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Research article

Single tube amplification and detection of male date palm using polymerase chain reaction and loop-mediated isothermal amplification technique

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Abstract

Date palm (*Phoenix dactylifera* L.) is a dioecious fruit species. Date palm seedlings take about 5 yr until their first flowering, which is too long to wait to identify those plants with pistillate flowers that are inappropriate for commercial production. The objective of this research was to develop a method for sex specification of date palm using polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) incorporating fluorescence detection. A PCR-fluorescent and LAMP technique incorporating fluorescent dye succeeded in sex identification of date palm. The male tubes emitted fluorescence under ultraviolet light (positive), while the female tubes did not emit fluorescence (negative). The use of fluorescent dye integrated in PCR and LAMP could eliminate the electrophoresis step and thereby markedly shortened the time required for analysis. The techniques were developed from previous research and had high accuracy. These techniques amplified and detected within a single tube, were much faster and used less equipment than existing traditional methods. Furthermore, it could reduce the cost by at least USD 5 per sample.

Introduction

The date palm (*Phoenix dactylifera* L.) belongs to the Arecaceae family, is a heterozygous perennial fruit tree that is dioecious and is a long-living evergreen tree that can grow for over 30 yr (Johnson, 2011)). The date palm is an important fruit crop in semi-arid regions and the major date producers in the world are located in the Middle East and North Africa and export globally (Zaid, 2002). *P. dactylifera* can be grown in almost all tropical countries, including Thailand where it grows well (Intha and Chaiprasart, 2018). Sex determination of date palm at the seedling stage is important for efficient production, as well as identifying the sex ratio for dioecious group plantings as determined by the X/Y sex chromosomes,

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with the male being heterozygous (XY) and the female being homozygous (XX) (Al-Mahmoud et al., 2012). However, being dioecious requires insects, wind or humans to transfer the pollen from male flowers to female flowers on separate plants.

Sex identification of the date palm using morphology at the seedling stage is impossible until the first inflorescence appears in the flowering stage which may not occur until around 5–7 yr after planting (Al-Qurainy et al., 2018). The spathes and inflorescence of the male and female plants have different appearances and components as detailed in Intha and Chaiprasart (2018). Specifically, the male spathe is characterized by an enlarged torpedo shape. The male florets consist of sepals and petals and also the stamens on which the pollen will appear in the blooming stage. The female spathe is characterized by an elongated shape and is often smaller than the male spathe. The female florets are round and the stigma is clearly visible due to the lack of petals and pollen in the blooming stage. The shape of the spathe may vary according to genetic diversity. Classifying the gender using morphological characteristics at the seedling stage is not possible because the reproductive organs have not yet been expressed. However, polymerase chain reaction (PCR)-based markers can be used to identify the sex in juvenile date palm plants (Adawy et al., 2014). Loop-mediated isothermal amplification (LAMP) remains challenging as a mean of classifying the sex of the date palm; however, it can be amplified and detection is faster than conventional PCR. In addition, LAMP may be a solution to avoid time-consuming gel electrophoresis as well as being simpler than PCR with a single tube technique to amplify and detect of DNA that only requires a constant-temperature incubator (Nagamine et al., 2002).

Sex identification based on the DNA sequence differences between male and female date palms has been developed using PCR for amplification of a target. The PCR technique is widely used in molecular biology but this technique requires gel electrophoresis for checking the products of the PCR amplification which can take a long time and require additional special equipment. The sex identification of date palm has required the use of gel electrophoresis (Al-Mahmoud et al., 2012; Al-Qurainy et al., 2018; Intha and Chaiprasart, 2018) to illustrate the polymorphisms of DNA bands. The aims of the current research were: to detect the PCR products based on a fluorescence technique that could identify male date palms; to design the LAMP primers for amplification of the male DNA and to detect LAMP products using a fluorescence technique. This technique should not require the use of gel electrophoresis and should be shorter than reported techniques.

Materials and Methods

Plant material and genomic DNA isolations

Young leaves of mature male and female were collected from the Kaset Piboon Farm, Phitsanulok, Thailand at the flowering stage as reference material. Four cultivars (KL1, Barhi, Deglet Nour and Khadrawi) of date palm were used as reference material for the PCR experiment. Five cultivars (KL1, Barhi, Deglet Nour, Khadrawi and Medjool) of date palm were used as reference materials for the loop-mediated isothermal amplification. All leaf samples were kept at -20°C until genomic DNA isolation. The genomic DNA was isolated using the CTAB method, then was assessed for concentration and purity using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific; USA) and samples were diluted in TE buffer to 50 ng/µL for each sample and kept at -20°C for use as a DNA template in the PCR and LAMP procedures.

Polymerase chain reaction primers

Male specific primers, F: CGGCAATAGCACCATAG TAAATTGCCTA, and R: GCTAACTTGGTGCACGGA TCTCT, were developed from original research (Al-Mahmoud et al., 2012; Intha and Chaiprasart, 2018). These primers were designed to amplify a malespecific fragment. The forward and reverse primers were synthesized and were diluted to 1 µM for PCR amplification.

Loop-mediated isothermal amplification primers

Accession MH668906 (Torres et al., 2018) with a length of 1,449 nucleotides was acquired from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) and these sequences were used in the design of the LAMP primers. The LAMP primers were designed using the Primer Explorer V.5 software (Eiken Chemical; Japan) and were diluted to 10x (2 µM F3 and B3, 16 µM FIB and BIP, 8 µM Loop B and Loop F).

Polymerase chain reaction and loop-mediated isothermal amplification process

Each PCR reaction comprised of 1 µL of genomic DNA, 1 µL each of the forward and the reverse primers, 12 µL of EmeraldAmp® PCR Master Mix (Takara Bio; Japan) and the master mix was made up to a total volume of 25 µL using sterilized water. The PCR reagent was incubated at 94°C for

2 min followed by 35 cycles of 94°C for 30 s, 57.7°C for 30 s, 72°C for 1 min and finally 72°C for 5 min and then held at 4°C indefinitely. Tubes 1–4 were samples from males of KL1, Barhi, Deglet Nour and Khadrawi, respectively. Tubes 5–8 were samples from females of KL1, Barhi, Deglet Nour and Khadrawi, respectively, and tube N was a negative control comprised of all reagents but without any genomic DNA of date palm.

All the PCR products were added with 5 µL of fluorescent loading dye (DNA safety dye GSD-100; [Natural Immunity; Japan]: loading dye [1:20, volume per volume]) and mixed by vortexing for 5 s.

The LAMP reagent was prepared from 12.5 µL of LavaLAMP™ DNA Master Mix (Lucigen; USA), 8 µL of Nuclease-free H_2O, 2.5 µL of 10x Target-Specific Primer Mix, 1 µL of genomic DNA and 1 µL of green fluorescent dye. Tubes 1–5 were samples from males of KL1, Barhi, Deglet Nour, Khadrawi and Medjool, respectively. Tubes 6–10 were samples from females of KL1, Barhi, Deglet Nour, Khadrawi and Medjool, respectively, and tube N was a negative control without any genomic DNA of date palm with all reagents. The reaction was conducted at 69°C for 45 min and held at 4°C indefinitely.

Polymerase chain reaction and loop-mediated isothermal amplification analysis

The PCR and LAMP products were visualized under ultraviolet light. The male samples were expected to detect fluorescence, while the female samples were expected to quench it. Then, all the PCR and LAMP samples were run using 1.2% agarose gel electrophoresis with 100 V and were photographed using a SmartView Pro 1200 Image System (Major Science; Taiwan) to confirm the experimental results.

Results and Discussion

Polymerase chain reaction results

The PCR products in tubes 1–8 and N were detected under ultraviolet light to assess sex specificity based on fluorescence. Fluorescence was detected from the male tubes (1–4), while it was not detected from any of the female tubes (5–8) nor the negative control tube (N). Fluorescence could clearly observed with the naked eye, a digital camera or even a cell phone camera (Fig 1). After all the PCR products had been run using electrophoresis, the results showed that the male lanes (1–4) contained a single DNA band at around 320 bp, while the female (5–8) and negative control (N) lane had no DNA bands (Fig 2). These results indicated that the electrophoresis results were consistent with the fluorescence detection of the PCR product. Conventional electrophoresis was used to detect PCR products which clearly showed the cause of the fluorescence was due to the increased amount of DNA. The pairs of primers were developed from Intha and Chaiprasart (2018). Forty samples (20 males and 20 females) from five cultivars (Deglet nour, Barhi, Hayani, Medjool, Tunisia) were used as reference materials. Therefore, the reduced number of samples in this research was appropriate.

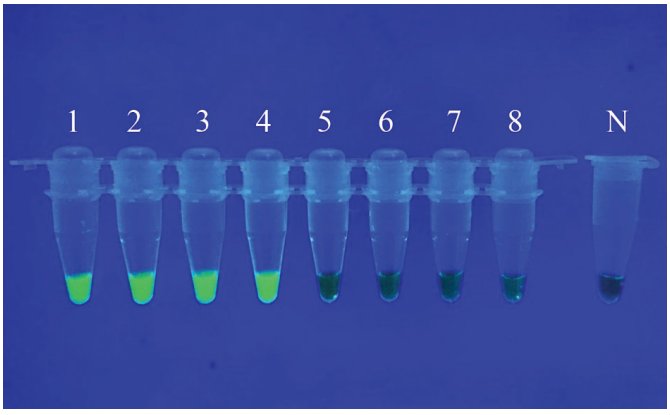

Fig. 1 Sex specification of date palm using polymerase chain reaction-fluorescent-based assay showing fluorescence under ultraviolet light in males of KL1, Barhi, Deglet Nour and Khadrawi (tubes 1–4, respectively) but none in either females of KL1, Barhi, Deglet Nour and Khadrawi (tubes 5–8, respectively) or the negative control without genomic DNA of date palm (tube N)

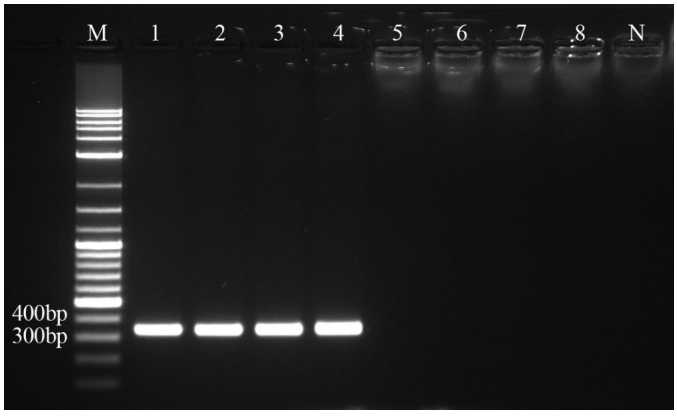

Fig. 2 Verification of the polymerase chain reaction (PCR) product of the PCR-fluorescent based assay using 1.2% agarose gel electrophoresis, where males of KL1, Barhi, Deglet Nour and Khadrawi (lanes 1–4, respectively) had the expected single-band pattern (approximately 320 bp) that was absent in females of KL1, Barhi, Deglet Nour and Khadrawi (lanes 5–8, respectively) and negative control without genomic DNA of date palm (lane N)

Loop-mediated isothermal amplification results

The nucleotides from accession MH668906 were designed for the LAMP primers (Fig 3, Table 1) using the Primer Explorer V.5 software (Eiken Chemical; Japan). The LAMP primers were designed to amplify a large number of DNA loops in males only. Therefore, the product was easily detected by fluorescence. The males (tubes 1–5) glowed under the ultraviolet light, while the females (Tubes 6–10) and negative control (tube N) did not (Fig 4). The amplified DNA loop was identified using electrophoresis. The results showed that the males (lanes 1–5) had various sizes of DNA bands with the same pattern, while the females (lanes 6–10) and the negative control (lane N) did not (Fig 5). These results indicated that the fluorescence of the LAMP products was accurate and reliable.

The results indicated that the PCR and LAMP provided accurate diagnosis. In particular, the fluorescent technique was able to specify the male date palm without requiring gel electrophoresis. The developed PCR-fluorescent and LAMP techniques allowed for amplification and detection within a single tube.

General PCR requires gel electrophoresis for analysis of the PCR products followed by the DNA banding pattern comparison that takes at least 40 min. The sex specification of date palm using DNA markers requires the gel electrophoresis (Al-Mahmoud et al., 2012; Dhawan et al., 2013; Adawy et al., 2014; Mohamed and Sami, 2015; Al-Ameri et al., 2016; Al-Qurainy et al., 2018; Sawwa et al., 2020). Verification of the PCR product using electrophoresis can be done as well. The current results were consistent with the research of Al-Mahmoud et al. (2012) and Intha and Chaiprasart (2018).

Name: glycerol-3-phosphate acyltransferase 3 (GPAT3) mRNA, complete cds
Accession: MH668906

ATGCCTATGTCCTCTCCTGTGGCAACTGCTTGTCATGGAGGGGAAGAGAGGCGCA
GCATTGCCTGCGAATTTGAG/GGTGCCCTCCTCATCTCAAAGAGACTCTTTGCCTA
CTTCATGCTTGTTGCTCTTGAAGCTGGTGGCCCCATCAGGGCTCTTACACTGCTCC
TAGTCTTCCCTCTCCTATGGTTGCTCGAGCTCGTCGGCCTTGAGAGGATGGCTCTT
CAACTGATGATCTTTGTATCCACGGCTGGTCTTAGGGTCGATGATCTGAAGGCTG
TGGCAAAGGCCACCCTGCCCAGATTTTATTTGGAAGACCTTAGGCAGAGAGCAT
ATCAGGTTTTCTCGAGCTATGAGGGGAAGAAATTTGTGGTTACTTGTATCCCCAG

F3 F2 Loop F F1 B1 Loop B B2 B3

Fig. 3 Nucleotide sequences from accession MH668906 and location of the loop-mediated isothermal amplification primers (underlined with type in bold superscript) used to amplify date palm male DNA

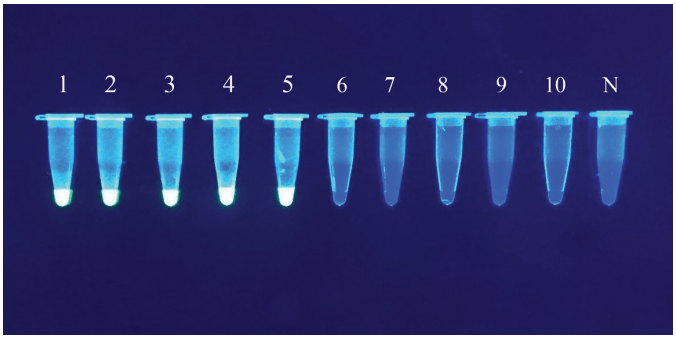

Fig. 4 Sex specification of date palm using loop-mediated isothermal amplification technique and fluorescence under ultraviolet light in males of KL1, Barhi, Deglet Nour, Khadrawi and Medjool (tubes 1–5, respectively) with no fluorescence for females of KL1, Barhi, Deglet Nour, Khadrawi and Medjool (tubes 6–10) nor in the negative control without genomic DNA of date palm (tube N)

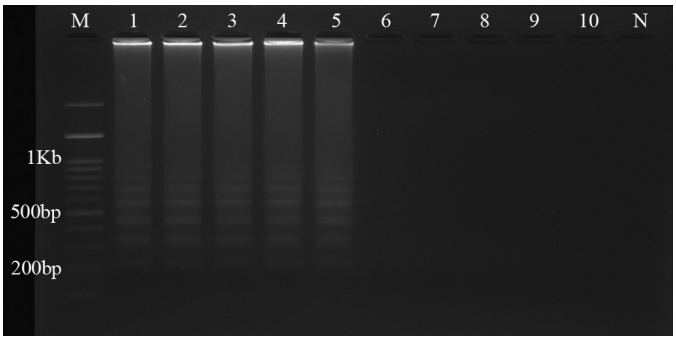

Fig. 5 Verification of loop-mediated isothermal amplification product using 1.2% agarose gel electrophoresis, with males of KL1, Barhi, Deglet Nour, Khadrawi and Medjool (lanes 1–5, respectively) showing many DNA bands amplified and detected but these are absent in females of KL1, Barhi, Deglet Nour, Khadrawi and Medjool (lanes 6–10, respectively) and in the negative control without genomic DNA of date palm (lane N)

Table 1 Nucleotide sequences of loop-mediated isothermal amplification primers

No.	Type	Sequence
1	F3	GCATTGCCTGCGAATTTGAG
2	B3	CCACAGCCTTCAGATCATCG
3	FIP	ATGGGGCCACCAGCTTCAAGGGTGCCCTCCTCATCTCA
4	BIP	TCCTATGGTTGCTCGAGCTCGTACCAGCCGTGGATACAAAG
5	Loop F	CAAGCATGAAGTAGGCAAAGA
6	Loop B	CTTGAGAGGATGGCTCTTC

The PCR-fluorescent technique added the fluorescent compound in the PCR tubes after the reaction had been completed, reducing the time in the detection steps and without any electrophoresis requirements. The developed technique was faster than conventional PCR or in the original research (Intha and Chaiprasart, 2018). To date, there have been no published reports of the use fluorescent dyes for the detection of PCR products in term of "glow/no glow" differentiation using conventional PCR. Fluorescence has been mostly used as a fluorophore-labeled probe (Melinger et al., 2016) in real-time PCR (Wilson et al., 2011). A fluorophore is a fluorescent chemical compound that re-emits light upon light excitation (Stockert and Blázquez-Castro, 2017; Chinnappan et al., 2019). It is sometimes used alone, as a tracer in fluids, as a staining dye for certain structures, as a probe or indicator, as a marker (or dye, or tag or reporter) for affine or bioactive reagents (antibodies, peptides, nucleic acids). In the current research, fluorophores have played important roles in a nucleic acid reporter that could re-emit light upon ultraviolet excitation when mixed in the PCR products. For example, RedSafe™ (iNtRON Biotechnology, USA) absorbs light at a wavelength of 514 nm and then re-emits it at a wavelength of 537 nm (iNtRON Biotechnology, 2021), while SYBR Green I (Thermo Fisher Scientific; Australia) absorbs light at a wavelength of 497 nm and then re-emits it at a wavelength of 520 nm. When a fluorophore is added to the PCR product, it inserts itself between the double-stranded DNA (the PCR product) and then emits light after being stimulated under ultraviolet light. If there is no PCR product, the fluorophore does not emit light or does so at a low light intensity. (Zipper et al., 2004).

LAMP is widely used as DNA markers because DNA can be synthesized and verified by using basic tools such as a water bath, dry bath and an ultraviolet transilluminator. It also takes less time and has higher specificity than the PCR technique so nowadays it has become more popular. The LAMP technique has not previously been reported for use to classify sex in dioecious plants. It has been found useful in the detection or diagnosis of pathogens in living organisms such as *Ehrlichia canis* (Muangchuen et al., 2014), *Fusarium oxysporum* f. sp. Ciceris (Ghosh et al., 2015), Dengue virus (Lau et al., 2015) and Leptospira (Najian et al., 2019). In addition, the LAMP technique can be used to classify the sex in animals such as dairy cattle (Khamlor et al., 2015) and wild passerine (Koch et al., 2019).

Glycerol-3-phosphate acyltransferase 3 (GPAT3) has 1,449 long base pairs and play an important role in male reproductive organs and male fertility and is a gene necessary for flower development in date palm and other monocotyledons (Torres et al., 2018). The GPAT3 gene nucleotide sequence was used to design primers for the LAMP technique. This resulted in the specificity of the nucleotide sequence on the DNA strands of the male date so it could amplify DNA in male date palms, but not in female date palms. This was consistent with Torres et al. (2018) who reported that GPAT3 was absent in female date palms.

The developed PCR-fluorescent and LAMP techniques have provided new tools for specification of the sex of date palms, allowing amplification and verification in the same tube. The results could be seen using the fluorescence (male)/non-fluorescence (female) contrast without the need for gel electrophoresis. These novel techniques are simple, time-saving, cheaper (reducing the cost by at least USD 5 per sample) and highly accurate and should be suitable for sex specification in large numbers of date palms.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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