

# Validation of PCR-based Markers Associated with Sex Determination in Date Palm (*Phoenix dactylifera* L.)

Zarmeena Amjad<sup>1</sup>, Zulqurnain Khan<sup>1\*</sup>, Muhammad Shan<sup>1</sup>, Plosha Khanum<sup>1</sup>, Amjad Saeed<sup>2</sup> and Gulzar Akhtar<sup>3</sup>

<sup>1</sup> Institute of Plant Breeding and Biotechnology, MNS University of Agriculture, Multan

<sup>2</sup> Department of Forestry, Range and Wildlife Management, The Islamia University, Bahawalpur

<sup>3</sup> Department of Horticulture, MNS University of Agriculture Multan

\* Correspondence: [zulqurnain.khan@mnsuam.edu.pk](mailto:zulqurnain.khan@mnsuam.edu.pk)

**Abstract:** *Phoenix dactylifera* (Date palm) is one of the essential commercial and economical fruit crops which mainly grows in hot arid areas. A good source of food, shelter, and energy, date palm is widely known due to its unique characteristics. The dioecious nature of date palm makes it challenging to predict their sex until they flower almost 5 to 8 years after plantation. Researchers have developed many strategies to determine sex, including morphological study, biochemical studies, molecular-based studies as sequence characterized amplified regions (SCAR), randomly amplified polymorphic DNA (RAPD) marker, and polymerase chain reaction (PCR) assays, but still more strives are integral. To foretell sex at the seedling stage, specific PCR primers were employed in this study. These primers were designed on sex-linked regions that determine their gender. Date palm DNA was extracted through the CTAB method and amplified through PCR using sex-linked specific primers. PCR products have run on gel electrophoresis using 1.2% agarose to score the bands for sex determination. Four primers were employed in this study to determine the sex of date palm plants. Two primers were SCAR markers, and one was a sex-linked tetra primer. Tetra-primer amplified the double band fragment of 430 and 320bp respectively in male plants, while a band of 430bp was amplified in female plants. The other primer named ALAMER1 was amplified by the single band fragment of 186bp in males with no band in females. The fourth primer, named SCARdp, amplified the band fragment of 354bp in males. These SCAR markers were established to be male-specific. Sex-linked markers provide a platform for robust, efficient, and accurate determination of sex in date palms.

**Keywords:** sex identification, gender determination, molecular markers, sex-linked marker, date palm

**Citation:** Amjad, Z.; Khan, Z.; Shan, M.; Khanum, P; Saeed, A.; Akhtar, G. Validation of PCR-based Markers Associated with Sex Determination in Date Palm (*Phoenix dactylifera* L.). *Pakistan Journal of Biochemistry and Biotechnology*, 2022, 3 (1). <https://doi.org/10.52700/pjbb.v3i1.70>

Received: October 28, 2021

Accepted: January 6, 2022

Published: June 30, 2022

## 1. Introduction

Date palm is a dioecious monocotyledonous fruit tree, i.e., male and female plants are separate, and seeds have a single cotyledon. It is an essential and economical fruit crop in hot arid areas where the humidity is relatively low and water scarcity is high. The native fruit crop of Arabian golf and Arabian conquerors introduced this fruit crop into the subcontinent. For a long time, the date palm has been supposed to be a cultivar obtained from the hybridization of two or more wild species. Recent genetic data shows that date palm is obtained from the domestication of its wild population [1]. Date palm cultivation history is still inconclusive due to finding difficulty in the status of wild or feral populations of date palm [2]. As date palm is a dioecious plant, only female trees can produce fruits, so it is more economical to cultivate them and increase the yield. Breeders have a significant problem in identifying sex at the early seedling stage so that female trees could be cultivated as only female trees can produce date fruits. Moreover, the selection of superior males is also crucial. So, sex determination at the early seedling stage is significant

as breeders face difficulty identifying gender until it reaches the age of 5, their reproductive age.

Date fruit has high nutritional value and is an imperious constituent of the diet. It contains carbohydrates (total sugar 44% - 88%), fats (0.2- 0.4%), fibers (6.4-11.5%), proteins (2.3-5.6 %), minerals, and vitamins [3]. It also contains antioxidants that play an important role in preventing cardiovascular disease [4]. Additionally, the stem and leaves of the date palm can also be used as shelter and animal food. Because of its anti-inflammatory, anti-diabetic, anti-tumor, and antioxidant properties, the date fruit has been used widely in therapeutic medicines [5, 6]. It was found that sex chromosomes existed in date palms based on cytological experiments with chromomycin dyeing [7]. Morphologically, it is not easy to distinguish male and female date palm plants at the seedling stage. However, some morphological parameters can be used in sex identification, like a flower of male and female date palm plants. Male flowers are yellow, while female flowers are white. Leaves of male date palm plants are narrow, while leaves of female date plants are more comprehensive and have more pores. Spikelet number in males is relatively high compared to the female date palm. But these parameters can be observed when the plant reaches its reproductive stage [8].

Few biochemical attributes were different in males and females and can be used as critical attributes for sex determination in date palms. In female plants, sugar content is higher than male plants. Total phenolic content in females is found to be lower as compared to entire phenolic contents in males. Myo-inositol content and amino acid content is found to be higher in female while low in the case of females. In females, Ash and proline content is lower, while relatively high ash and proline content was found in male plants. Ash and proline are essential parameters for sex determination in date palms [9]. Molecular markers are a more helpful tool for sex identification in date palms as molecular markers involve the plant's DNA. Morphological and biochemical parameters have some limitations, like the chemical composition of compounds could be changed in harsh or unfavorable conditions. Water scarcity, the harsh environment could change the chemical composition of compounds present in the plant, so biochemical and morphological parameters are not the best option for date palm sex identification. The best option for sex identification in date palms is a molecular marker. These markers could be designed so that they can attach to the sex-linked region and thus can quickly identify date palm sex [10-12].

A wide range of popular molecular markers has been in use to show the differences between male and female plants that include the RAPD [13, 14], AFLPs [15], ISSR-SCAR [16], RAPD\_SCAR [17, 18], and SSR markers [19]. SCAR markers were usually found to be male-specific [20]. These markers have been widely used and help distinguish male and female plants. In dioecious plants, sex identification is economically beneficial. To enhance *Pistacia* yield, the separation of male and female is significant. The investigator characterized the four male parents and four female *Pistacia* parents for RAPD PCR amplification. Only two primers (FPK1106 and FPK105) clearly distinguish male from female among 32 primers [21].

Sex identification of papaya seedlings through RAPD has also been carried out [22]. Date-SRY (Sex determining region Y) partial sequences were taken and amplified by nested polymerase chain reaction (PCR). The result indicates that date palm sex was identified in all plants tested. These amplified regions showed the close-match with papaya sequences. Furthermore, they designed a set of primers that amplify the SRY-gene sequence in the hopes of identifying male date palms. Using the RAPD approach, they looked at four male and four female Egyptian date palm plants. They discovered that four females of Egyptian date palm (Amhat, Siwi Zaghoul, and Samany) shared 87.5 to 89.9% of their DNA. A study revealed that the banding pattern suggests that four females and

three males are genetically connected [23]. That is why, in the current investigation, we employed sex-linked specific primers.

## 2. Materials and Methods

### 2.1. Specimens' collection

Seedling specimens were collected from cultivated date palm varieties after two months of seed germination. These seedlings were then stored at -88 degrees for future use.

### 2.2. DNA extraction

DNA of date palm extracted through CTAB method [24]. Take the 200mg of date palm leaf sample and grind it in 600µl of CTAB buffer until a fine paste. Transferred the fine paste into a microfuge tube and added 50µl of beta-mercaptoethanol into the tube. The mixture was incubated for about 45 minutes at 65°C in a water bath. After incubation, centrifuged the sample mixture in a centrifuge machine at 14,000rpm for 10 minutes. Took the supernatant, transferred it to a clean microfuge tube, added 400µl Chloroform: Iso Amyl Alcohol (24:1), and mixed it with gentle inversion. After mixing, centrifuged the tube for 5 minutes at 14,000rpm. Transferred the upper aqueous layer to a clean microfuge tube, which contained the DNA. Added the 400µl of ice-cold/chilled ethanol into a microfuge tube. After it, gently inverted the chilled ethanol containing microfuge tube 3-4 times so the DNA could be precipitated and keep them at -20-degree temperature for 1 hour. After 1 hour, centrifuged the chilled DNA sample at 14,000rpm for 30 seconds, discarded the supernatant, and kept the DNA pellet. Washed the DNA pellet with 200µl of 70% ethanol and centrifuged it at 14,000rpm for 10 minutes. Removed all the supernatant and let the DNA dry for approximately 10 minutes. Added 20µl of autoclaved distilled water into each microfuge tube and spun it for 1-2 minutes to completely dissolve the DNA and then store them at -20 degrees.

### 2.3. DNA quantification

Nanodrop was used to quantify the isolated genomic DNA. DNA quality was checked by running all DNA samples on 1% agarose gel. DNA of good quality was selected for PCR assay. The stock purified DNA was used to make working dilutions of DNA samples with heavy bands of > 200ng of 15ng/L.

### 2.4. PCR amplification

Four primers were used for amplification, and their sequences were listed in table 1. PCR amplification was performed with extracted genomic DNA of date palm seedlings.

#### 2.4.1. PCR assay for markers

The first step of the PCR program is denaturation which was carried out at 94°C for 8 min, which was followed by 36 denaturation cycles at 94°C for 1 min, the third step was annealing at 56°C (SCARdp), 58°C (ALAMER1) and 56-58°C (Tetra) for the 30s, the fourth step is a 1:30s extension at 72°C, followed by a 5-minute extension at 72°C. Then on 1.5% agarose gel, amplification products were separated.

**Table 1:** List of primers used for the sex determination of date palm.

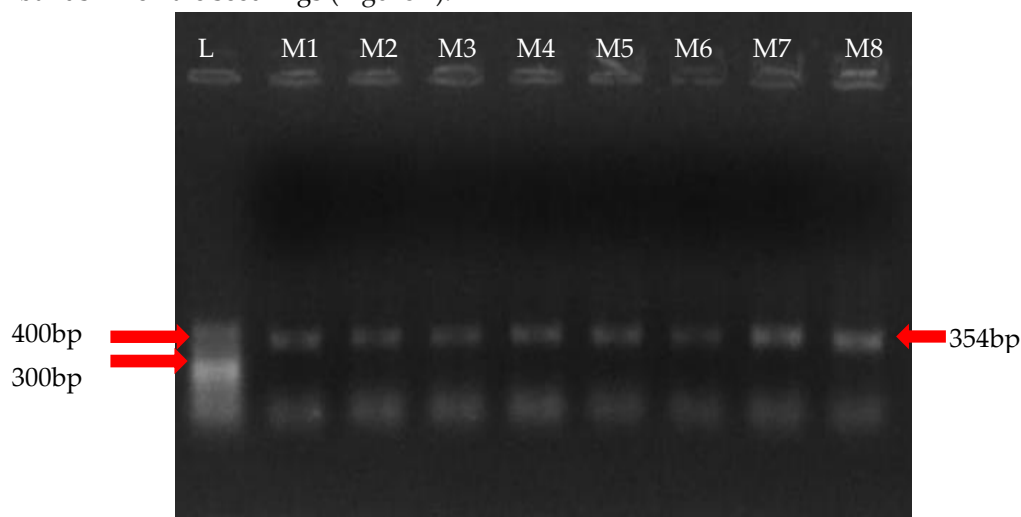
Primer Name	Primer Sequence	Tm	References
ALAMER1F	CGTGGGATGAGGTAGTTTGG	62.4	[25]
ALAMER1R	CTCGCGATGCAAACCAACCAA	60.4	[25]
SCARdpF	TTTTGGGCTTGTCTAGCATC	58.4	[17]
SCARdpR	GTTCTGCAAATTAAGAGAAAAGGT	58.3	[17]

1F	GCATTAGCACCATAGTAAATTGT	57.4	[26]
1R	GTCCAATCAGAGTGCCTCAA	62.7	[26]
2F	GCAATAGCACCATAGTAAATTGCCTA	61.4	[26]
2R	CGCTAACTTGGTGCACGGATCTCT	66.3	[26]

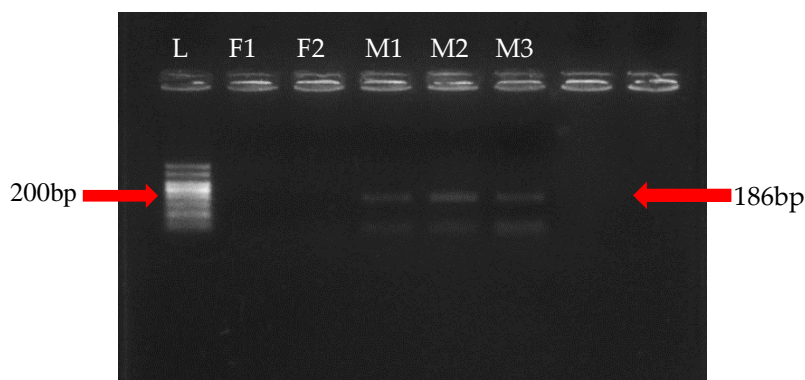
### 3. Results

#### 3.1. Validation of SCAR markers

Two pairs of SCAR markers were validated in this study. One was SCARdp, and the other one was ALAMER1. The SCARdp (SCARdpF 5'-TTTTGGGCTTGTCTAGCATC-3' and SCARdpR 5'-GTTCGCAAAATTAAGAGAAAAGGT-3') produced the single, distinct band of 354bp in male plants (Figure 1). The other SCAR marker, ALAMER1 (ALAMER1F 5'-CGTGGGATGAGGTAGTTTGG-3' and ALAMER1R 5'-CTCGCGATGCAAACCAACCAA-3), produced the band of 186bp in male plants with no bands in female seedlings (Figure 2).



**Figure 1:** Profile of male date palm seedlings using SCAR primer pair. 354bp fragment is amplified in male plants. Lane M1-M8 represents all male plants. Lane L represents a 50bp DNA ladder.

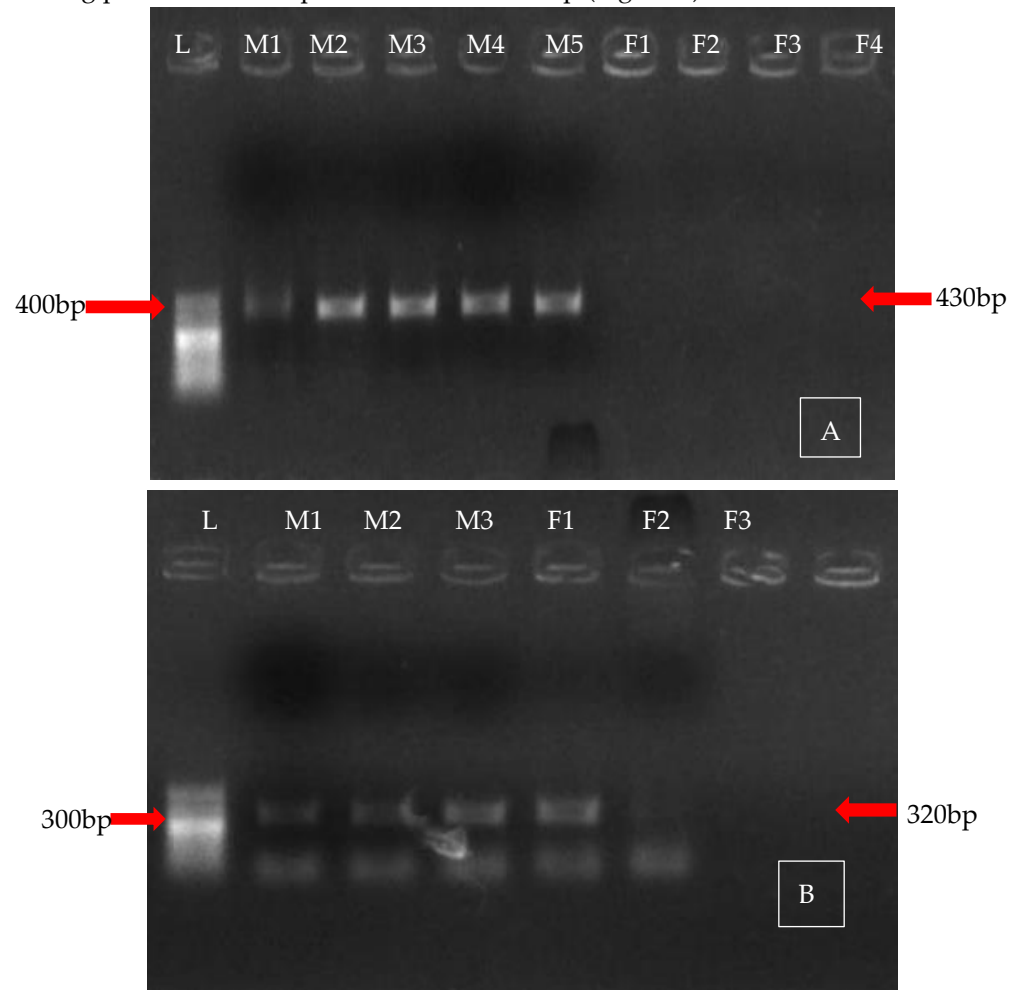


**Figure 2:** Using the newly developed ALAMER1 marker, date palm seedlings were screened for male and female plants. The expected male band length is approximately 186bp fragment. Male seedlings were identified by the presence of a band, whereas the absence of a band identified female seedlings. Lane F1-F2 has no band amplification,

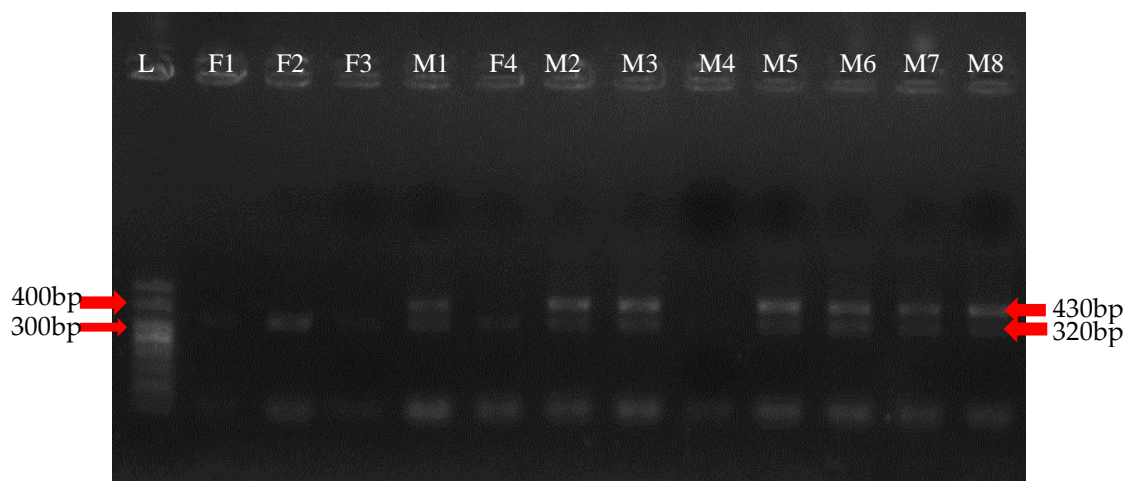
representing it as female, while in lane M1-M3, a band of 186bp was amplified. Lane L represents a 50bp DNA ladder.

### 3.2. Validation of Tetra-prime

Tetra-primer were validated in this study to determine sex at the seedling stage. The first pair of tetra-primer 1F-1R produced a clear band of 430bp in males with no bands in female seedlings (Figure 3A), while the other pair 2F-2R produced the band of 320bp in both male and female plants (Figure 3B). When these primers were used in the tetra-primer form, they produced the single, clear band of 320bp in female seedlings with double banding patterns in male plants of 430 and 320bp (Figure 4).



**Figure 3:** 1F-1R and 2F-2R primers were used to create PCR analyses for sex identification in date palm. **A:** initial primer pair (1F-1R) that was utilized to distinguish the sex of date palm. The male-specific segment is roughly 430bp long, while the female does not have any bands. Lanes M1-M5 represents male plants with an amplified fragment of 430bp. **B:** PCR analyses of the other primer group 2F-2R were utilized to distinguish the sex of date palm. The length of the DNA band, which is around 320 bp, might be used to identify both the sex of date palm as a positive DNA banding pattern. L represents a 50bp DNA ladder.



**Figure 4:** PCR analyses for female and male specimens using the 1F-1R and 2F-2R primers combined with tetra-primers. The male specimen highlighted the dual-band, but the female specimen expressed a monoband. Lane L represents the 50bp DNA ladder.

#### 4. Discussion

Sex determination is an essential and fundamental developmental process of all sexually propagating plants and has high economic value. Sexual characteristics dictate the cultivation and breeding methods of commercial crops. The early date palm sex characterization is crucial for commercial production because commercially female plants are of high economic value as they bear the fruits. A well-balanced sex ratio is required for a productive date palm farm. Male-associated markers are relatively prevalent in plants like *Phoenix dactylifera* and cannabis Sativa, which is not surprising. Markers indicating maleness in plants when sex has not been identified suggest the presence of either sex chromosomes that have not been differentiated by cytological procedures or genes involved in sex characterization. The conventional mean of sex identification includes the developed flower characteristic analysis at the mature stage. Flowering in date palm may take five years, which is quite a long time. Date palm spathe morphology and inflorescence pattern can differentiate but only in mature plants. The male spathe is wider and swollen but is small as compared to the female. Female spathes have more uniform characteristics. The male flower contains influential petals and stamens and a sweet and pleasant scent. Female plants have a stigma but no petals or stamens, whereas male plants do [26]. So, morphologically, there is no apparent difference to distinguish whether the plant is male or female at an early age. Moreover, the maturation age of date palm is almost 5 years, and we cannot determine its sex till it becomes a mature plant and bears fruit. Economically, the female date plants are vital as they bear the fruit and are more valuable for breeders and cultivators. So, for breeders' sake, it is important to distinguish both sexes at an early seedling stage to save resources and produce female fruit-bearing plants. As morphologically, we cannot distinguish, so these laboratory-based techniques are helpful.

SCAR markers are reported to distinguish sex and are used in molecular profiling. In *Hippophae rhamnoides*, males and females are distinguished using SCAR markers [13]. As male and female date palms' morphology is the same, these SCAR markers herein developed can determine sex. The SCAR marker is male-specific and is present in male plants but absent in female plants. One of the SCAR markers validated is SCARdp. In Figure 1, a fragment of 354bp length was obtained in all-male plants. Our result follows [17], who developed this marker and used this for sex identification in date palm seedlings. This study used a specific SCAR marker (ALAMER1) to evaluate sex at the early seedling stage. The marker differentiated the male and female seedlings based on male-specific having fragment length of 186bp fragment. This marker showed reproducible results and identified the male and female samples. In Figure 2, lane F1-3 represents a female plant with no

amplification, while lane M1-3 shows a band pattern of 186bp fragment. This result was supported by the work [25]. In this study, we used a sex-linked primer which was a tetra-primer. This primer includes two primer pairs, 1F-1R and 2F-2R. The initial primer pair (1F-1R) was utilized to distinguish the sex of date palm in Figure 3a. The male-specific segment is around 320bp long, while the female has no bands. In the second figure 3b, PCR analyses of the other primer pair 2F-2R, which was utilized to distinguish the sex of date palm, revealed a 400bp band fragment in both males and females. The predicted dual band is shown in male specimens, while in female specimens, the predicted monoband is seen (Figure 4). The double bands were 400 and 300bp length, while the single band was 400bp length. This result is the following reported results which showed the same band pattern using this marker. The findings of [26] confirmed our results.

### Conclusions

Finally, the SCAR marker and tetra primer developed might identify gender in date palm seedlings. It will save time, cost, and sources as the reproductive age of the plant are 5-7 years. As a result, plant breeders may use this technology as a potential gender identification tool.

### Endorsement

Dr. Sultan Habibullah Khan and Dr. Aftab Ahmed, Center for Advanced Studies in Agriculture and Food Security (CAS-AFS), University of Agriculture, Faisalabad (UAF), Pakistan, and Dr. Sadia Bushra (CABB-UAF), provided technical assistance and guidance throughout this study.

### Finance

PI (Zulqurnain Khan) used his personal finances to complete the project.

### Interest conflicts

There are no conflicts of interest declared by the authors.

### Ethics confirmation

This article does not include any experiments with humans or animals conducted by any authors.

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