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Morphological Marker for Gender Identification in Date Palm (*Phoenix dactylifera L.*) at the Seedling Stage Using RAPD-PCR Analysis

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Abstract

Date palm (*Phoenix dactylifera* L.) is an important fruit crop in arid and semi-arid regions in Nigeria. At the seedling stage, male and female trees cannot be clearly distinguished until about four years after planting when the palms first flower. This research was aimed to differentiate gender at seedling stage among date palm accessions obtained from NIFOR in Nigeria using morphological marker and a specially developed DNA RAPD marker technique. A PCR based method was found to be successful using extracts from young leaves. The male determinant marker was able to distinguish a male-specific fragment at 200bp while male and female individuals were identified on a fragment of 400 bp. The male determinant marker generated fragments that consisted of one banding patterns in male DNA extracts and no banding pattern in female. When the 21 date palm seedlings were screened, 12 were identified as male and 9 as being female. Morphologically, the male seedlings were observed to have a larger girth size and a higher number of lower leaf spines while the female seedlings were observed to possess a smaller or thinner girth size with little number of lower leaf spines. The primers was repeated for other cultivars and the same DNA banding patterns and morphological differences were observed. The developed sex determinant marker could be used for gender identification at the seedling stage of date palms. Plant breeders and growers may adopt this marker as a tool for gender identification in date palm seedlings before planting in the field.

Keywords: Morphological; Rapd; PCR; Primers; Sex Determination; BP (base pairs)

Introduction

Date palm (*Phoenix dactylifera* L.) belongs to the family Arecaceae (2n=36). Research related to date palm is greatly restricted, owing to the lack of measures to identify its gender at the seedling stage. Date palm cultivation is more cost-effective through the cultivation of female plants than male plants. An increase in the number of female date palm plants per hectare may result in an increase in date production, thereby making the plantation more profitable. An increase in the number of female date palm plants per hectare may result in an increase in date production, thereby making the plantation more profitable. The high genetic diversity is very important in plants for their survival in their natural habitat [1]. The seedlings produced may be either male or female, and no reproducible technique is currently available for gender determination in germinated seeds of date palm. Several efforts have been directed recently to establish a method for the early detection of seedling gender before transplanting them in the fields. However, no methodology has so far been developed for gender identification at the seedling stage (Hafiz *et al.,* 1980). Molecular markers have gave an efficient method for sex identification at an early stage of plant, these markers include Random Amplified Polymorphic DNA (RAPD) [2], In addition, Khanam *et al.,* (2012) reported that random amplified polymorphic DNA (RAPD) marker has been used widely and efficiently to characterize many date palm varieties for better understanding and analyzing the genetic relationships and genetic diversity among and within date palm varieties.

Materials and Methods

Plant material and genomic DNA extraction

Leaf samples (3 weeks to 1 month old) of date palm seedlings were collected from the twenty-one (21) different accessions under study at the Biological Sciences experimental field in the Federal University of Technology, Minna, Nigeria and stored at −80°C. The DNA was extracted using a CTAB (Cetyltrimethyl ammonium bromide) based procedure using DNeasy plant mini kit (Qiagen, Germany), according to the manufacturer's instructions. Quality/Quantity of DNA was assessed by electrophoresis on 0.8 % agarose gel and run in 1X TBEbuffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0).Analysis was carried out using a SmartView Pro 1200 Imager System (Scientific Biotech, Taiwan) and band intensity was compared with a100 bp DNA Ladder RTU (Genedirex®, Taiwan). All DNA samples were diluted to a concentration of 50 ngμL−1 using an elution buffer and stored at -20 °C.

Primer Selection and DNA amplification

A range of combinations of six primers was used, together with PCR amplification across the young leaf samples of the twenty-one date palm accessions. DNA amplification was performed on Applied Biosystems Veriti96-well Thermal Cycler with the following programme. First denaturation at 94 °C for 5 min., followed by 40 cycles of denaturation for 1 min at 94 °C, annealing at 36 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Amplification products were analysed by gel electrophoresis on 0.80 % agarose gel in 1x TBE buffer (Tris/borate/ethylenediaminetetra acetic acid). Gels were stained with ethidium bromide and visualised under UV light. Each amplification reaction was performed using a single primer and repeated thrice to verify the reproducibility of the results.

S/N	PRIMER NAME	PRIMER BASE SEQUENCE
1	OPT –10	5^1 – CCT TCG GAA G –3^1
2	OPT – 05	5^1 – GGG TTT GGC A –3^1
3	OPT – 01	5^1– GGG CCA CTC A –3^1
4	OPH – 04	5^1 – GGA AGT CGC C –3^1
5	OPH – 06	5^1 – ACG CAT CGC A–3^1
6	OPH –10	5^1 – CCT ACG TCA G –3^1

Table 1: The Names and Base Sequences of the Primers used for this Study

Development of sex determinant primers

In the development of the specific markers for identifying male and female date palms, two sets of primers were employed. One set of two primers (the dominant marker) was self-designed using KU323795.1 as a reference male sex determination sequence, which was used to amplify the male sex-specific DNA target band. The second primer set was used as a positive control in the reaction. Table below documents primer details.

Primer Target		Primer sequence	Primer Name
Male determinant	Forward	CTCTTCCAATGTTTCTTTCTTGTG	Reference. Sequence
	Reverse	CTACCACTGGCTTCTGCTAAC	KU323795.1
Male/female	Forward	GCATTAGCACCATAGTAAATTGT	Positive Control
Determinant	Reverse	GTCCCAATCAGAGTGCACTCAA	

Table 2: Names and Base Sequences of the Sex Determinant Primers used on the Twenty-One Date Palm Accessions

Identification of male specific band from RAPD profile

A total of six (6) decamer oligonucleotides (RAPD primers) were used with the bulk DNA of male and female plants for selection of male-specific band. The designed male specific primer was further used with individual DNA samples of male and female plants for reproducibility testing and selection of male-specific band.

Validation of male specific band from RAPD profile

The designed primers were further used for the amplification of the genomic DNA of eight (four each) known commercial varieties namely Deglet noor and Tirgal and two accessions each of the previously determined sexes were repeated in order to authenticate the efficacy of the developed primers.

Results and Discussion

Results

RAPD-PCR amplifications and development of marker

In the preliminary study, each of the six primers showed some variations in banding patterns in the amplified fragments of the accessions of unknown male and female genotypes. Almost all of the primers provided patterns that were unrelated with sex. Furthermore, the PCR profile showed that the self designed primer combination of male determinant of Forward (CTCTTCCAATGTTTCTTTCTTGTG) and Reverse (CTACCACTGGCTTCTGCTAAC) fragments, when amplified, was able to distinguish a male-specific fragment of approximately at 200bp (base pairs). All other primers, when amplified with sex-specific fragments, could not provide clear identification of either male or female seedlings. Thus, a second combination of primers was needed to provide co-dominant markers for the identification of the both male and female individuals on a fragment of approximately 400 bp. Hence these markers could generate fragments that consisted of one banding patterns in male DNA extracts and no banding pattern in female DNA extracts.

Seedling population

An examination of gender identification for the 21 six months old seedling of date palm genotypes was observed to have a very good differentiation of male and female genotypes (Plate VIII). The analysis of the patterns indicated that there were twelve (12) male and nine (9) female seedlings within the total of 21 (Plate IX).

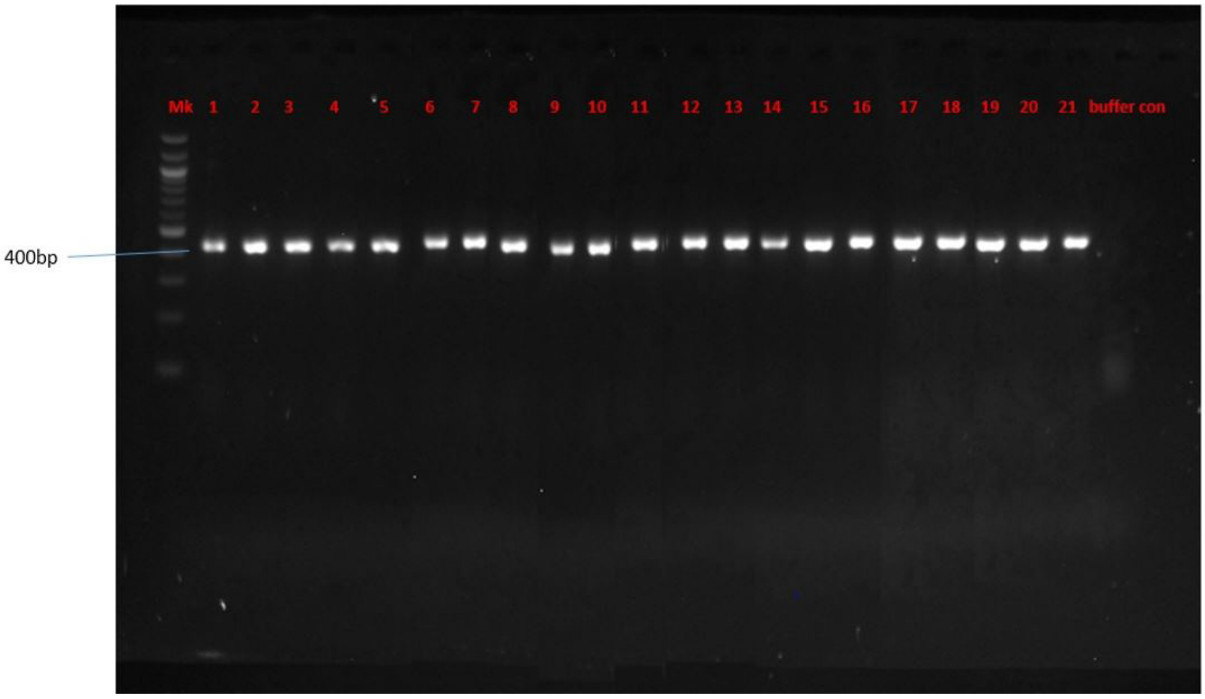

Plate I: Agarose gel electrophoresis of the PCR product of both male and female of the date palm accessions. An amplification of 400bp indicates positive amplification. This indicates good and quality extracted DNA. Loading arrangement: (1)R13P1 (2) R4P12 (3)R5P8 (4)R24P9 (5)R5P20 (6)R3P22 (7)R1P18 (8)R7P1 (9)R13P5 (10)R13P9 (11)R14P21 (12)R4P29 (13)ZARIYA (14)R16P31 (15)R2P4 (16)R9P2 (17)R6P21 (18)R9P21 (19)R5P24 (20)R5P6 (21)R1P10

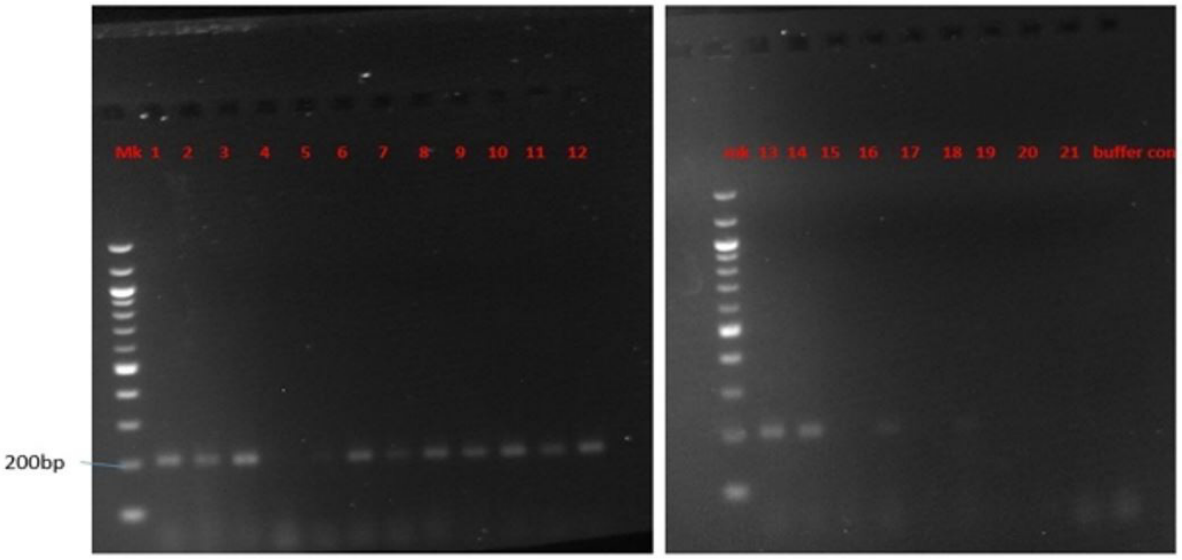

Plate II: Agarose gel electrophoresis of the PCR product differentiating male date palm seedlings from the female using male specific primers. An amplification of 200bp indicates male loading arrangement: (1)R13P1 (2)R4P12 (3)R5P8 (4)R24P9 (5)R5P20 (6)R3P22 (7) R1P18(8)R7P1 (9)R13P5 (10)R13P9 (11)R14P21 (12)R4P29 (13)ZARIYA (14)R16P31 (15)R2P4 (16)R9P2 (17)R6P21 (18)R9P21 (19) R5P24 (20)R5P6 (21)R1P10. Samples 1 2 3 6 7 8 9 10 11 12 13 and 14 tested positive to the male primers

Lane number	Male Lane number	Female lane number
1-----12	1, 2, 3, 6, 7, 8, 9,10	4, 5
	11, 12,	
13----21	13, 14.	15, 16, 17, 18, 19, 20, 21

Table 3: Seedling Population of Twenty-One Date Palm Accessions using Male Specific Primer in the DNA Amplification Procedure

RAPD-PCR amplification and validation of male-specific primers

RAPD-PCR of DNA extraction of two commercial varieties namely Ajwah and Tirgal (each with four accessions, totaling eight) and two each of the already determined sexes of the accessions were amplified and determined (Plate X), to further authenticate the efficacy of the male specific primers, the commercial varieties (Ajwah and Tirgal) and the earlier identified male and female accessions were repeated for reproducibility. All the eight commercial varieties indicated male loading while the earlier identified accessions (R5P8 and R13P1 for male while R24P9 and R5P20 for female) gave same sexes of previous results respectively, (Plate XI).

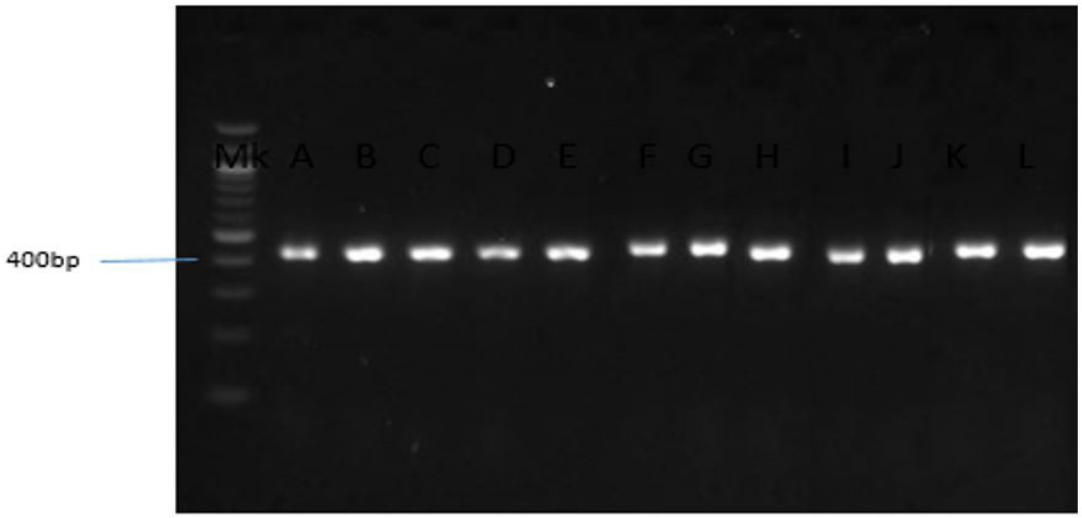

Plate III: Agarose gel electrophoresis of the PCR product of both male and female Date palm. An amplification of 400bp indicates positive amplification: (A) R5P8 (B) R13P1(C) R24P9 (D)R5P20 (E) Ajwah (F) Ajwah (G) Ajwah (H) Ajwah (I) Tirgal (J)Tirgal (K) Tirga (L) Tirgal

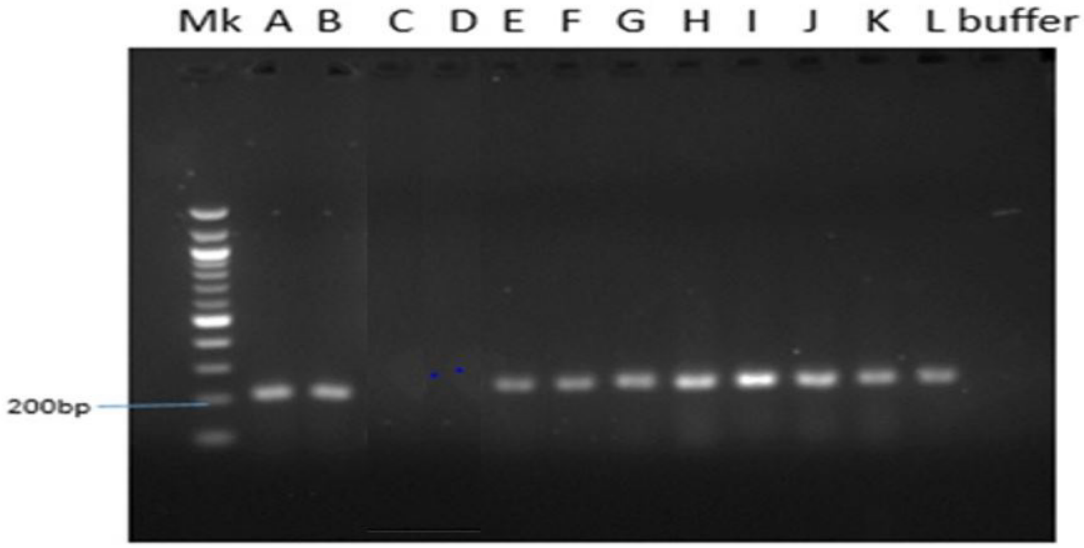

Plate IV: Agarose gel electrophoresis of the PCR product differentiating male date palm seedlings from the female using male specific primers. An amplification of 200bp indicates male loading arrangement: (A) R5P8 (B) R13P1(C) R24P9 (D)R5P20 (E) Ajwah (F) Ajwah (G) Ajwah (H) Ajwah (I) Tirgal (J)Tirgal (K) Tirgal (L) Tirgal

Lane number	Male Lane number	Female lane number
A-----L	A, B, E,F,G,H,I,J,K,L,M,N,	C, D

Table 4: Seedling population of twelve date palm accessions using male specific primer in the DNA amplification procedure

Morphological variations in male and female genotypes

Out of the twenty-one accessions studied, eight accessions, that were identified as male samples from the results of the molecular characterisation were morphologically similar. They were all observed to have a larger girth size and possess a higher number of lower leaf spines (Plates XII and XIII). In the female genotypes, however, they were observed to possess a smaller or thinner girth size with little number of lower leaf spines (Plates XIV and XV).

Plate V: Morphological identification of male accessions. Arrows in accessions R13P1 and R4P12 show large girth; arrows in accessions Zariya, R5P8, R3P22 and R1P18 showed a high number of lower leaf spines

Plate VI: Morphological identification of male accessions. Arrows in accessions R13P5 and R4P29 show large girth size; arrows in accessions Zariya, R13P9, R14P21 and R7P1 show high number of lower leaf spines

Plate VII: Morphological identification of female accessions, arrow in accessions R4P29 and R1P10 show absence of lower leaf spines; arrows in accessions R9P2 and R9P12 show smaller girth size

Plate VIII: Morphological identification of female accessions. Arrow in accession R6P20 show smaller girth; arrows in accession R15P6 indicate absence of lower leaf spines

Discussion

In Nigeria, sex determination in date palm has been a major challenge in the development of breeding programme as it has been difficult to differentiate morphologically between male and female date palm at the seedling stage (i.e. prior to first flowering). In this study, the use of sex-determinant primers were developed which has been proved as a reliable technique for sex determination in date palm. Out of a total of 21 seedlings, one from each accession, twelve (12) were observed to be male and nine (9) were female. The sex determinant primers produced positive results when tested on commercial varieties and pre-determined genotypes. The ability to identify gender by DNA methods provides a distinct advantage as it is difficult to distinguish sex in the early seedling stage from morphological differences. Sex-specific DNA markers have been used to differentiate sexes in date palm cultivars [3]. It was reported that the use of sex specific DNA markers such as RAPD primers has been successful in different varieties of date palm at seedling stage to identify sex-linked markers in the crop. A number of sex-specific DNA markers have been identified in several dioecious plants using RAPD primers [4, 5].

In this study, morphological differences observed among the male and female genotypes using molecular characterisation is an indication that morphological parameters are under genetic control. [6] reported genetic and morphological variations in the leaf of *Populus simonii* and *P. nigra* plants in China. The male-specific RAPD primer developed in this study was used to differentiate between male and female plants at the seedling stage.

Molecular marker-based techniques are reliable in determining the sex of dioecious plants as they are stable and independent of age and environment. In addition, DNA identification of male and female genotypes provides a fast and reliable approach to sex differentiation in plants regardless of reproductive age. [7] and [5], noted that SCAR marker has been used for sex-determination in many dioecious plant species in which male and female plants look similar at the vegetative stage. [8] reported the successful use of biochemical studies for gender identification of date palm plants. [9] reported the use of male-specific SCAR markers which were developed to differentiate male and female plants such as *Humulus scandens, Rumex nivalis* and *Phoenix dactylifera* using molecular marker profiling.

Findings of this study provide a baseline information needed for genetic and morphological studies of on date palm [10-13]. Dioecism and the long time taken to attain sexual maturity have led to selection programmes based on clonal propagation of females from good date palm cultivars [14-15]. This procedure promotes genetic uniformity, accelerates the process of genetic erosion and makes crops vulnerable to environmental stresses. Plant breeders may adopt sex determining markers as a potential tool for gender identification of date palm seedlings before their plantation in field.

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