Asian Journal of Agriculture and Biology
https://doi.org/10.35495/ajab.2024.253

Original Article

An RT-RPA–CRISPR/Cas12a-based assay for robust sexual Identification of dioecious plants

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Received: 01 December 2024 / Accepted: 23 January 2025 / Published Online: 22 February 2025

Abstract

Dioecious plants, like Date palm (*Phoenix dactylifera*) and Jojoba (*Simmondsia chinensis*), are a type of plant that do not have sexual identification in the same way that animals or humans do. Sexual identification in plants typically involves determining the sex of individual plants within a dioecious species. Date palms and jojoba are dioecious, with male and female trees, and their sexual identification is important in agriculture for proper pollination, but until now it doesn't involve methods like RPA (Recombinase Polymerase Amplification) or CRISPR. We successfully developed a CRISPR/Cas12a-based fluorescence test for the sexual identification of date palm and jojoba as models for dioecious, with RPA and CRISPR-Cas12a, which could be used as a simple, accurate, and fast detection method in agricultural fields due to its advantages of high-sensitivity. This investigation uses our proprietary technology to detect sub-kilobase non-repetitive genomic loci, CRISPR-FISH. In this study, we designed 16 different sgRNAs using full-length Human SRY as the substitute target in date palm and jojoba, given the extremely high homology between Human SRY and Date Palm SRY gene. These sgRNAs were labeled them 3 Atto565 dyes per sgRNA to visualize them at a single-cell level. Fluorescence in situ hybridization (FISH) has been employed as a potent and practical method for directly identifying certain DNA segments inside the genome. In this method, plant chromosomes are physically mapped using ribosomal DNA genes (45S and 5S rDNA) as markers to examine genomic organization. The initial result for SRY staining in a substitute cell line, the Human A549 cell, a male cell line containing a single copy of SRY was promising albeit with high background staining. Prominent dots (red) in the nuclei region were observed, but with high background staining in both nuclei and cytosol. This refined technique is perfect for quickly localizing tiny DNA segments and single-copy genes. Additionally, PRINS is a quicker and less expensive option than FISH. Furthermore, the successful application of the assay for Sexual identification of some Dioecious plant samples highlighted its potential for rapid and accurate sex detection in agricultural settings. In summary, this RPA-CRISPR/Cas12a diagnostic method offers a potentially valuable technological solution and management for Dioecious plant sex-determination at very early stage.

Keywords: Dioecious plants, Date palm; Jojoba; female; male; sex determination; FISH; CRISPR/Cas12a, RPA-CRISPR/Cas12a

How to cite this article:

Solliman MED and Mohasseb HAA. An RT-RPA–CRISPR/Cas12a-based assay for robust sexual Identification of dioecious plants. Asian J. Agric. Biol. 2025: 2024253. DOI: https://doi.org/10.35495/ajab.2024.253

Introduction

In dioecious plants like date palm and jojoba, there are separate male and female plants. Identifying the sex early in the plant's life cycle is critical, as only one sex may be economically valuable. For instance: Date palms only female trees produce edible fruit. While jojoba female plants are favored for seed production due to the high-value oil content. Traditionally, sexual identification is done later in development when flowers or sex-specific traits become apparent, which can be costly and time-consuming. The development of a robust and sensitive diagnostic method is of utmost importance to control the sexual identification of *Phoenix dactylifera* plants within a dioecious species (species with separate male and female plants),so to determine the sex of a date palm tree, you would use traditional botanical methods like visual inspection of reproductive structures, rather than advanced molecular techniques like PCR and other molecular methods might come into play when researchers are working on breeding programs to select for desirable traits, and for basic sexual identification of the plants (Solliman et al., 2023a, 2023b). Reverse transcription followed by PCR (RT-PCR), polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), loop (or RT-loop) mediated isothermal amplification (LAMP and RT-LAMP), and microarrays are just a few of the techniques that have been developed (Baranwal et al., 2020; Hadidi, 2019; Hadidi et al., 2020; Rubio et al., 2020). According to Clark and Adams (1977), ELISA is dependent on the binding of virus-specific antibodies to the virus coat protein.

RT-RPA (Reverse Transcription-Recombinase Polymerase Amplification) is a rapid, isothermal amplification method that can process samples without requiring high-end lab equipment. Unlike PCR, RPA operates at a consistent temperature (typically 37–42°C), making it portable and field-friendly. Cas12a, a CRISPR-associated protein, has a collateral cleavage property that, upon binding to its target sequence, will cut nearby non-target DNA or RNA. This property is harnessed with fluorescent probes to produce a visible signal when the target sequence is present, allowing for sensitive detection. The natural immune-like system that protects bacteria and archaeal species from foreign invaders through molecular immunity is the source of clustered regularly short palindromic repeat-associated systems, or CRISPR-Cas. In eukaryotic species, CRISPR-Cas has been widely used for transcriptome regulation, genome engineering, and molecular immunity (Ran et al., 2013; Hsu et al., 2014; Ali et al., 2015; Aman et al., 2018; Mahas et al., 2019). Target identification and cleavage in a few CRISPR systems activate their non-specific endonuclease activity, which has been used to identify nucleic acids (Aman et al., 2020). For example, the popular nucleic acid detection platforms DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) and SHERLOCK (Specific High-sensitivity Enzymatic Reporter UnLOCKing) use the collateral activities of Cas13 and Cas12, respectively, when using ssRNA or ssDNA reporters (Chen et al., 2018; Gootenberg et al., 2018; Li et al., 2018a, 2018b; Kellner et al., 2019). Numerous studied have reported that biotin- or fluorophore-labeled can be used to generate endpoint or quantitative measurements. LAMP and RPA techniques have been successfully integrated with CRISPR/Cas technology for rapid and portable viral nucleic acid detection (Kellner et al., 2019; Broughton et al., 2020). To enable sensitive and rapid detection of SARS-CoV-2, the causal agent of COVID-19, we recently developed the in vitro Specific CRISPR-based Assay for Nucleic Acids detection (iSCAN), which combines the specific detection and subsequent collateral activity of CRISPR/Cas12 with target sequence amplification via RT-LAMP (Guo et al., 2023; Ali et al., 2020; Xiong et al., 2020; Chen et al., 2018; Zetsche et al., 2015). However, due to their complexity and inefficiency, these techniques have typically not been used to identify T-DNA insertion sites. Sequences that fall under predetermined limits can be amplified using conventional PCR. To amplify DNA sequences that flank regions with known sequences, several techniques have been developed, including unpredictably primed PCR (Chen et al., 2018; Zetsche et al., 2015), Inverse PCR (Triglia et al., 1988; Ochman et al., 1988), and targeted gene-walking PCR (Parker et al., 1991; Solliman et al., 2019). Sequences that fall under predetermined limits can be amplified using conventional PCR. To amplify DNA sequences that flank regions with known sequences, several techniques have been developed, including unpredictably primed PCR (Dominguez and Lopez-Larrea, 1994), Inverse PCR (Triglia et al., 1988; Ochman et al., 1988), and targeted gene-walking PCR (Parker et al., 1991; Solliman et al., 2019, 2023b).

To identify the sex of a date palm tree, one has to examine the reproductive structures of the tree. Female date palms produce fruit (dates), while male

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date palms produce pollen. Male flowers tend to be smaller and have different characteristics than female flowers. Experienced growers can identify the sex of date palms based on these visual cues. While CRISPR technology can be used in plant breeding to modify specific genes or traits, it's not used for sexual identification. Techniques like DNA fingerprinting or genetic markers can be employed to confirm the sex of a date palm, but this is typically done for research or breeding purposes. RPA (recombinase polymerase isothermal amplification) is rapidly impacting the field of molecular diagnostics. Since its invention, the technique has been used to detect microorganisms associated with humans and veterinary, food industry, and agricultural fields. Because RPA is currently working effectively as an alternative to polymerase chain reaction, other platforms can be combined with RPA to yield improvements over the advantages of high sensitivity and isothermal reactions (Chen et al., 2018; Zetsche et al., 2015). After specifically recognizing and cleaving the target sequence, some CRISPR/Cas systems, such as Cas13, Cas12a, and Cas14, exhibit incidental nonspecific side-cutting activity and have been developed for nucleic acid detection. For example, by cutting fluorescently labeled nucleic acids to generate fluorescent signals, CRISPR-based diagnostic procedures have been successfully applied to a variety of bacteria and viruses and have good prospects (Guo et al., 2023; Xiong et al., 2020). The final finding of this studies can be utilized and used as a cytological marker and Sexual identification of some dioecious plant samples to differentiate between male and female trees at an early stage.

Material and Methods

Experimental design

CRISPR-Cas12a (Cpf1) proteins are RNA-guided enzymes that bind and cut DNA as components of bacterial adaptive immune systems. Like CRISPR-Cas9, Cas12a has been harnessed for genome editing based on its ability to generate targeted, double-stranded DNA (dsDNA) breaks. CRISPR-associated (Cas) nucleases have transformed the area of genome editing in recent years. Cas9 and Cas12 provide previously unheard-of versatility by using an RNA guide to cleave double-stranded (ds) DNA targets close to a brief sequence known as a protospacer adjacent motif (PAM). More compact versions would ease delivery and broaden use, though. Here, we demonstrate how Cas12a's indiscriminate single-stranded DNA (ssDNA) cleavage activity is released by RNA-guided DNA binding, ultimately leading to the total degradation of ssDNA molecules (Figure 1). We find that other type V CRISPR-Cas12 enzymes also exhibit target-activated, non-specific ssDNase cleavage. By combining Cas12a ssDNase activation with isothermal amplification, we use a method termed DNA Endonuclease Targeted CRISPR Trans Reporter, which achieves point-of-care testing and attomolar sensitivity for DNA detection according to Guo et al. (2023); Xiong et al. (2020); Chen et al. (2018); Zetsche et al. (2015).

According to the sequence information provided and GenGank database (*Phoenix dactylifera* SRY related sequences: GenBank: KJ873056.1 and GenBank: KC577225.1)., we analyze the genome sequence of *Phoenix dactylifera* SRY. As is shown in Figure 2, the location distribution of the two sequences on the same genome.

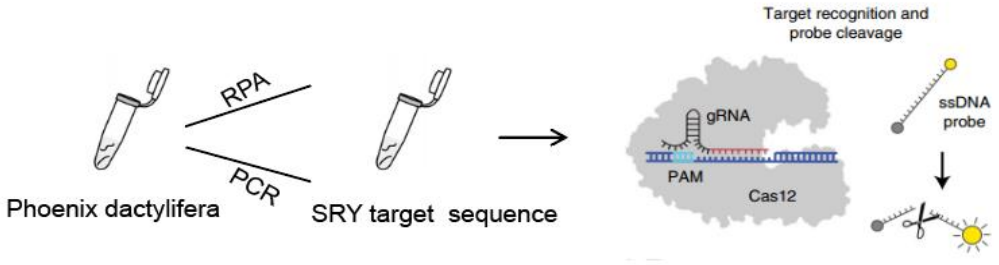

Figure-1. Schematic diagram of the experimental design process.

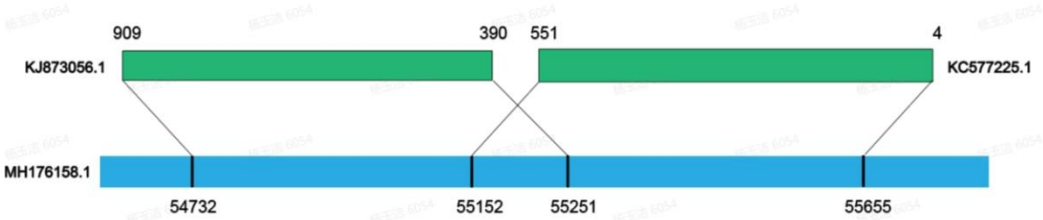

Figure-2. Schematic diagram of gene location.

Experimental primers and sgRNA:

Primers (Table 1) and sgRNA targeted for Cas12a (Table 2) were designed according to the two sequences respectively.

Table-1. Primer-related sequences used in the experiment.

Primer's name	Sequence
SRY-Pd-F2	GAAATGGAGGGAACAGGGTA
SRY-Pd-F3	CGGGCGACAATACCACCAT
SRY-Pd-F4	AATTGGTTGGCACTTTAGG
SRY-Pd-R3	GTTGGTGCTGGGTCCTGAT
SRY-Pd-R4	ATGGGCTTGATGAGGGATT
SRY-UPd-F1	CCCACTAACACCATCACTT
SRY-UPd-F2	ATCACTTTCCCGTATTGCC
SRY-UPd-R2	CGAATCTCCGAAATCTATG

Table-2. sgRNAs used in the experiments as well as ssDNA probe-related sequence.

Name	Sequences
sgRNA-4	mU*mA*mA*UUUCUACUAAGUGUAGAUGGAGAUUCGAACAAAUUGGU*mU*mG*mG
sgRNA-5	mU*mA*mA*UUUCUACUAAGUGUAGAUGGGUUAUACAAUAUAUCUUU*mU*mC*mC
ssDNA probe	FAM-TTATT-BHQ1

Experimental instruments and reagents

Table-3. Part of the experimental instruments and reagents.

Instruments and Reagents	Manufacturer	Cat number
Fluorescence quantitative PCR instrument	Kunpeng gene	Archimed R4
NEBuffer r2.1（10×）	NEB	B6002V
TwistAmp® Basic	TwistDx	TABAS03KIT
Lba Cas12a	NEB	M0653S
2 × Taq Master Mix	Vazyme	P112-01

https://doi.org/10.35495/ajab.2024.253

Extraction of the genome

The genome of the Date palm and Jojoba plants was extracted for subsequent experiments according to Solliman et al., 2019, 2023b.

PCR amplification

The extracted genome was used as the target sequence, and it was amplified by PCR using synthesized primers with 2 × Taq Master Mix in a thermocycler as shown in Tables 4 and 5. Then PCR products were detected by the CRISPR/Cas12a system.

Table-4. PCR reaction procedure.

Temperature	Time	
95℃	3 min	
95℃	15 sec	34 cycles
54℃	15 sec	
72℃	25 sec	
72℃	5 min	

Table-5. PCR reaction system.

Components	Volume (μL)
SRY-Pd-F2(10μM)	2
SRY-Pd-R4(10μM)	2
2×Taq Master Mix	25
gDNA	1
ddH_2O	20

RPA amplification

RPA amplification was performed with the extracted genome, optimized primers, and TwistAmp® Basic reagent. The specific reaction steps are as follows: Prepare reaction mix in a 1.5ml tube as is shown in Table 6, then add the reaction mix to a TwistAmp Basic reaction, Pipette to mix. Add 2.5 μl of 280mM Magnesium Acetate (MgOAc) (supplied) and mix well to start the reaction. Incubate at 39°C for 20 minutes before subsequent detection.

Table-6. RPA reaction system.

Components	Volume (μL)
SRY-Pd-F2(10μM)	2.4
SRY-Pd-R4(10μM)	2.4
Primer free Rehydration buffer	29.5
gDNA	2
ddH_2O	11.2

CRISPR/Cas12a assay

Products obtained by PCR and RPA were used as the target sequences of the CRISPR/Cas12a detection system. The reaction system is shown in the following table 7. The results can be detected by fluorescence quantitative PCR instrument and blue light irradiation.

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Table-7. Composition of Cas12a detection system.

Components	Volume (μL)
10×NEBuffer r2.1	2
Lba Cas12a (1μM)	1
Amplified DNA	1
sgRNA (10μM)	0.5
ssDNA probe (5μM)	2
RNase inhibitor	0.5
DEPC H_2O	13

Fluorescence in situ hybridization (FISH):

Gathering and preparing samples of the genome of the *Phoenix dactylifera* plant and peripheral blood samples from healthy men were acquired with informed consent. The lymphocytes from the medium-term cultures were extracted using standard cytogenetic procedures. To prepare metaphase spreads, lymphocytes were suspended in a fixative solution (3:1, methanol: glacial acetic acid) and subsequently spread onto sterile slides. The slides were air dried for PRINS or FISH after being progressively dehydrated for two minutes in ethanol baths containing 70, 85%, and 100% ethanol. Fish, PRINS, and Primers We employed four SRY gene-specific primers (Wuzhong Biomedical Industrial, China) (Solliman et al., 2019). The primer sequences were found to be specific for their targeted targets using a Blast search (Table 1).

Results:

The date palm (*Phoenix dactylifera* L.) is a dioecious plant species characterized by separate male and female sex organs on distinct individual plants. Accurate sex determination of date palm seedlings holds paramount importance in the context of augmenting fruit production by selecting female plants. However, conventional techniques for sex differentiation are labor-intensive and often necessitate several years to yield precise outcomes. In this investigation, we introduce a sex determination approach for date palms employing Polymerase Chain Reaction (PCR), integrating a male-specific primer targeting SRY-related sequences, in conjunction with SRY-related sequences from humans as a positive control. SRY is acknowledged for its pivotal role in male fertility. Our findings reveal that SRY amplification exclusively transpired in male date palm samples, whereas female samples exhibited no amplification of SRY. Consequently, the presence of one distinct PCR band signifies a male plant, whereas a no band designates a female plant. To ascertain the effectiveness of these primers, we applied them to all date palm samples obtained from Saudi Arabia's different regions, successfully ascertaining the sex of each specimen. This PCR-based sex determination method provides a swift and dependable means of early-stage sex identification in date palms, thereby facilitating enhanced date palm cultivation practices and heightened fruit production.

First, the quality and concentration of obtained genome DNA (Table 8) were verified on agarose gel, as shown in Figure 3. Except for Date seeds 1B gDNA, others are adequate.

Table-8. Relevant genomes that have been obtained.

	gRNA
1	Date seeds 1A gDNA
2	Date seeds 1B gDNA
3	Date seeds 1C gDNA
4	Date seeds 2A gDNA
5	Date seeds 2B gDNA
6	Date seeds 2C gDNA
7	Jojoba gDNA

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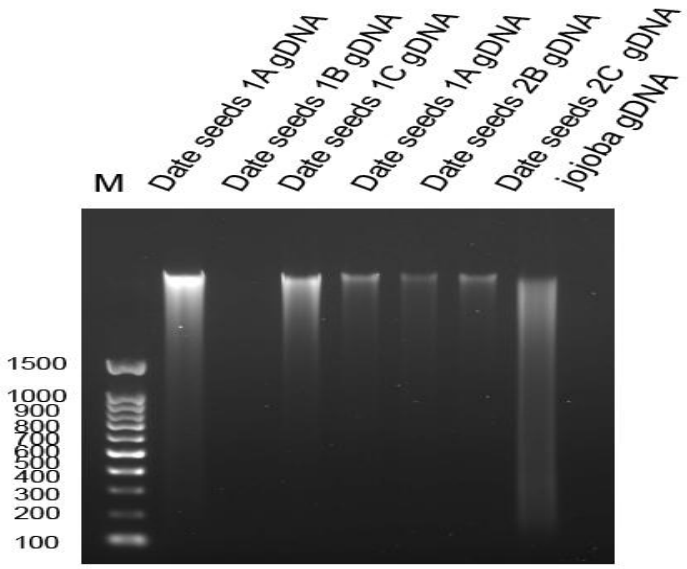

Figure-3. gDNA validation by agarose gel separation.

Using the validated genome described above, further experiments were performed. As is shown in Figures 5 and 6, PCR products of SRY were amplified in Date seeds 1A gDNA and Date seeds 1C gDNA, which were identified as female seeds, and Date seeds 2A gDNA, Date seeds 2B gDNA and Date seeds 2C gDNA were not amplified, which was identified as female seeds. The amplified PCR products were sent for sequencing, and the sequence alignment results were all correct.

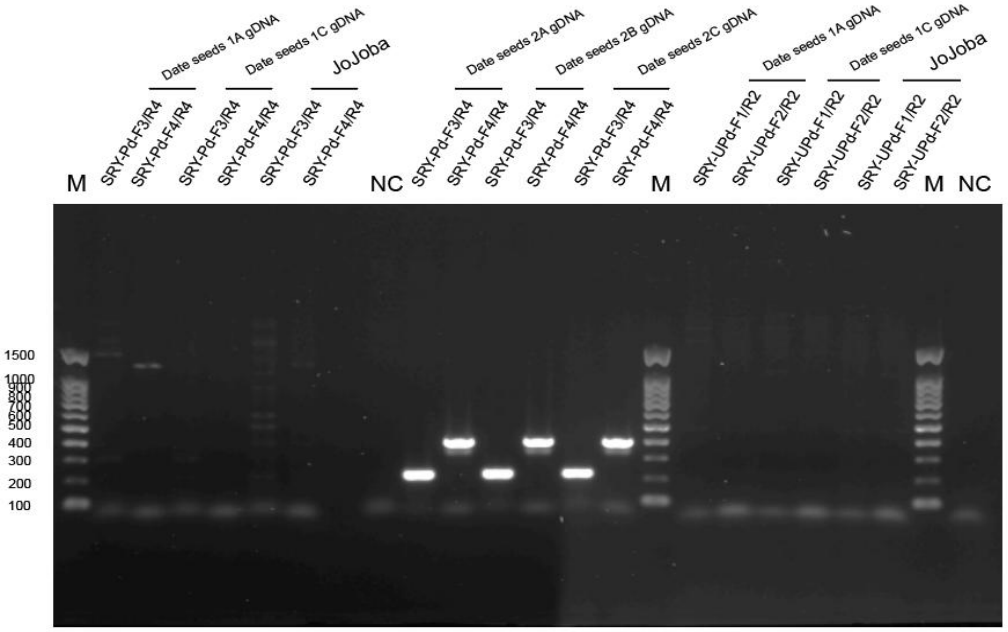

Figure-4. Agarose gel detection of PCR products

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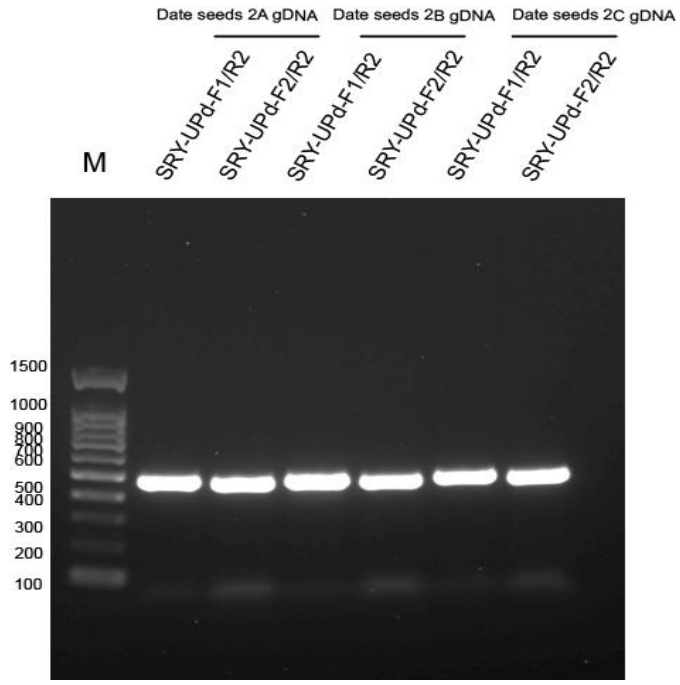

Figure-5. Agarose gel detection of PCR products

After the verification of the above experiments, the male genome and the female genome have been distinguished. Date seeds 2A gDNA (male) were selected for further experiments. PCR and RPA amplification were performed respectively (the amplified fragments contained PAM sequences required for cas12a detection), as shown in Figures 6 and 7, both PCR and RPA amplification results showed the expected length of the target band with clear brightness. Sanger sequencing alignment results of the eight samples in the two figures were all correct.

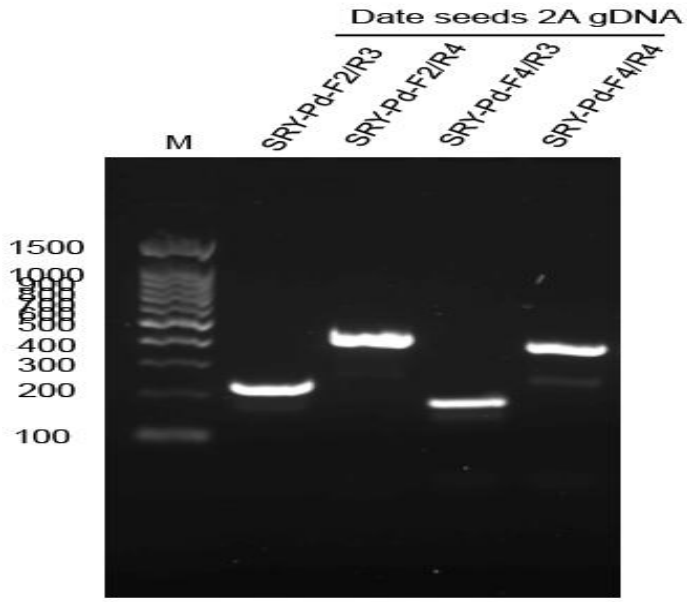

Figure-6. Agarose gel detection of PCR products

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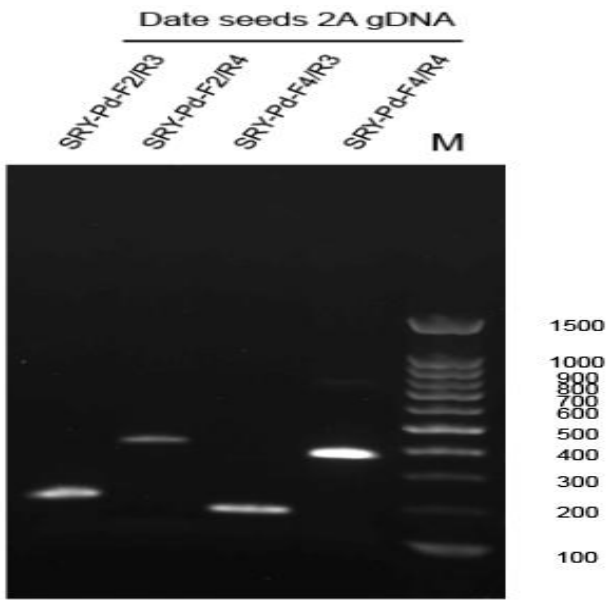

Figure-7. Agarose gel detection of RPA products

The genomes of female and male *Phoenix dactylifera* were amplified by PCR and RPA respectively to obtain the corresponding amplification products, which were used as the target sequences of CRISPR/Cas12a detection. Two target sites were designed for detection, corresponding to two guide RNAs: sgRNA-4 and sgRNA-5.

The detection system was prepared according to Table 7, mixed with shaking, briefly centrifuged, and detected by fluorescence quantitative PCR instrument at 37°C with fluorescence signal collection per minute. Finally, the sample tubes were placed under blue light. Fluorescence brightness results could be seen obviously.

SRY fragments were amplified by PCR from genomes of female and male *Phoenix dactylifera* respectively, and DEPC H_2O was used as a negative control. As is shown in Figure 8 and Figure 9, the male target sequence was recognized by CRISPR/Cas12a detection system, and trans-cleavage activity of Cas12a was stimulated, which cleaved the single-stranded DNA probe and generated fluorescence. Nevertheless, the fluorescence signals of female genomes were not detected. It is suggested that the detection system successfully distinguishes female *Phoenix dactylifera* from male *Phoenix dactylifera*.

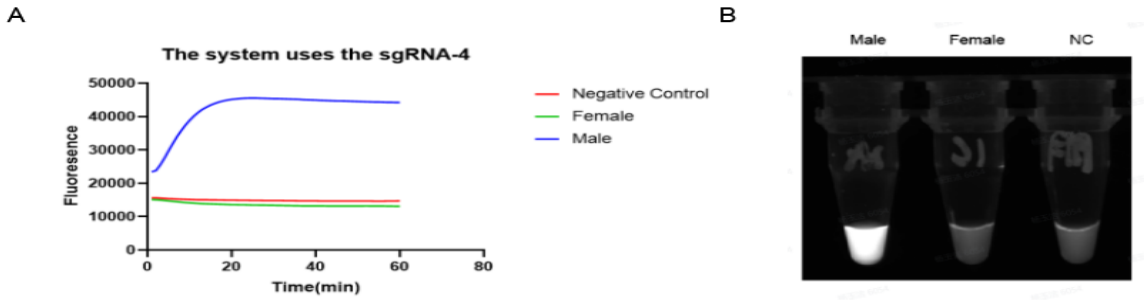

Figure-8. Experimental results of CRISPR/Cas12a detection using PCR amplification products as target sequences (The system uses the sgRNA-4). (A) Detection of fluorescence quantitative PCR instrument. CRISPR/Cas12a detection system recognized the SRY (male) sequence and activated its trans-cleavage activity to cleave the single-stranded DNA probe and generate fluorescence (blue line). When samples were female (green line) as well as DEPC H_2O (Red line), trans-cleavage activity was not activated, and the single-stranded DNA probe was not cleaved. (B) Blue light exposure results. When the target sequence was male, the fluorescence brightness of the detection system was much stronger than that of the other two experimental groups.

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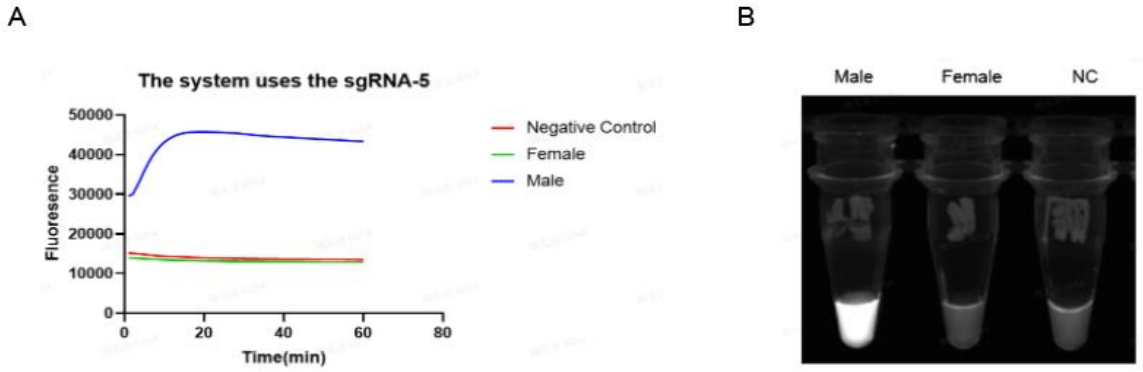

Figure-9. Experimental results of CRISPR/Cas12a detection using PCR amplification products as target sequences (The system uses the sgRNA-5). (A) Detection of fluorescence quantitative PCR instrument. CRISPR/Cas12a detection system recognized the SRY (male) sequence and activated its trans-cleavage activity to cleave the single-stranded DNA probe and generate fluorescence (blue line). When samples were female (green line) as well as DEPC H_2O (Red line), trans-cleavage activity was not activated, and the single-stranded DNA probe was not cleaved. (B) Blue light exposure results. When the target sequence was male, the fluorescence brightness of the detection system was much stronger than that of the other two experimental groups.

SRY fragments were amplified by RPA from genomes of female and male *Phoenix dactylifera* respectively, and DEPC H_2O was used as a negative control. As is shown in Figure 10 and Figure 11, the male target sequence was recognized by CRISPR/Cas12a detection system, and trans-cleavage activity of Cas12a was stimulated, which cleaved the single-stranded DNA probe and generated fluorescence. Nevertheless, the fluorescence signals of female genomes were not detected. It is suggested that the detection system successfully distinguishes female *Phoenix dactylifera* from male *Phoenix dactylifera*.

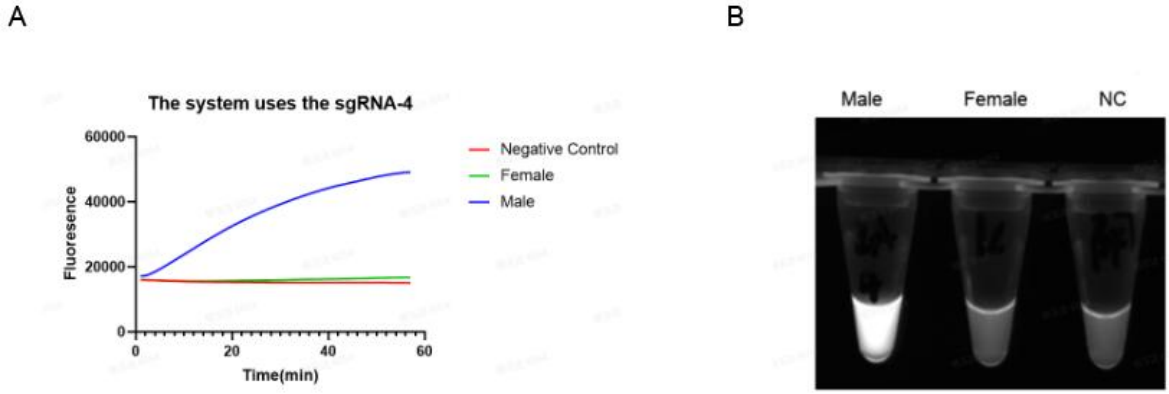

Figure-10. Experimental results of CRISPR/Cas12a detection using RPA amplification products as target sequences (the system uses the sgRNA-4). (A) Detection of fluorescence quantitative PCR instrument. CRISPR/Cas12a detection system recognized the SRY (male) sequence and activated its trans-cleavage activity to cleave the single-stranded DNA probe and generate fluorescence (blue line). When samples were female (green) as well as DEPC H_2O (Red line), trans-cleavage activity was not activated, and the single-stranded DNA probe was not cleaved. (B) Blue light exposure results. When the target sequence was male, the fluorescence brightness of the detection system was much stronger than that of the other two experimental groups.

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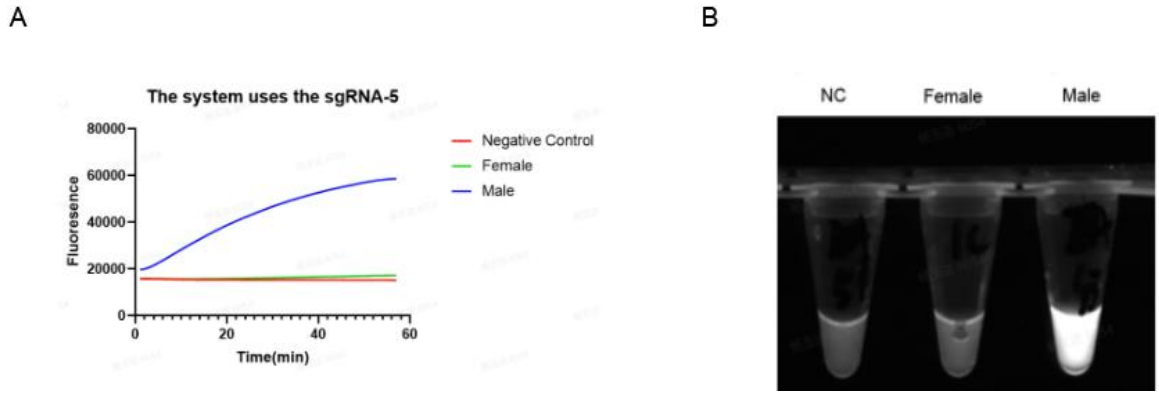

Figure-11. Experimental results of CRISPR/Cas12a detection using RPA amplification products as target sequences (The system uses the sgRNA-5). (A) Detection of fluorescence quantitative PCR instrument. CRISPR/Cas12a detection system recognized the SRY (male) sequence and activated its trans-cleavage activity to cleave the single-stranded DNA probe and generate fluorescence (blue line). When samples were female (green) as well as DEPC H_2O (Red line), trans-cleavage activity was not activated, and the single-stranded DNA probe was not cleaved. (B) Blue light exposure results. When the target sequence was male, the fluorescence brightness of the detection system was much stronger than that of the other two experimental groups.

SRY in situ detection

SRY gene detection in Humans and date palms using FISH:

To develop a primed in situ labeling (PR1NS) method that can identify single-copy genes more successfully. Four primers of the sex-determining region Y (SRY) gene and the TSA TM Biotin System were incorporated in the detection of the SRY gene based on the conventional PRINS method, together with innovative reagents and techniques such as the TaqStart antibody. As a control, fluorescence in situ hybridization (FISH) was utilized to find the SRY gene. Outcomes There were fifty metaphases scored. In every metaphase, PRINS labeling revealed signals for the SRY at band Yp11.3 on the Y chromosome. These signals were identical to those derived from the FISH data. In addition, FISH might be useful to detect the translocated SRY gene and localize the signal with ease.

The initial result for SRY staining in a substitute cell line, the Human A549 cell, a male cell line containing a single copy of SRY was promising albeit with high background staining as shown in the next figure. We can see prominent dots (red) in the nuclei region, but still, high background staining in both nuclei and cytosol.

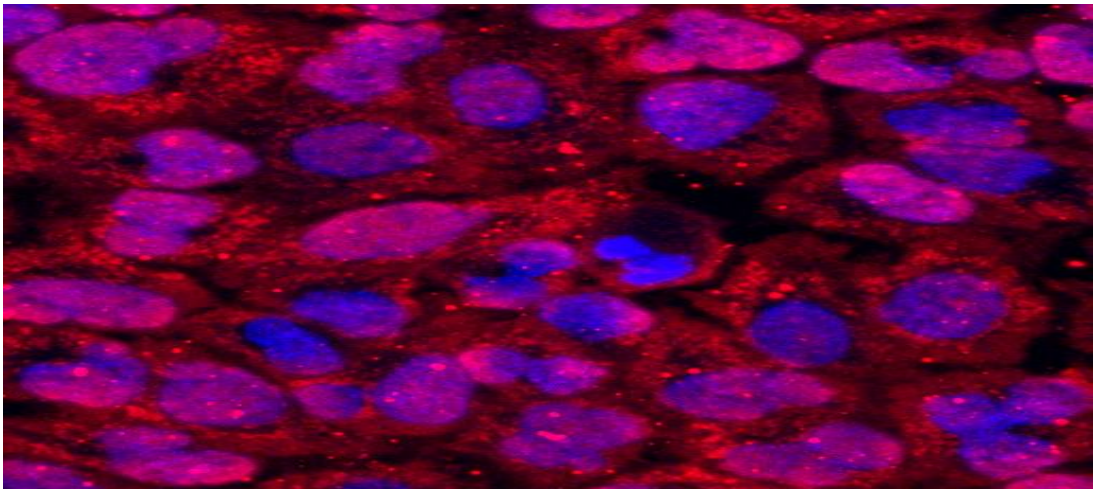

Figure-12. FISH with interphase nuclei and metaphase chromosomes of a male with date-SRY probe. Note the presence of the SRY signals in all the male cells.

Our second try with a lower concentration of sgRNA staining improves the background staining a lot as shown in the following figure. There is a significant background reduction in cytosol and a large fraction of cells shows single dot staining. This is indicative of specific staining of single-copy SRY staining in A549 cells.

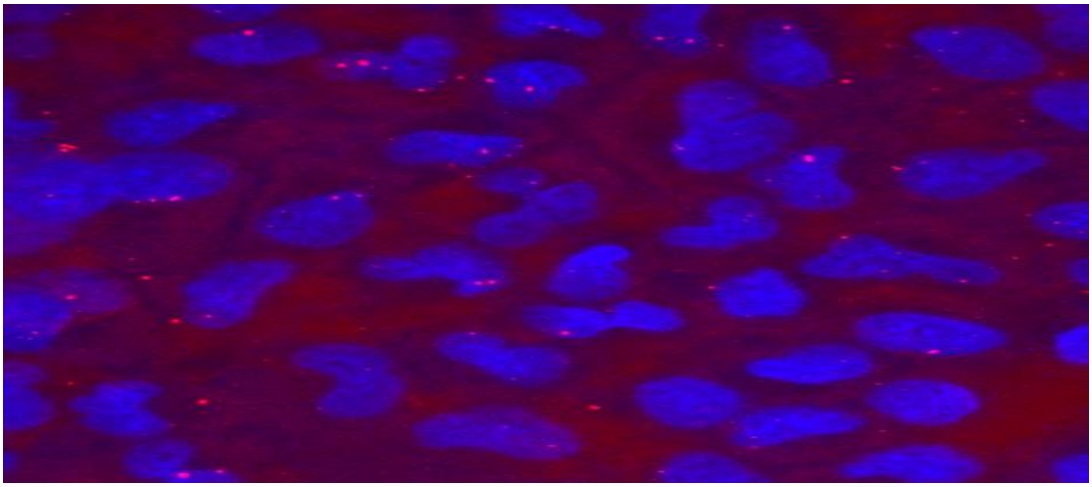

Figure-13. Fluorescence in-situ hybridization (FISH) utilizing the date SRY probe, which binds to the y chromosome's centromere and SRY gene concurrently, inside the human interphase nuclei and metaphase chromosomes. Demonstrates that the interphase nuclei contain both the X (green dot) and Y (red dot) chromosomes.

The SRY gene is located on the Y chromosome, which is physically normal. The X chromosome was absent from certain cells. Here, only exemplary cells with various karyotypes are displayed. Tandem duplication of the SRY gene is suggested by a single localized signal (copy number 16).

DISCUSSION:

Our recently created methods for clearly choosing the female date palm and jojoba plant at a young age are highly significant and useful to breeders as they reduce the overall plantation expenses related to the other methods of cultivating the non-productive male date palm and jojoba plants. The early sex determination of date palms and jojoba would also create new opportunities for global genetic improvement program implementation, date palm and jojoba genotype seeding, and the restoration of biodiversity in date palms and jojoba groves.

The outcomes of our research also pertain to the above-discussed utilization of a sex-specific gene sequence in determining a date palm and jojoba sex. The gpat3 primers are also associated with a technique for identifying a date palm and jojoba sex by looking for particular primers inside the genome. The process is defined in some embodiments by amplifying a subset of the date palm and jojoba-tested genomic DNA using gpat3 gene-specific forward and reverse primers to determine the date palm and jojoba-tested sex.

Thus, SRY in humans is comparable to that in mice and jojoba plants. Consequently, a plant model of SRY function has been created. This has proven crucial in identifying how gpat3 interacts with other genes to determine male sex. Since date palm and jojoba were first discovered historically, we have made tremendous strides toward understanding sexual dimorphism; the SRY gene in males has been shown to be the cause of the difference between the sexes. As of late, we have learned more about the molecular processes involved in determining sex.

Potential Applications:

Rapid identification of plant sex allows for early culling of non-productive plants (e.g., male date palms), optimizing space and resources., Conservation: For endangered dioecious species, the assay can help in selective breeding and management programs. This assay provides a model for applying CRISPR-based detection methods in other areas of plant biotechnology, particularly for traits that lack early visible markers.

https://doi.org/10.35495/ajab.2024.253

Sullivan et al. (1993) define the amelogenin sex test as the sole sex test included in commercially available PCR kits for determining gender in humans, despite the fact that many PCR-based techniques can distinguish a sample as coming from a male or female. To identify the hitherto unidentified 5' and 3' flanking sections of the DNA, several alterations were created. PCR was often performed using genomic DNA fragments that had been digested by a restriction enzyme(s) following ligation (Mohasseb et al., 2020), cloning into a vector (Solliman et al., 2020), or ligation to partly or double-stranded (Solliman et al., 2019).

As previously stated, the current effort is concerned with molecular methods unique to the sex of date palm and jojoba plants and with applying these methods to differentiate between male and female plants. The early selection of female plants made possible by the approaches has the advantage of lowering the overall plantation expenditures related to the cultivation of the non-productive male jojoba plants. Furthermore, our methods are "universal" in the sense that they work with date palm and jojoba of any origin, variation, or cultivar (Solliman et al., 2023a, 2023b).

Conclusion

We successfully developed a CRISPR/Cas12a-based fluorescence test for the sexual identification of *Phoenix dactylifera* with RPA and CRISPR-Cas12a, which could be used as a simple, accurate, and fast detection method in agricultural fields due to its advantages of high-sensitivity and isothermal reactions. We will continue to optimize the staining condition for the SRY sgRNA probe and include control cell lines like the female cell line Hela cell. After the initial optimization, we will start to work on the nuclei isolated from Date Palm seedlings with the previous methods of nuclei extraction and staining method developed for this project. This improved method is ideal for rapidly localizing single-copy genes and small DNA segments. And PRINS is a cost-and-time-effective alternative to FISH. In conclusion, we have shown that a CRISPR/Cas12a-based detection assay coupled with nucleic acid amplification can detect plant sex with high specificity and sensitivity. The RT-RPA–CRISPR/Cas12a assay represents an innovative solution for dioecious plant management. By combining the rapid amplification capabilities of RPA with the sensitivity of CRISPR/Cas12a detection, this approach enables fast, accurate, and field-deployable sexual identification, paving the way for enhanced agricultural efficiency and resource management.

Acknowledgment

Authors gratefully acknowledge Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (KFU250409), for supporting this research work.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: This work was supported by Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (GRANT KFU250409) and The APCs were funded by the same organization.

Contribution of Authors

Both authors contributed equally to this research, and both read and approved the final manuscript.

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