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# Rapid and reliable protocol for the DNA extraction and identification of gender in Date palm seedlings

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## Abstract

Date palm is a dioecious species, bearing fruits in the female plants only. Identification of gender at the seedling stage can enhance productivity by transferring only female plants to the field from the nursery. For this purpose male-specific region in the genome is amplified using polymerase chain reaction (PCR), following DNA extraction. In such an investigation where the sample size is large, the maximum chances of errors occurring are at the DNA extraction step. Although many protocols for the extraction of DNA are available, their drawbacks are either a higher turnaround time, resources utilized or the purity for downstream processes. Therefore, we have optimized the DNA extraction method from the hard tissue material that allows the assessor to extract the DNA in a relatively shorter time period, with less resources utilized, and reliable for downstream processes like PCR. The efficiency of the optimized DNA extraction method in the present investigation was assessed using 225 Date palm seedlings, from which DNA was extracted, followed by gender identification using PCR. Here, we propose a hassle-free protocol for the DNA extraction and PCR amplification in Date palm cultivars. Out of 225 samples, 106 samples were found to be females (47.11%), and the remaining 119 samples (52.89%) were males. The proposed protocol can be utilized for the large-scale gender identification in Date palm seedling using less time and resources.

**Key words**: *Date palm, Dioecious plants, DNA test, Gender identification*

## Introduction

Date palm (*Phoenix dactylifera* L.) is a member of Arecaceae family bearing male and female flowers on separate plants. It is a monocot fruit tree with distinctive anatomical feature and a great degree of adaptation to different environments (Krueger, 2021). It is cultivated in tropical and sub-tropical regions of the world which includes Egypt, Saudi Arabia, Iran, Algeria, Iraq, Pakistan, Sudan, Oman, U.A.E. and Tunisia are top 10 date producing countries (FAOSTAT, 2022). In India, the tree is widely cultivated in Rajasthan and Gujarat (Pareek, 2015). Dates are rich in carbohydrates and dietary fibre (Benmeziane-Derradji, 2019), while seeds also provide carbohydrates, oil, fibre, and protein (Alkhoori et al., 2022). Fruits grow only on female plants, which flower after a 4–8-year juvenile phase. Early gender detection is crucial to save resources otherwise spent on male trees. DNA based molecular markers are not influenced by environmental factors, stage of the plant or plant parts, and therefore are more reliable than biochemical and other morphological markers. Sequencing of the male specific region in Date palm cultivars revealed potential region from which markers can be designed and used for the identification of the gender. Two of such markers (male specific and gender neutral) were identified (Torres et al., 2018). This marker system has been used to identify sex in Date palm seedlings (Jani et al., 2018). Other researchers have used different markers which are also gender determining (Intha and Chaiprasart, 2018). A list of sex-linked molecular markers including Random Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplified Region (SCAR), Simple Sequence Repeat (SSR), Start Codon Targeted (SCoT) markers, Restriction Fragment Length Polymorphism (RFLP), and Amplified Fragment Length Polymorphism (AFLP) has also been reviewed (Naqvi et al., 2021).

DNA-based identification of gender requires DNA isolation, and PCR amplification with the male-specific and gender-neutral markers. The procedure for the isolation of high-purity DNA is a fundamental step and requires the involvement of several resources and time which becomes limiting factor when considerable number of plants are handled. Few manufacturers have developed a direct protocol for the DNA extraction from the leaves followed by amplification of the DNA using modified polymerase which is resistant to many PCR inhibitors secreted from the plant cells. Such kits have disadvantages of being costly and providing less quality and quantity of DNA that restricts the downstream applications. Alternative to that, widely used extraction method have several steps which demands time in order to get a DNA with high purity (Modi et al., 2017). However, the time can be reduced in such a way that final quantity and quality of the DNA are not compromised substantially. In the present investigation, we have optimized the protocol for the DNA extraction from Date palm seedlings considering time, cost, resources and reliability for the large-scale gender identification.

## Material and Methods

*Plant material*

Seedlings of local cultivar of Date palm were raised in the Farmer's Nursery in Mankuva village of Kachchh district, Gujarat, India (23°13'10.5"N, 69°31'43.4"E). A total of 225 seedlings were raised till 5-6 leaves were emerged (Figure 1), which took nearly 6-7 months. Seedlings were tagged and 3-4 cm long leaf was cut with the razor blade and placed in a sampling bag. After sampling, the leaves were immediately transported to the laboratory under cooling conditions.

*DNA extraction*

Isolation of total DNA was carried out using cetyl trimethyl ammonium bromide (CTAB) method proposed by Modi et al. (2017) for the clonal fidelity testing in Date palm. However, minor modifications were made. These modification steps are mentioned briefly in Table 1. Entire process of the DNA extraction is summarized graphically in Figure 2.

**Table 1: Modifications in the method used by Modi et al. (2017) for DNA extraction from Date palm leaves**

| Sr. No. | Parameters/ Steps | Method used by Modi et al. (2017) | Current Method |
|---|---|---|---|
| 1 | Crushing | Used liquid nitrogen to make fine powder | Done without liquid nitrogen and crushed using 1 ml tip after chopping |
| 2 | Incubation | 65℃ for 60-90 minutes | 65℃ for 10 minutes |
| 3 | Chloroform: Iso-amyl alcohol wash | Two times | Single time |
| 4 | After precipitation of DNA with 100% ethanol | 3 Hours of incubation | No incubation |

*PCR, gel electrophoresis and analysis*

PCR reaction was carried out with 2 sets of primers as mentioned in Jani et al. (2022). Master-mix was prepared by taking 10.0 µl of 2X PCR master-mix (EmraldAmp MAX PCR Mastermix, Takara); 0.2 µM of gender specific primers and 0.05 µl of gender-neutral primers (as final concentrations); 1 µl of isolated DNA; and nuclease free water to make the final volume to 20.0 µl. The PCR reaction was conducted by initial denaturation at 98℃ for 1 minute followed by 30 cycles of denaturation, annealing and extension at 98, 58 and 72 ℃ for 10 seconds, 20 seconds, and 30 seconds, respectively. Final extension step was carried out at 72℃ for 1 minute in a T100 Thermal Cycler (Bio Rad). All the products were run in a Mini-sub cell GT cell electrophoresis apparatus (Bio Rad), 1.5% of agarose was dissolved in 1X TBE buffer (89 mM of Tris-Cl, pH 8.0; 89 mM of Boric acid and 2mM of Ethylene diamine tetra acetic acid as final concentrations). To this gel, 10 µg of ethidium bromide (per 100 ml) was added and distributed evenly throughout the gel. The master-mix was pre-loaded with loading and tracking dyes therefore the samples were directly loaded on the well.

**Figure 1: Nursery-raised, 6-7 months old, Date palm seedlings at the time of sample collection**

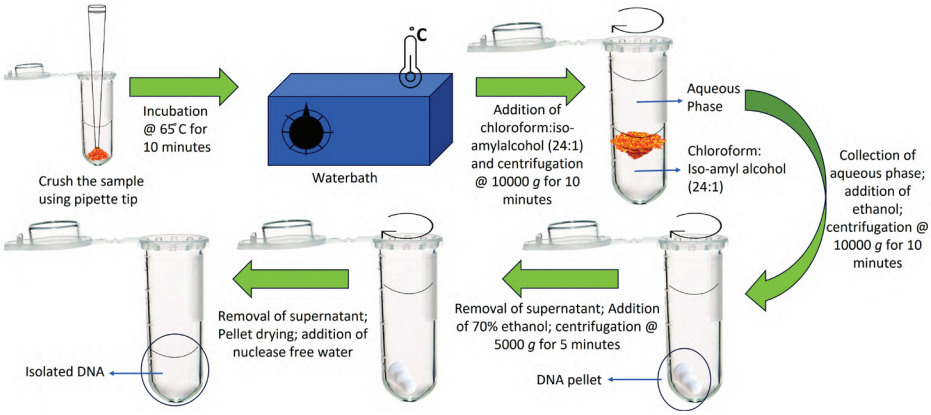


**Figure 2: Schematic representation of the DNA extraction procedure from the leaf sample of Date palm**

Gel was analyzed using ChemiDoc Transilluminator (Bio Rad). Two sets of primers *viz.*, male specific (Torres et al., 2018) and internal control (Jani et al., 2022) were used. A product of around 150 bp was the indicator of success of PCR reaction (gender neutral product) and a product of around 450 bp was an indicator of male specific amplification. Therefore, samples containing two amplicons (150 bp and 450 bp) were considered as male and the sample containing only single amplicon (150 bp) were considered as female samples. All the samples were PCR amplified in four batches (Batch 1: 1-60 samples; Batch 2: 61-121 samples; Batch 3: 122-178 samples; and Batch 4: 179 to 225 samples) and for each batch, no template control (N) and positive control (P) were also run. Nuclease-free water and the DNA from confirmed male sample were used for N and P, respectively.

## Results and Discussion

DNA extraction step in the present investigation is very crucial process and further analysis with PCR depends on the quantity and quality of the DNA. Crushing of the casually chopped samples made the extraction solution slightly green which is an indication of release of cellular constituents like pigments and other biomolecules. Further confirmation of the release of nucleic acids was done when addition of the absolute ethanol was conducted. Few bubbles along with the small threads were visible. After centrifugation (ethanol precipitation step), an exceedingly small amount of white pellets were visible which were completely dissolved in nuclease free water. The protocol was modified to reduce the time of incubation at two different steps, i.e., at the CTAB extraction step; and at the alcohol precipitation step. This modification made the DNA extraction procedure rapid.

Gel electrophoresis was conducted to visualize the PCR product(s) as 150 bp (in both the genders) and 450 bp (in males only). All the samples showed 150 bp products which indicated that PCR was successfully run. Intensities of these amplicons were ample which showed that lower concentration of the gender-neutral primers is enough to amplify the DNA. A total of 119 samples showed 450 bp product which indicated male samples.

**Table 2: Tabular representation of the difference between the optimized protocol and the other available protocols/ products for DNA extraction. Cost mentioned here is calculated based on the current market price.**

| Parameters | Method used by Modi et al. (2017) | DNA extraction kit from different reputed brands | Direct Plant PCR kit | Optimized protocol |
|---|---|---|---|---|
| Quantity (ng/µl) | 100 – 500** | 100 – 3000** | Cannot be determined | 20 – 100* |
| Quality ($A_{260/280}$) | 1.8 to 2.0** | 1.8 to 2.0** | Cannot be determined | 1.6 – 1.8* |
| Downstream applications | PCR, RFLP, Loop Mediated Isothermal Amplification (LAMP) | PCR, RFLP, LAMP, Sequencing | PCR | PCR, LAMP |
| Turnaround Time (TAT) per sample | 6 Hours | 2-3 hours | 10-20 minutes | 1.5 hours |
| Numbers of steps involved | 14 | 16 | 3 | 12 |
| Cost (as on 10th December, 2025)*** | ~ ₹. 50.00 | ~ ₹. 300.00 | ~ ₹. 130.00**** | ~ ₹. 35.00 |

* These parameters were not tested in all the samples, however the range was determined by checking random samples.

** From the available literature or from the claims made by reputed brands

*** Cost of wear and tear of the instruments, electricity and manpower were considered the same for all the protocols, and therefore, excluded.

**** The cost includes up to PCR step.

Intensities of male specific amplicons were adequate to conclude about the gender. These results showed that 47.11% and 52.89% samples were found to be females and males, respectively (Figure 3). These values revealed that the gender ratio was observed to be nearly 50:50. Moreover, no repetition of samples for DNA extraction as well as PCR were required. Therefore, the proposed protocol for the gender identification in Date palm cultivars is highly dependable. Jani et al. (2025) also identified 280 seedlings for the gender from across different regions of nearby locality and found that the primers are 100% reliable for the early sex determination in Date palm.

## Conclusion

The present investigation was conducted to optimize the reliable protocol for the NDA isolation from hard tissue like Date palm for the identification of gender using molecular markers. The accuracy in CTAB method for the DNA extraction is remarkably high but faces several bottlenecks such as time consuming and carry over contamination as far as gender identification in enormous number of samples are concerned. We targeted different steps of DNA extraction and PCR amplification to make the protocol rapid and more reliable without error. Several steps bring high quantity DNA provided the time required for the incubation was given. We reduced the time and compromised the DNA quantity in our protocol.

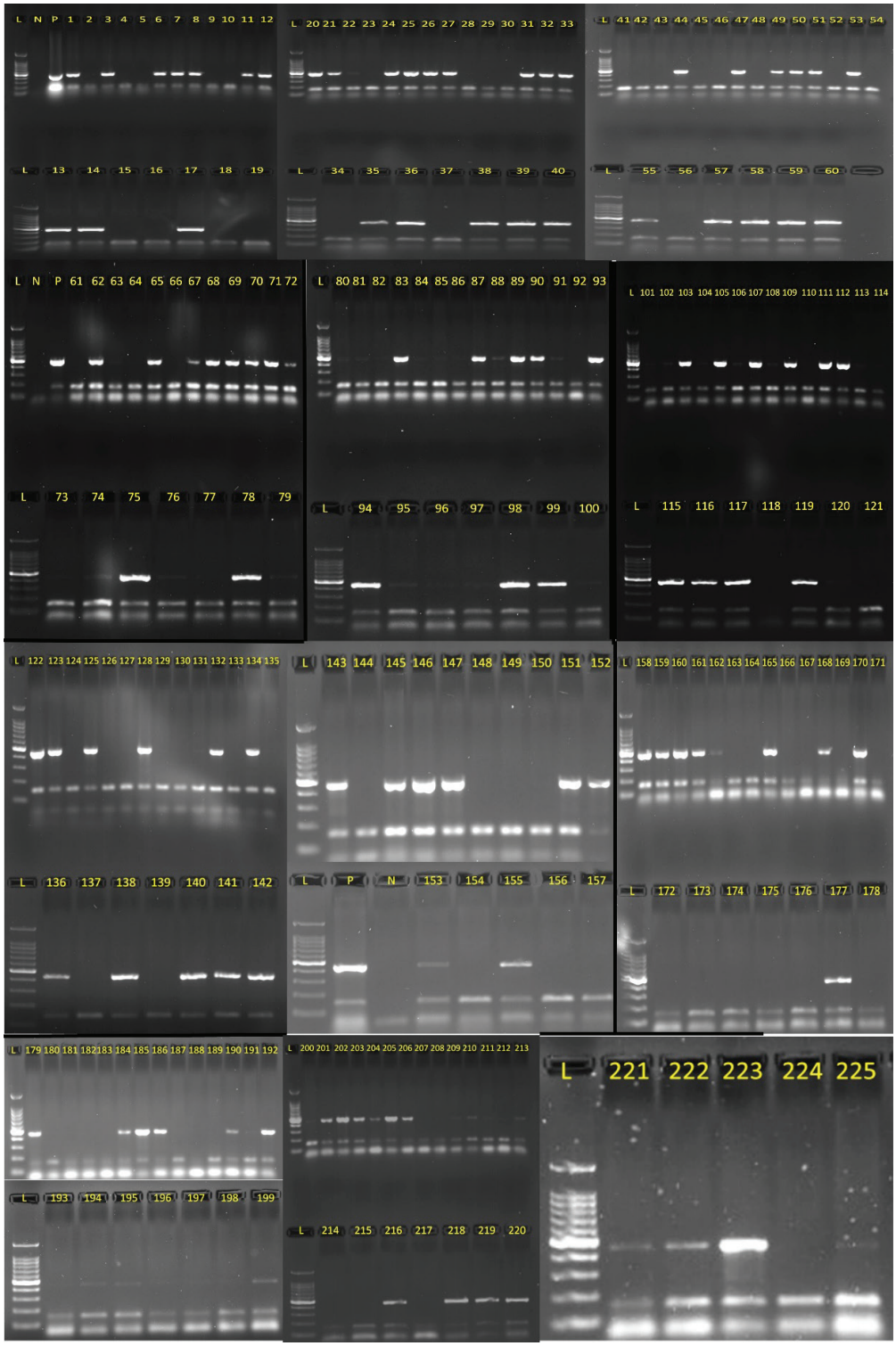


**Figure 3: Visualization of gender neutral and male specific amplicons in Date palm samples on 1.5% agarose gel (L – 100 bp ladder; N – No Template Control; P – Positive Control).**

In the present investigation, we did not check the quantity and quality of the DNA extracted. However, all the samples showed amplicons and gender prediction could possible in a single attempt, which implied that quality control step, at least for the PCR based assays, can be skipped. Ideal ratio of male: female samples in seedlings is considered as 50:50 and we have achieved the same ratio as 53:47 which is nearly 50:50. Minimizing the incubation time in different steps of DNA extraction and assurance of no carry over contamination of male samples along with amplification makes the protocol rapid and reliable.

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