits and geographical origin. These results will be helpful for an efficient screening, management and use of male date palm genetic resources in selection and breeding program.

REFERENCES

Abdulla, M., and O. Gamal (2010). Investigation on molecular phylogeny of some date palm (*Phoenix dactylifera* L.) cultivars by protein, RAPD and ISSR markers in Saudi Arabia. Aust. J. Crop Sci. 4: 23–28.

Adawy, S. S., Hussein, H. A. Ebtissam, El-Khishin, H. Dina, Moharam and El-Itriby A. Hanayia. (2004). Genetic variability studies and molecular fingerprinting of some Egyptian date palm (*Phoenix dactylifer*a L.) cultivars: II. RAPD and ISSR profiling. Arab J. Biotech., 5(2):225-236.

Ainsworth, C., J. Beynon, and V. Buchanan-Wollaston (1996). Techniques in plant biology: The practical manual. Wye College, University of London, UK.

Armstrong, J. S., A. J. Gibbs, R. Peakall, and G. Weiler (1994). The RAPD Distance package, Ver.1.03. Research School of Biological Sciences, Australian National University, Canberra, Australia.

Caetano-Anollés, G. (1998). DAF Optimization Using Taguchi Methods and the Effect of Thermal Cycling Parameters on DNA Amplification. Bio Techniques 25:472-480 http://www.cropsci.uiuc.edu/faculty/gca/GCA.data/PDF/Biotechniques 98.

Croy, E.J., T. Ikemura, A. Shirsat and R.R.D. Croy (1993). Plant Nucleic Acids. Plant Molecular Biology. Bios Scientific Publishers. Blackwell Scientific Publications. UK.

Eissa, E. A., A. B. A. El-Razek, S. F. El-Sharabasy, and R.M. Rizk (2009). Morphological and molecular genetic characterization of soft date palm (*Phoenix dactylifera* L.) cultivars in Egypt. Egypt. J. Genet. Cytol. 38: 269–284.

Hussein, E.H.A., S.S. Adawy, S. E. M. Ismail and H.A. El-Itriby (2005). Molecular characterization of some Egyptian date palm germplasm using RAPD and ISSR markers. Arab J Biotechnol 8: 83-98.

Jaccard P. (1908). Étude comparative de la distribution florale dans une portion des Alpes et des Jura. Bulletin del la Société Vaudoise des Sciences Naturelles 37: 547–579.

Jan, H. U., M. A. Rabbani, and Z. K. Shinwari (2011). Assessment of genetic diversity of indigenous turmeric (*Curcuma longa* L.) germplasm from Pakistan using RAPD markers. J. Med. Plants Res. 5(5): 823–830.

Jubrael, J. M. S., S. M. Udupa, and M. Baum (2005). Assessment of AFLP-based genetic relationships among date palm (*Phoenix dactylifera* L.) varieties of Iraq. J. Amer. Soc. Hortic. Sci. 130(3): 442–447.

Khanam S, A. Sham, J. L. Bennetzen, and M. A.M. Aly (2012). Analysis of molecular marker-based characterization and genetic variation in date palm (*Phoenix dactylifera* L.) – a review. Aust. J. Crop Sci. 6(8): 1236–1244.

Lapitan, V. C., D. S. Brar, T. Abe, and D. R. Edilberto (2007). Assessment of genetic diversity of Philippine rice cultivars carrying good quality traits using SSR markers. Breed Sci. 57(4): 263–270.

Maryam, M. J. Jaskani, F. S. Awan, S. Ahmad, and IA. Khan (2016). Development of molecular method for sex identification in date palm (*Phoenix dactylifera* L.) plantlets using novel sex-linked microsatellite markers. Biotech. 6: 22 DOI 10.1007/s13205-015-0321-6

Metwaly, H. A. A., Z. A. M. Abou-Rekab, A. A. Abd El-Baky, and A. A. El-Bana (2009). Evaluation of some seeded date palm trees grown in Fayoum Governorate. B. chemical characteristics, p. 701–715. 4th Conf. Recent Technol. Agric.

Moghaieb, R.E.A., A.A. Abdel-Hadi, M.R.A. Ahmed and A.G.M. Hassan (2010). Genetic diversity and sex determination in date palms (*Phoenix Dactylifera* L.) based on DNA markers. Arab J. Biotechnol. 13: 143-156

Mohamed, M. A. El-A. (2004). Evaluation and selection of some seedling date palm males and their effects on fruiting and fruit quality of "Seewy" date palms, p. 35–99. M.Sc. Thesis, Fac. Agric., Cairo Univ., Egypt.

Moustafa, A. A., Z. A. Ibrahim, S. A. S. El-Yazal, and M. A. El-Anwer (2006). Evaluation and selection of some seedling date palm males grown at Fayoum Governorate. Abstr. 3rd Int. Date Palm Conf. Abu Dhabi, United Arab Emirates Feb. 2006.

Nguyen, H. P. (2005). DNA Isolation and RAPD Analysis of Cladophora, Spirogyra, Bryopsis, with focus on Derbesia. [online]. LBS 145, Spring 2005, Michigan State University. US. https://www.msu. edu/~nguyen81/lbs145.htm

Pharmawati, M., G. Yan, and I.J. McFarlane (2004). Application of RAPD and ISSR markers to

analyse molecular relationships in *Grevillea* (Proteaceae). Aust. Syst. Bot. 17: 49–61.

Ponnuswami, V., R. Jagadeesan, A. Ramesh Kumar, M. Prabhu, and S. Makesh (2008). Genetic relationship and diversity in palmyrah accessions based on RAPD markers. Amer-Eur. J. Sust. Agric. 2(2): 165–171.

Reddy, M. P., N. Sarla, and E. A. Siddiq (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica. 128: 9–17.

Semagn, K., A. Bjornstad and M. N. Ndjiondjop (2006). An overview of molecular marker methodsfor plants. African J. Biotechnology. 5 (25): 2540-2568.

Simmons, M. P., and C. T. Webb (2006). Quantification of the success of phylogenetic inference in simulations. Cladistics. 22: 249–255.

Sinha, R. P., M. Dautz and D. P. Häder, (2001). A Simple and Efficient Method for the Quantitative Analysis of Thymine Dimers in Cyanobacteria, Phytoplankton and Macroalgae. Acta Protozoologica, 40: 187-195.

Smith, J. S. C. and O. S. Smith (1989). The description and assessment of distances between inbred lines of maize. II. The utility of morphological, biochemical and genetic descriptors and a scheme for testing of distinctiveness between inbred lines. Maydica. 34: 151–161.

Sonboli, A., A. Gholipour, M. H. Mirjalili, and M.A. Rad (2011). Molecular characterization of Iranian *Dracocephalum* (Lamiaceae) species based on RAPD data. Acta Biol. Szeged. 55(2): 227–230.

Trethowan, R. M., and A. M. Kazi (2008). Novel germplasm resources for improving environmental stress tolerance of hexaploid wheat. Crop Sci. 48: 1255–1265.

Williams, I. (1999). Biogeographic Fine-tuning Helps Weed Biocontrol. Biocontrol News and Information: Volume 20 (2).

Xuemei, P., C. Yan, Y. Ziyi, Z. Weiming, L. Qitang, and L. Changmei (2012). DNA sequence and RAPD information re-affirms the taxonomic relationships between *Apocynumvenetum* L. and *Poacynumpictum* (Schrenk) Baill. Pakistan J. Bot. 44(4): 1261–1266.

Younis Rania, A. A., M. Ismail Omayma, and S. S. Soliman (2008). Identification of sex-specific DNA markers for date palm (*Phoenix dactylifera L.*) using RAPD and ISSR techniques. Res. J. Agric. Biol. Sci. 4(4): 278–284.