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Identification and Validation of Sex Specific DNA Markers in Date Palm (*Phoenix Dactylifera L.*) Using RAPD Markers

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Abstract – Date palm (Phoenix dactylifera L.) is a dioecious long-lived & mono-cotyledoneous woody perennial belonging to Palmae (Arecaceae) family with chromosome number 2n=36. Date palm (Phoenix dactylifera L.) is an important fruit crop mostly grown in the regions of Africa, the Middle East and South Asia. In India date palm cultivation has taken hike in production many dry areas are farming for export quality date in regions of Gujarat and Maharashtra. In India, nearly 0.3 million ha of land can be effectively utilized for date palm cultivation & the extremely dry areas comprising of Jaisalmer, Barmer and Western parts of Bikaner and Jodhpur districts are the potential regions for its cultivation.Date fruit is rich in sugar, iron, potassium, calcium and nicotinic acid. Fresh dates per kg provide approximately 3,150 calories. The dry flesh of the ripe date contains about 80% sugar, 2.5% fibre, 2% protein and 2% each of fat, minerals and pectic substances. There are many other products which can be prepared using date fruits such as syrups, jams, ice cream, alcoholic beverages and soft drinks & the leaves of palm are used in the manufacture of paper. Main issue in cultivation of date palm is yield which is reduced by increased in no of male plants during cultivation. During establishment of plantations, male and female cannot be clearly distinguished until five years after cultivation. Date palm is slow growing and it is difficult to determine the sex of the trees before the flowering. This major problem for farmers does not allow them to cultivate sufficiently large number of productive female trees with only a minimal number of male trees as one male is sufficient to pollinate 50 females when grown together. Thus the establishment of an efficient date palm selection programme requires the identification of suitable markers for the early assessment of the sex of the progeny. Molecular analysis of genotypic markers offers an attractive, reliable alternative method in identifying plants at early growth stage. So we screened for 30 RAPD primers for identification of male and female sex specific primers in which highly polymorphic primers found in OPB-20, OPB-15 and OPA-05 compared to other RAPD primers. Three primers we found capable of efficiently differentiating the male genotypes from the females of date palm. The ability to differentiate most of the tested male and female genotypes by the RAPD markers suggested that this technique is practically applicable for date-palm genotypic identification

Key Words: Date Palm, Early Sex Determination, RAPD, Sex Specific Primers, Molecular Analysis

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INTRODUCTION

The date palm (Phoenix dactylifera L.), 2n=36, is a dioecious long-lived monocotyledonous plant, which belongs to the family Arecaceae. It is one of the excellent candidate crops in arid and semiarid regions of the world with high tolerance to environmental stresses. In addition to its valuable fruit, the tree is cultivated for fuel, fiber and as shelter for ground crops. The annual world production of dates has reached 6-8 million mt (metric tons), representing a market exchange value of over 1 billion USD. (El Hadrami and Al-Khayri, 2012).

It has the distinction of being one of the oldest fruit trees in the world. It is a palm with slender trunk, 70-100 ft in height and can tolerate a high salinity level of up to 22,000 parts per million. It has become an important plantation crop due to the highly nutritious nature of the fruit which it bears for 40-50 years. Date palm (Phoenix dactylifera L.) is an important fruit crop mostly grown in the arid regions of Africa, the Middle East and South Asia. In Pakistan, date palm is considered third major fruit crop after citrus and mango Pakistan is the fifth largest dates producer in the world. Date palm is a long-lived, dioecious, monocotyledonous fruit tree having (2n=2x=36) chromosome number and wind

pollinated member of the Arecaceae family. Date palm is the major fruit crop of arid climate region, cultivated mainly in North Africa, South Asia, USA and Australia. It covers a surface area of about 800,000 ha and is important – directly or indirectly for the life of about 100 million inhabitants in the world

Date fruit is rich in sugar, iron, potassium, calcium and nicotinic acid. One kg fully ripe fresh dates provide approximately 3,150 calories. The dry flesh of the ripe date contains about 80% sugar, 2.5% fibre, 2% protein and 2% each of fat, minerals and pectic substances. Thus, date fruit can help supplement the dietary needs of desert people where very few nutritive foods are available. In California, diced date, date paste and sugar are manufactured for use in breakfast and bakery. Although dates are cultivated for their fruits, other parts of the palm are also used e.g. trunks for building, leaves for thatch and the spathes for palm wine. The secondary products generated from date fruits are syrups, jams, ice cream, alcoholic beverages and soft drinks. Wild date palm, Phoenix sylvestris is widely used in parts of India for sugar making. Arabs who eat dates on regular basis show an extremely low incidence of cancer and heart disease. The leaves of palm also have the potential for use in the manufacture of paper. (Singh et al, 2004)

Like a number of other agriculturally important crops such as nutmeg, pistachio, cannabis and papaya, date palm is also dioecious. This species is slow growing and it is difficult to determine the sex of the trees before the first flowering, when they are five years of age. This major problem for farmers does not allow them to cultivate sufficiently large number of productive female trees with only a minimal number of male trees as one male is sufficient to pollinate 50 females when grown together. Thus the establishment of an efficient date palm selection programme requires the identification of suitable markers for the early assessment of the sex of the progeny (Quenzer et al., 1998) and long yield-life (100years) and referred as the “tree of life” in the Bible. It is a multi-purpose tree, being highly regarded as a national heritage in many countries. Date palm having high nutritional value, productivity

The correct identification of palms based on physiological and morphological markers is usually not possible until fruits are produced. Moreover, the characterization and evaluation of genetic diversity based on a large set of phenotypic data is often difficult to assess due to environmental influences. Molecular analysis of genotypic markers offers an attractive, reliable alternative method in identifying plants at early growth stage. Molecular techniques based on DNA have been very successful in the analysis of a variety of crop plants. Techniques of genome fingerprinting include several techniques, one of which is randomly amplified polymorphic DNA (RAPD). RAPD is a powerful technique which can be used to separate, identify and determine the specific genomes or to estimate the phylogeny among the individual genomes. Traditionally, assessment of genetic diversity has been based on the differences in morphological and agronomic traits or pedigree information in different crops.

Biological macromolecules, such as DNA-based techniques have successfully been used for the analysis of genetic relationships and classifications of plants, which are difficult to be determined by the classical taxonomic methods due to environmental variations. Hence, DNA fingerprinting has proven to be the most suitable method for accurately identifying date palm cultivars and for analyzing their genetic diversity and phylogenetic relationships. DNA typing in plants is primarily used for identification of gene assortment, protection of biodiversity or germplasm conservation and identifying markers associated with specific traits. Genetic preservation is dependent on understanding the amount and distribution of the genetic diversity present in the existing germplasm.

REVIEW OF LITRATURE

The relation between two female date palm cultivars (Sewi as semi dry date and Amhat as moist date) and three pollinizers. The pollen grains were selected from male pollinizers located at Giza, Aswan and El-Wadi (New Valley) governorates, Egypt. Also, RAPD technique was used to compare among the five date palm genotypes. Many traits such as fruit set, bunch weight, yield per palm and both physical and chemical fruit properties were studied. The results showed that Giza and Aswan pollinizers were the most suitable for pollinizing Sewi and Amhat female, respectively. At the molecular level, 9 primers were used for RAPD analysis. These primers gave a total of 55 different alleles for all genotypes. The most Polymorphic Information Content (PIC) value and polymorphism percentage that detected by OPB-07 and OPO-14 markers showed the high score (8 bands) with polymorphism (87.5% and 50%), respectively. Primers OPB-10 and OPO-19 revealed low level from bands (4bands) with 75%. Also, OPA-02 and OPO-13 and revealed six fragments with 50 and 83.33 polymorphism, respectively. (Mostafa *et al*. 2016)

Isolation of the high molecular weight DNA from fibrous unopened pale yellow leaves of ten date palm cultivars. DNA was extracted with CTAB method using liquid nitrogen and supernatant was kept for an overnight for precipitation of DNA at 40^0C. Quantification of extracted DNA was checked with spectrophotometer and on 0.8% agarose gel electrophoresis. DNA was further diluted for 10ng/µl and optimized for RAPD-PCR amplification. Mirbhar *et al*. (2014)

These relationships were also seen in olive (*Olea europaea L* using two DNA marker methods; The data analysis revealed that both ISSR and RAPD-

PCR succeed to classify the five examined cultivars into two clusters. Elsheikh *et al*. (2014). A number of sex-specific molecular markers have been identified in several dioecious plants like *Pistacia vera* (Hormaza et al. 1994), *Actinidia chinensis* (Gill et al. 1998), *Salixviminalis* (Gunteretal.1998),*Piperlongum* (Banerjeeet al. 1999), *Myristica fragrans* (Shibu et al. 2001), *Actinidia deliciosa* (Shirkot et al. 2002), *Cannabis sativa* (Torjek et al. 2002), *Mercurialis annua* (Khadka et al. 2002), *Calamus simplicifolius (*Yang et al. 2005), *Carica papaya* (Parasnis et al. 2000; Deputy et al.2002; Gangopadhyay et al. 2007), and *Simmondsia chinensis* (Agrawal et al. 2007; Agarwal et al. 2011;Inceetal.2010). In datepalm,some putative sex-linked random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers have earlier been reported (Singh et al. 2006; Younis et al. 2008), but these could not be verified on use of more number of genotypes. Recently, Mohsenzadehand Pasalari(2010)have report RAPD marker, which produced a male plant-specific 520-bp fragment in "*Zamardan*," an Iranian date palm cultivar. The sequence-characterized amplified region (SCAR) markers, generally developed from RAPD and ISSR fragments, are locus-specific and more reliable and more reproducible for molecular identification (Paran and Michelmore 1993). SCAR markers linked to sex-specific genes have been developed in many dioecious plants like *C. papaya* (Bedoya and Nunez 2007), *P. vera L*. (Yakubou et al. 2005), and *Cycas circinalis* (Gangopadhyay et al. 2007), and *Ginkgo biloba L*. (Liao et al. 2009). In the present study, we have developed a sex-specific SCAR marker for reliable sexing of date palm plants

In an investigation, identification & evaluation of genetic variability between cultivars of date palm on the basis of morphological & biochemical markers are difficult, time-consuming & provide limited information. The aim of this study was to detect genetic variability in different cultivated date palm populations around the city of Manama in Baharin. RAPD was applied to study the genetic diversity in date palm plants. Pathak *et al*. (2008) the genetic diversity among 15 different cultivars of date palm at the experimental farm of Qatar University using simple sequence repeat (SSR) marker and find out of the genetic similarity and diversity among the well-known Qatar date palm cultivars. DNAs were extracted from the young fresh leaves. Among 16 primer paired tested for their ability to generate expected SSR banding patterns in Qatar date palm genotypes,10 primers successfully produced clear single band in most of the studied genotypes so, far, 6 SSR primer did not amplified clear bands in our genetic materials even using different PCR conditions. The amplified SSR band ranged from 100-200bp.A total of 40 alleles with an average of 4 alleles per locus were scored. Hamon *et al*. (1997)

MATERIALS AND METHODS

MATERIALS

Plant material and genomic DNA isolation

For the investigation, a total of eight genotypes of date-palm (*Phoenix dactylifera L*.) were used for assessing the molecular polymorphism. Out of these four are male (unknown variety) and four are female. Leaves samples of various genotypes were collected from Bageshwari Sugarcane Industry, Partur. The leaves samples were wrapped in aluminum foil and stored at -80^{0}C deep-freezer and that obtaining leaves samples were used as source of DNA. The DNA was extracted from eight date palm genotype leaves and seeds by using CTAB extraction method (Murray and Thompson 1980). Quality of DNA was assessed by electrophoresis on 0.8 % agarose gel and its quality and quantity was evaluated in Nanodrop. DNA samples were used for quantification and absorbance was measured at 260nm and 280 nm using micro-cuvette. (Nanodrop Technologies, Wilmington, Dalware) to dilute DNA stock as 50 ng/ µl d_3H_2O to use in PCR amplification.

PCR Amplification:

The PCR reactions were set in 12 µl reaction volume, Master Mix was prepared with the template DNA 2.5 µl, PCR Buffer with 1X $MgCl_2$ 3 µl, dNTP mix (0.25 Mm) 2 µl, Primer (10 pmol) 1.5 µl, Taq DNA Polymerase (1U/ µl) 1 µl, Distilled water 2 µl Taq DNA polymerase 0.2 and 5.8 lL of nuclease-free water. Amplification was carried out in veriti 96-well fast thermal cycler under the following conditions:

Table no 1: PCR program for amplification of DNA

Step No.	Steps	Temperature (°C)		Time
1	Initial denaturation	94⁰		2 min
2	Denaturation	94⁰	40 cycle	1 min
3	Annealing	37⁰		30 sec
4	Extension	72⁰		2 min
5	Final extension	72⁰		2 min
6	Hold	4°C		∞

All amplified DNA products were resolved on agarose gel (1.5%) and visualized under UV light. Gel elution for DNA purification was performed using gel elution kit.

RESULT

The results obtained from study conducted for the molecular diversity of eight date palm genotypes have been presented as under.

Genomic DNA Isolation:

The DNA was extracted by using CTAB-based method from date palm leaves & seeds. The procedure developed by Murray and Thompson (1980) was followed with minor modification.

Quantification of DNA:

The quantification of isolated DNA was done by using Nanodrop. The Nanodrop gives ratio of DNA: RNA and gives actual quantity of DNA. A260/280 ratio provides indication of protein, and polyphenols and carbohydrates contamination. The quantity of isolated DNA was ranged from 222.5 to 2406.7 µg/ml and absorbance ratio at A260/280 ranged from 1.6 to 1.9 indicating good quality DNA samples. Concentration of each DNA sample is given in table no 5

Table no.2: DNA concentration and Absorbance Ratio of eight date palm genotypes

Genotypes	Quantity (µg/m	Absorbance Ratio
Male 119 (1)	222.5	1.9
Male 194 (2)	541.9	1.98
Male 192 (3)	880.3	1.97
Male 121 (5)	812.1	1.9
Female 9800 (1)	2406.7	1.99
Female 189 (2)	1498.8	2.0
Female 161 (3)	684.9	1.85
Female 133 (4)	790.5	1.7

Quality Estimation of DNA:

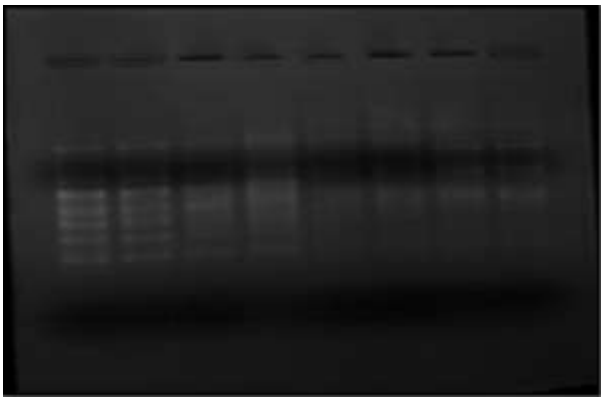
The quality of isolated DNA was checked by using agarose gel electrophoresis. After electrophoresis, the purity of isolated DNA is shown in plate no 2.

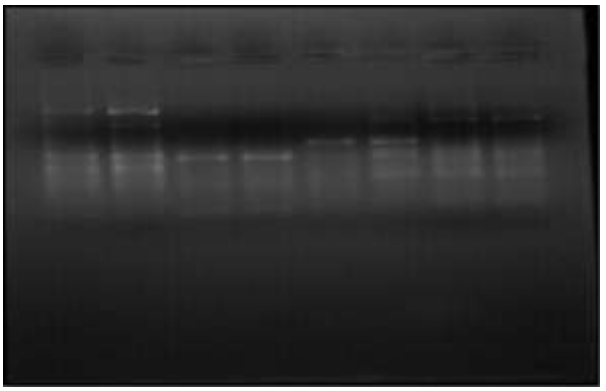
Isolated DNA samples from date palm leaves

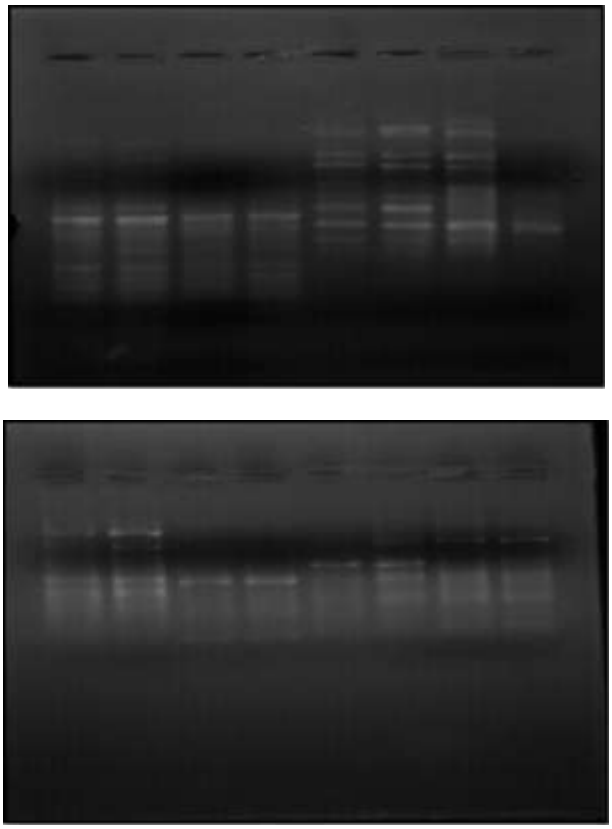
RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD):

Screening of primers

The genomic DNA extracted from each plants were subjected to polymerase chain reaction, the thirty random primers from OPA, OPB & OPC series were used. Molecular characterization of nine plants of date palm was carried out. The purpose of this study was to reveal diversity among nine different genotypes of date palm. In RAPD assay total five primersO were selected for pooled analysis from thirty primers. The results obtained using five primers have been discussed below,







RAPD profile of date palm with primer OPB-16. OPB -7 and OPB-20, OPB -15

Table no. 7. Series of RAPD marker

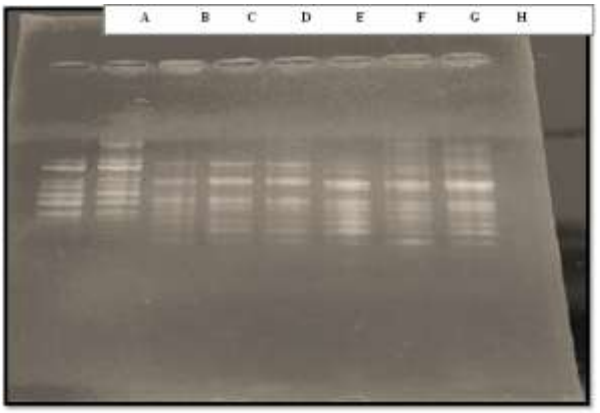
Sr. No.	Primer code	Sequence 5' to	Presence of poly-morphic band
1.	OPA – 2	TGCCGAGCTG	-
2.	OPA – 9	GGGTAACGCC	-
3.	OPA – 6	GGTCCCTGAC	-
4.	OPA -20	GTTGCGATCC	-
5.	OPA-13	CAGCACCCAC	-
6.	OPA-5	AGGGGTCTTG	+
7.	OPA-1	CAGGCCCTTC	-
8.	OPA-7	GAAACGGGTG	-
9.	OPA-14	TCTGTGCTGG	-
10.	OPA-8	GTGACGTAGG	-
11.	OPA-15	TTCCGAACCC	-
12.	OPA-17	GACCGCTTGT	-
13.	OPA-10	GTGATCGCAG	-
14.	OPA-4	AATCGGGCTG	-
15.	OPB-2	TGATCCCTGG	-
16.	OPB-16	TTTGCCCGGA	+
17.	OPB-4	GGACTGGAGT	-
18.	OPB-15	GGAGGGTGTT	+
19.	OPB-8	GTCCACACGG	-
20.	OPB-19	ACCCCCGAAG	-
21.	OPB-7	GGTGACGCAG	+
22.	OPB-20	GGACCCTTAC	+
23.	OPB-14	TCCGCTCTGG	-
24.	OPB-18	CCACAGCAGT	-
25.	OPC- 19	GTTGCCAGCC	-
26.	OPC-7	GTCCCGACGA	-
27.	OPC-10	TGTCTGGGTG	-
28.	OPC-4	CCGCATCTAC	-
29.	OPC-9	CTCACCGTCC	-
30.	OPC-20	ACTTCGCCAC	-

Validation of identified polymorphic primers

Among these thirty primers five primers were highly polymorphic given below in table no: 8

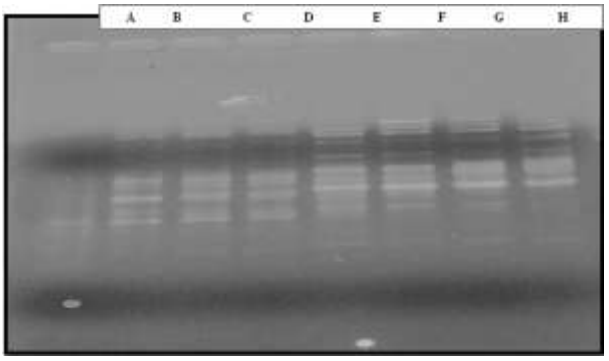
Table No. 8. Validation of identified polymorphic primers

Sr. No.	Primer	No. of bands	No. of polymorphic bands	Polymorphism %
1	OPB-16	9	4	44.44%
2	OPB-7	9	3	33.33%
3	OPB-20	10	5	50%
4	OPB-15	5	1	20%
5	OPA-5	4	3	75%



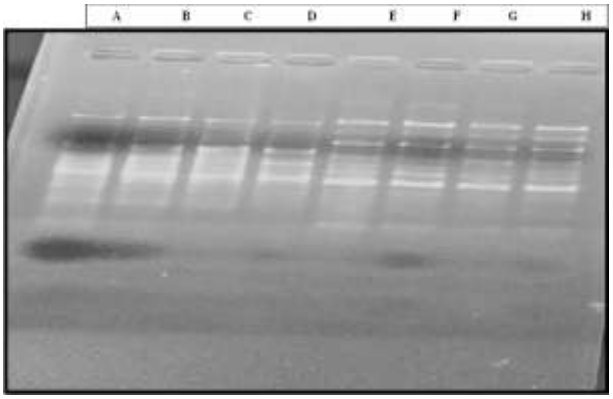
RAPD profile of date palm with primer OPB -16 (Female Specific primer).

1. LANE A, B, C, & D are female species
2. LANE E, F, G, H are male species



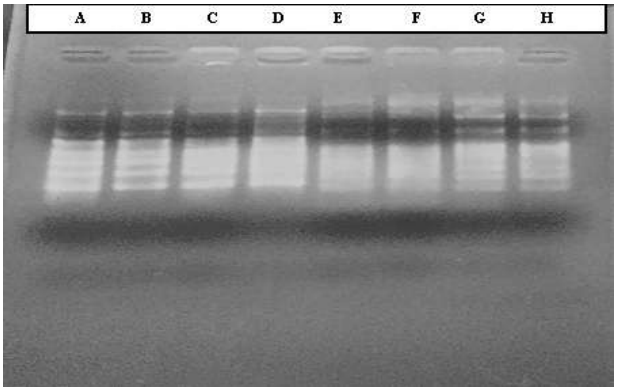
RAPD profile of date palm with primer OPB-20.

1. ANE A, B, C, & D are female species
2. LANE E, F, G, H are male species



RAPD profile of date palm with primer OPB-15 (Male specific primer).

1. LANE A, B, C, & D are female species
2. LANE E, F, G, H are male species



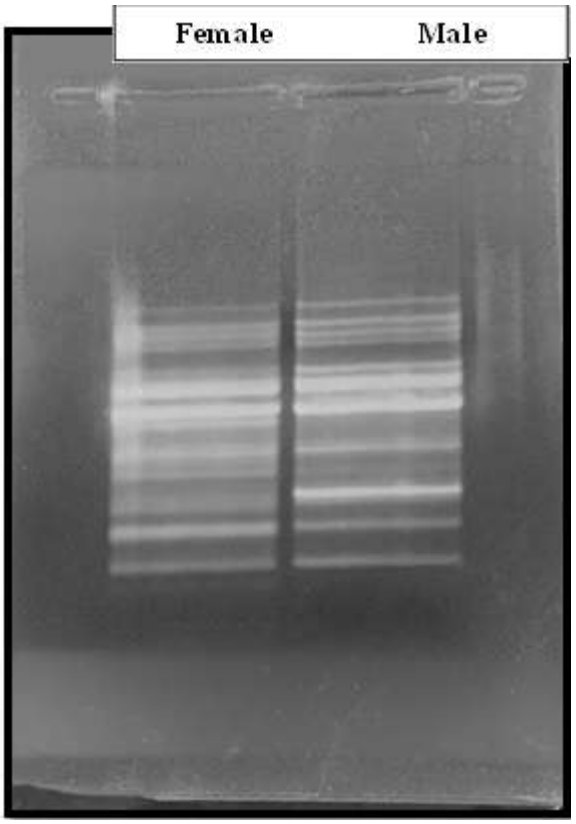
RAPD profile of date palm with primer OPA-05.

1. LANE A, B, C, & D are female species
2. LANE E, F, G, H are male species

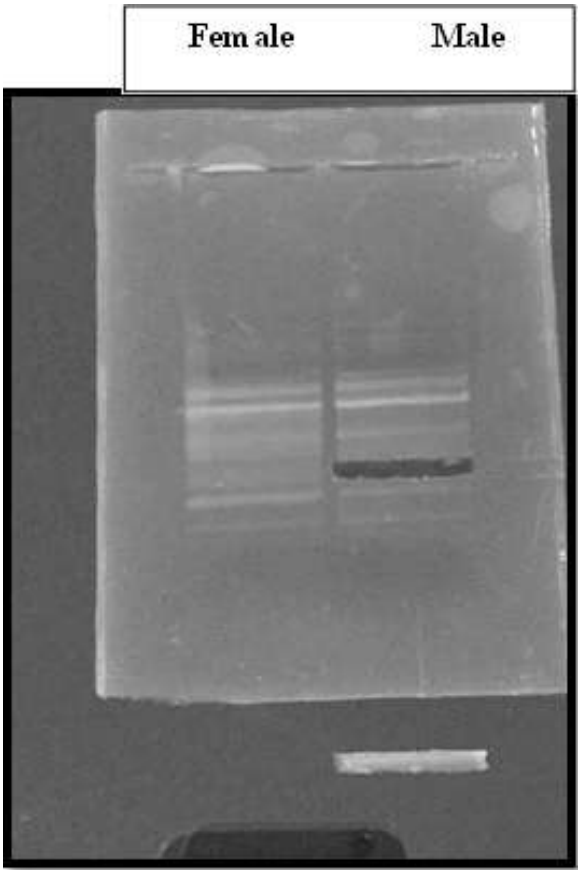
Gel elution for purification of DNA

OPB 20

OPB 20 gives male specific polymorphic banding pattern in date palm so bulk pooling of all male & female DNA samples were performed to elute the polymorphic male specific band for purification of band eluted from gel for development of SCAR markers.



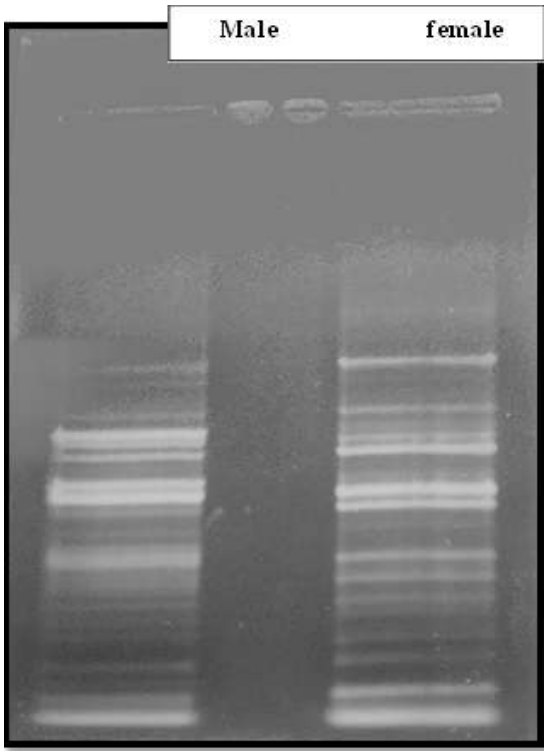
RAPD profile of bulk mixture of male and female date palm sample using OPB – 20



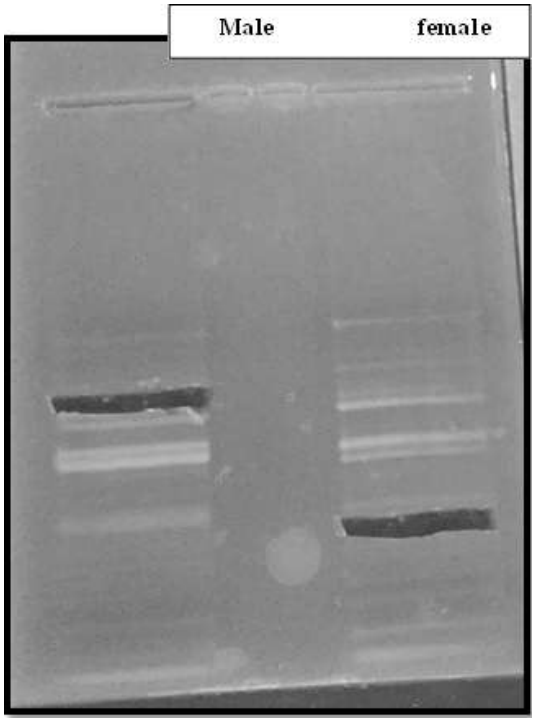
Illusion of male specific polymorphic band obtained from gel by using OPB 20.

OPB 15

OPB 20 gives female specific polymorphic banding pattern in date palm so bulk pooling of all male & female DNA samples were performed to elute the polymorphic female specific band for purification of band eluted from gel for development of SCAR markers.



RAPD profile of bulk mixture of male and female date palm sample using OPB – 15



Elusion of female & male specific polymorphic band obtained from gel by using OPB 15.

DISCUSSION

Date palm (*Phoenix dactylifera L.)* is important crops in several developing countries, and this sequence provides a resource that may be vital for their improvement. For instance, it would have been extremely difficult to identify the gender specific RAPD markers we report without the availability of a draft genome sequence. In this work, the utility of RAPD markers in the sex determination analysis of Date Palm germplasm was studied. The optimal number of primers, required to discriminate among genomic DNA of eight cultivars, depends on the level of polymorphism generated by type from the palm samples. At the polymorphism level, thirty RAPD primers were screened a high level of polymorphism was generated utilizing the 5 RAPD primers. Among them 37 bands were monomorphic and 16 were polymorphic. The maximum numbers (05) of the polymorphic bands were observed for primer OPB-20 and minimum number (1) band was observed for primer OPB-15. The polymorphism percentage ranged between 20 to 75. Bulk pooling of DNA mixtures of male and female date palm plants with selected 3 primers(OPB 20), (OPB 15)& (OPA 5) from validated PCR primers. Gel elution of polymorphic bands obtained and stored for further development of SCAR markers.

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