



Original article

Identification and sequencing of Date-SRY Gene: A novel tool for sex determination of date palm (*Phoenix dactylifera* L.)



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ABSTRACT

Dioecism has always been an issue in many plant species with its numerous disadvantages, especially in woody trees such as date palms. As one of the most important crops in the Middle Eastern countries, researchers are having problems identifying of sex of the plant in its early stages of development. Hence, proper population stands in the male: female ratio for maintenance is almost impossible in the field for better production. In this study, sex determination of date palm (*Phoenix dactylifera* L.) were identified in regions of the Y chromosome (Date-SRY) gene, the pivotal gene that initiates sex determination, using a new technique and thus an economically desirable objective, which will significantly impact profits in seed based cultivations. Partial sequences of the Date-SRY were taken and amplified by nested polymerase chain reaction (PCR). According to the results, the exact sex of date palm was identified in all the tested plants, while amplified regions of the Date-SRY gene closely matched with the human and papaya sequences. In addition, a primer pair was designed to amplify the sequences of the SRY-date gene with confidence that it will identify male date palms. These primer sequences include SRY-date Forward 5'- cggccctctaagtatctgtgcaacg-3' (SRY-date F) and the SRY-date Reverse 5'- gttgcaacttcgaagcagag-3' (SRY-date R). The complete sequence of the DNA has been registered and deposited in GenBank (BankIt1598036 DPSRY1 KC577225 thenKJ873056).

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1. Introduction

Sex determination was usually identified with the physical separation of male and female plants from each other, and its mechanism in different crop species were documented in many

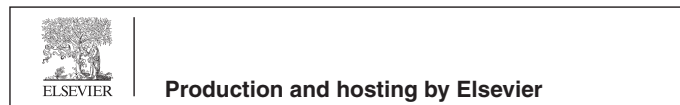
reviews (Juarez & Banks, 1998; Geber et al., 1999; Ainsworth, 2000; Matsunaga & Kawano, 2001; Negrutiu et al., 2001; Barrett, 2002; Charlesworth, 2002; Heikrujam, et al., 2015). It also plays a crucial role in the life cycle of plants that reproduce through sexual means. The system underlying sex determination in plants are largely unexplored, although recent studies of sex determination in many plant species - from ferns to maize - have been fruitful in identifying the diversity of genetic and epigenetic factors that are involved in determining the sex of the flower or individual. Furthermore, sex determination in non-crop plants is controlled by environmental factors; it comes with a pivotal role in population studies, the observation of the male and female ratios in population and the exploration of factors that manipulate sex distribution.

Due to its dioecious nature, breeding in date palm (*Phoenix dactylifera*) has not been practiced because the sex of the plant cannot be known until it reaches its reproductive stage (Bendiab et al., 1993). Until 1996 when the sex chromosomes were not

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distinguishable in plants, identification of differences between the heterochromatin region of chromosomes by chromomycin A3 staining of the root chromosome from male and female cells was the tool for male and female plant identification (Siljak-Yakovlev et al., 1996).

The sex of dioecious plants is usually tedious to identify, especially before flowering and during the early stages of its development. On the other hand, the identification of male and female flowers in fruit and seed bearing plants is becoming a priority with breeders, especially prior to propagation, since this would result in crop improvement and increased profits (Sarkar, et al., 2017). There are also a number of plant species where female flower representation is more important than males. These include *Borassus flabellifer* (George et al., 2007), *Carica papaya* (Parasnis et al., 2000), *Actinidia deliciosa* (Shirkot et al., 2002), *Eucommia ulmoides* (Xu et al., 2004), *Myristica fragrans* (Shibu et al., 2000), *Piper longum* (Manoj et al., 2005–2008), *Pistacia vera* (Hormaza et al., 1994), *P. dactylifera* (Younis et al., 2008), *Hippophae rhamnoides* (Persson & Nybom, 1998; Sharma et al., 2010), and *Simmondsia chinensis* (Agrawal et al., 2007; Sharma et al., 2008).

As one of the most important plantation crop in the palm family (Arecaceae), the date palm (*Phoenix dactylifera* L.) is an ornamental, dioecious, perennial, and monocotyledonous plant known for its distinct fruit. In dry tropical regions around the world such as western Asia and Africa, it plays a major role in socioeconomic conditions in terms of shelter, food, and fiber (Morton, 1987; Adawy et al., 2004; El Hadrami & El Hadrami, 2009; Jain et al., 2011; Srivastav, et al., 2013). However in Arab nations, it accounts for almost 60 percent of its production with more than 800 varieties (El-Juhany, 2010; Al-Abdoulhadi et al., 2011).

For decades of extensive date palm research, there have been numerous attempts to identify the sex of the plant, but most were unsuccessful, resulting to unpredictable number of female plants in the field that leads to uncertainty in the production potential of the crop. The number of female plants must be more than the male plants. Consequently, it is not possible to know the sex of the plant until it flowers and reaches the reproductive age spanning from five to ten years (Bendiab et al., 1993; Juarez & Banks, 1998; El Hadrami & El Hadrami, 2009). Breeding of the plant is also challenging due to the long phases of juvenility and dioecism. Because of the presence of low genetic diversity, methodologies that are simple and perfect for differentiation of the gender is absent even before the first flowering (Aberlenc-Bertossi et al., 2011). Thus, determining the sex of the date palm plant is necessary since the presence of a reliable method would significantly increase profitability in terms of seed based cultivation (Al-Khalifah et al., 2012).

There have been extensive efforts to understand the basis for sex identification and development using a wide range of methodologies that include biochemical and molecular markers that tend to be beneficial in the identification of male and female dioecious crop plants such as the date palm (Elmeer & Mattat, 2012; Al-Mahmoud et al., 2012; Heikrujam, et al., 2015, Al-Ameri et al., 2016). In recent years, the Inter Simple Sequence Repeat (ISSR) marker is gaining usage that offers several advantages over other dominant markers which are widely utilized for plant genome analysis (Abdel-Mawgood, 2012; Gaafar et al., 2014, Maryam, et al., 2016) but rarely used for sex identification in dioecious plants (Milewicz and Sawicki (2011); Aleksandrov et al. (2011); Nanda, et al., 2013; Heikrujam et al., 2014). Finally, research paper by Atia and Adawy (2015) show a novel set of sex-specific PCR-based markers reveals new and the most popular markers Hypothesis of sex determination in Date Palm.

Moreover, a wide range of popular molecular markers have also been used to show the differences between male and female plants that include the RAPD (Harvey et al., 1997, Shirkot et al., 2002,

George et al., 2007, Sharma et al., 2010), AFLPs (Vos, et al., 1995; Renganayaki et al., 2005; Danilova and Karlov, 2006; Wang et al., 2011), ISSR-SCAR (Korpelainen et al., 2008), RAPD-SCAR (Manoj et al., 2005–2008; Xu et al., 2004; Dhawan et al., 2013), ISSR and RAPD (Younis et al., 2008), and SSR markers (Cherif et al., 2013).

Among the techniques, the most commonly used method for sex determination in plants is the RAPD-PCR. In date palm, Younis et al. (2008) used the ISSR and RAPD markers to identify sex-specific DNA markers for the selection and identification of good male pollinators for utilization in breeding programs, thus increasing the yield and improving some quality traits of fruits. Thirty RAPD were screened and 20 ISSR primers on seven date palm cultivars (four females and three males) collected from the Com Ambo farm at the Aswan Horticultural Services for Ministry of Agriculture in Egypt and reported two male-specific and three female-specific RAPD markers and five male-specific ISSR markers. However, the utility of these markers were not applicable when tested on other cultivars collected from other locations (Dhawan et al., 2013).

Similarly, the researchers screened 100 RAPD and 104 ISSR primers on 25 female and 20 male date palm plants and reported one RAPD primer that produced a 1000-bp band that was specific only to male plants. With less reproducibility characteristics of RAPD primers and after cloning and sequencing of this DNA band, a SCAR primer pair was designed and tested on 45 date palm plants. The report suggested an amplification of one 406-bp band that was specific to both female and male plants, and one 354-bp band that was specific to male plants only. Although the SCAR primer pairs show positive correlation with male-specificity, it does not confirm the location of the primer pair on date palm sex chromosome, specifically male-specific chromosome. To identify sex-linked markers, Cherif et al. (2013) studied 52 male and 55 female geographically diverged date palm genotypes using three microsatellite (SSR) markers and reported three genetically linked loci that are heterozygous only in males and thereby confirming the existence of an XY chromosomal system with a non-recombining XY-like region in the date palm genome. The existence of an XY chromosomal system in date palm suggests the possibility of discovering Y chromosome-specific DNA marker for identification of male plants.

The sex-determining region (SRY) gene, crucial in mammalian sex determination, was identified in recent years and has been extensively studied. An individual with a functional SRY is considered male, while an individual without it is female. It is a potential candidate for the mammalian testis-determining factor and a tool for gender identification in the early stages of fetus development, especially when ultrasound method is not suitable. SRY appears to be part of a family with several autosomal representatives. Generally, the X and Y chromosomes determine an individual's sex. A female has two X chromosomes designated as XX, while a male comes with one X and one Y, designated as XY. However, there are cases where a female had XY chromosomes and a male with an XX blueprint. After the analysis of these individuals, it was revealed that some of the genes involved in sex determination included SRY as an important factor in the formation of testis, while Human SRY is located on the Y chromosome.

The SRY molecular factor is an attractive approach for sex determination and has already been successfully tested in mammals and in papaya. One of the existing molecular methods for sex determination is the polymerase chain reaction (PCR). Using two oligonucleotide primers that cross-hybrid into opposite strands and flank the region of interest in the target DNA, PCR is an in vitro and sterile method for the enzymatic synthesis of specific DNA sequences. The PCR-based sex determination identifies the presence (male) or absence (female) of the SRY gene. Although PCR-based sex determination of date palm is unattractive approach, suitable forward

and reverse primers specific to Y chromosome were not available for identification in this species. The goal of this study is to test PCR amplification of SRY as a method for determining sex in different varieties of date palm trees. A new pair of primers was also designed with the capacity to target a section of the SRY gene; this resulted in the amplification of a male-specific PCR fragment using routine PCR procedure. Conventional PCR technology was used to select the male plant, as this will guarantee explicit and fast sex identification process. The legitimacy and its appropriateness, coupled with the specificity of these primers were confirmed with DNA from a number of known samples of date palms.

2. Materials and methods

2.1. Plant material

2.1.1. Isolation of genomic DNA

For DNA isolation, young leaf tissues from randomly selected female and male plants (3–5 plants) of the selected date palm cultivars Burhi, Khalas and Sukkari were collected from the Al-Ahsa Oasis Farm, Kingdom of Saudi Arabia. Their Genomic DNA was then isolated on a mini-prep scale using CTAB (cetyl trimethyl ammonium bromide) method as reported by Murray and Thompson (1980). The quantity and quality of the nucleic acid were determined by measuring the absorbance at 260 nm and 280 nm using a Spectrophotometer. DNA concentration was quantified using the formula $A_{260} = 50 \mu\text{g/ml}$, while the purity of the DNA was determined by calculating the ratio of A_{260}/A_{280} for each sample.

2.1.2. Polymerase Chain Reaction (PCR) Materials

DreamTaq Polymerase, deoxynucleotide triphosphate (dNTP) and convergent primers used in this experiment were purchased from Fermentas, Thermo Fisher Scientific. Conventional method of PCR was used for amplification of the DNA fragment. Date palm male-specific PCR primers were designed based on the conserved SRY sequences of human and/or papaya Y chromosome using the Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The primer pair(s) was tested on DNA of male and female date palm genotypes using PCR amplification. Each PCR reactions was carried out in a 50 μl of the reaction mix containing 1 \times PCR buffer, 200 μM dNTP mix, 0.2 μM each of forward/reverse primer, 1.5 mM MgCl_2 , 1.5 U Taq DNA polymerase, and 100 ng of template DNA. The reaction conditions for PCR involved initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. To amplify male-specific SRY DNA fragment, SRY-150F (5-AGC AGT CAG GGA GGC AGATCA 3 V) was used as forward primer and SRY-245R was used as reverse primer as described by Lo et al. (1998).

The annealing temperature was standardized according to the specific T_m of the primer pair. After amplification, an aliquot of the reaction mixture was loaded onto a 0.8% agarose gel containing in 1X TBE buffer and checked by staining with ethidium bromide, and visualized under ultraviolet (UV) light. The gel picture was captured using Bio-Rad documentation system.

2.2. Isolation of SRY gene

2.2.1. Restriction digestion and End-filling with klenow

The genomic DNA of the date plant was digested overnight at 37 °C using 40 units of enzyme, making a final volume of 100 μl as described by Reddy et al. (2002). Enzymes used were BamHI and BglIII Sou3A, while the digested DNA was partially end-filled by dGTP and dATP. The end-filling reaction mixture contained dNTPs, 10X reaction buffer, klenow (1 μl) in reaction volume of 25 μl and

was incubated at 37 °C for 1 h. The purified DNA was extracted as described by Mohei El-Din (2002) and Mahmoud et al. (2009).

2.2.2. Adaptor annealing and ligation

The adaptors were generated according to the methods developed by Mohei El-Din (2002) and Mahmoud et al. (2009). For this purpose, oligonucleotides used were: 03301954 solliman-1 5'-AA TACGACTACTATAGGGCGGCCCGGGC-3' and solliman-2 (5'-C ACTATACCGCCGGCGGCCCGCT-3'.

Oligonucleotides solliman-1 and solliman-2 (synthesized by MacroGen, Korea) were re-suspended in sterile double-distilled water at a concentration of 100 pM/ μL . A volume of 20 μl for each adaptor was piped into a 0.5 mL Eppendorf microfuge tube and overlaid with mineral oil. The adaptors were then heated at 99 °C for 4 min in a beaker of water. The heat was removed, and the solution was allowed to cool for 1 h at room temperature. The annealed adaptors were decanted from under oil and stored at –20 °C.

After which, 10 μl of the genomic restriction digest was ligated to 1 μl of the annealed adaptors with 2 μl of T4 DNA ligase buffer and 2 μl of T4 DNA ligase (5units/ μL) in a 20 μl reaction. The ligation was incubated overnight at 12 °C and heat inactivated at 65 °C for 10 min. Also, 180 μl volume of TE (pH 8) was added to the ligation mix called the adaptor library. Excess primer-adaptor was removed by purification through QIAquick Columns (Qiagen).

2.3. Primary PCR amplification

The adaptor-ligated genomic DNA was diluted and used for the first PCR amplification in a 50 μl reaction volume. For amplification, the DNA was first denatured once for five minutes at 94 °C, followed by 35-cycles of PCR amplification. For each cycle, the DNA was denatured for 40 s at 94 °C, annealed for 40 s at 50 °C and elongated for two min at 72 °C. Adaptor primer T7 sequences are 5'- AATACGACTACTATAGGGC-3' and SRY-2 R (gene-specific primer, Date-SRY) was used for amplification. The sequence of the SRY-1 R primer is given: 5'- GGGCTGTAAGTTATCGTAAAAG GAGC-3'.SRY_2R; 5'-CCTAGCTGGTCACGTTGACCTTTTGTC-3'. Genomic DNA amplified with T7 alone served as negative control as methods developed by Mohei El-Din (2002) and Mahmoud et al. (2009).

2.4. Secondary amplification

Amplified products (10 μl each) were electrophoresed on a 0.8% agarose gel and processed to check for amplification. The remaining PCR products of the primary amplification were used as template after purification by phenol extraction. After precipitation, the DNA was re-suspended in 20 μl T10E1. Various dilutions of the excised band were PCR amplified using T7 and SRY-2R primers. The same reaction composition and cycle parameters used were as described for the primary PCR amplification.

2.5. DNA sequencing

Amplified DNA fragments of different sizes resulted from primary and secondary amplifications were sequenced by an automated sequencing machine such as Applied Biosystems 3730xl and 9 ABI 3700 in MacroGen Company, Korea.

2.6. Analysis of nucleotide sequence by homology and structural comparison of SRY-date to other SRY genes

Most of the sequence (DNA) analyses were performed using CLC Vector program and Genbank database. Homology searches were done using FASTA, while multiple sequence alignment was done using CLUSTALW (Hobohm & Sander 1995; Stultz et al., 1993).

3. Results

As a developmental process, the determination of sex especially in crop plants is of fundamental importance due to its economic implication. The present work reports on the molecular techniques used for determination of sex (male and female) in date palm cultivars. The emphases were on the strategy, which facilitate confirmation, and identification of the male and female plants.

3.1. - Development of a strategy to isolate date SRY gene

As a dioecious plant, date palm plant harbors XY sex chromosomes, thus has the potential to serve as an important model plant for sex determination. For the first time, the researchers investigated the existence of SRY-related sequences in Date palm cultivars. To isolate the date-SRY gene, PCR technique was used with the primers that were used to identify homologue of the conserved motif of the SRY gene from human. The analysts investigated the conserved sequences between forward and reverse primers of SRY for possible primers that produced smaller PCR products with the SRY primers in different regions of human and papaya genome. Henceforth, the investigators decided to use the same reverse primer SRY-1 R sequence as used in papaya (Yu et al., 2007, 2008). For forward primer, however, the researchers have chosen the adapter sequences as described in material and methods to the upstream from the reverse primers as mentioned by Moheï El-Din (2002) and Mahmoud et al. (2009).

The probes tested the amplification protocol using two annealing temperatures 48 °C and 55 °C. For male variety of date palm, no differences in amplification products between the two temperatures were observed (data not shown). In addition, the present protocol also utilized the use of a primer that enriched specific template prior to nested PCR. Also, one of the adapter strands was blocked at the 3' end by attaching an amine group. Using this method, a number of unknown 3' and 5' regions of known SRY genes were isolated earlier from humans and papaya (Yu et al., 2007, 2008).

3.2. - Isolation of date palm genomic DNA and amplification of putative SRY-gene using PCR

The genomic DNAs isolated from both male and female date palm plants were measured for their quality and quantity. The ratio of A260/A280 for each sample was around 1.8 and quantity of DNA among 10 samples varied from 320 µg/ml to 805 µg/ml (data not shown).

Quality undigested date palm genomic DNA, including Burhi, Khalas, and Sukkari were isolated from females and males of different cultivars grown in Al-Ahsa Oasis, King Saudi Arabia. The researchers ran it in 0.8% agarose gel in 1X TBE buffer stained with ethidium bromide as shown in Fig. 1. From visual observation of the DNA in the gel, quality and quantity of DNA looks great. DNA samples were digested with BamHI, BglII, or Sou3A restriction enzymes (data not shown). The digested ends were partially filled with A and G nucleotides to prevent self-ligation of the DNA fragments. These partially filled genomic DNA fragments were ligated with adapter-primers (ADOP-32 and ADOP-27) as the adapter-primers were designed to anneal with partially filled genomic DNA. The ADOP-32 primer contained sequences of the T7 primer.

The 3'-end of the ADOP-27 primer was blocked to prevent extension during PCR amplification (Moheï El-Din, 2002; Mahmoud et al., 2009). The ligated DNA served as a template, while the SRY sequences were PCR-amplified with T7 and SRY-1R primers. Along this line, amplified DNA fragments of different sizes were obtained. The amplified DNA was used as template for the

second PCR reaction with the T7 and SRY-2R primers (Fig. 2A). As an example, a band of amplified DNA of approximately 0.45 kb was observed in male plants of all three varieties of date palm tested in this investigation. The amplified DNA was purified and cloned into the pGEM-T vector to create pGEMT-SRY and verified through PCR method (Fig. 2B) (see Figs. 3A and 3B).

3.3. Sequencing of putative date palm SRY

Several primary amplified DNA fragments of different sizes were sequenced and the nucleotide sequence data of a 914-bp fragment is shown below.

1	gggggggatac cagttacca aat cggcctctaagatctgtgcgaacg gccagacatc
61	tttagaggcc acttctgcga ttctgaagc gacccttgag agcattcatc gagtggcttc
121	cgtcctcagc cgtggatggc tctcgagatc cccaatgcg acactctgag atcacctac
181	gactgggata ccggtgataa aatgctcact ggagccaagg gatggacctt cttcccgagg
241	gttcagagat ttcaggccat gcacataaat aaatgcacga gttataata tccactctc
301	ctgaaggcga aaatactgcc gaagacttgc agtttgcttc ccgctcatcc cgcttctgta
361	ctctgcttcg aagtgcaaac tggacaacag gtaagcgggg gaaatggagg gaacagggtg
421	tgcatagatt tcggagatc gaacaaattg gttggcactt taggggtata caatatact
481	tttccgttat aagccttaat ggaaggaaag gtgggggaaa tccattttgg attgagaaat
541	cgatgtgccg gtgcttaact tataatccag attgaaactt cgtgttcgtg atcaggaccc
601	agcaccaaca ccagcgtagg gggcgggcca caataccacc atagcggttc tagtagcagc
661	aaaaaatagt ggcatacaa acataagtag tagaacatga gtagcagaag tttaccggc
721	gcaataaccg gtggcactag tagcagaagc ataggctttt tgggtaccgg gcagaggcaa
781	ggcatcgaat ccctcatcaa gcccatccac atcaatagct cactaccac caacatcgaa
841	ataaggggat gactcgactg gcgaatcagc tgcaataatt ggactcgacc agtgcaactg
901	gaacaacagg taaa

3.4. - Putative SRY sequence analysis

The SRY fragment was 355-bp in length as shown in Fig. 7. GenScan and CLC analysis showed that DNA fragment had a 3' end homology to 3' ends of human and papaya SRY. The full-length open reading frame (ORF) was 355 bp in length. The SRY fragment assemblies and identification of sequence were carried out with CLC Genomics Workbench (CLC bio). BLAST results showed that the predicted ORF had 84% similarity to the published sequence amplified with human primers, indicating a high degree of similarity with human SRY (Fig. 6). The full-length sequence of SRY was obtained with a modified genomic "walking" method, which combined vectorette and suppression PCR "walking" and amplification. Full-length SRY was 355-bp includes the putative transcription start site and open reading frame. The open reading frame encoded for 182 amino acids and had 86% similarity to the published sequence amplified with papaya primers (protein_id = "AAB58342.1"), indicating a high degree of similarity

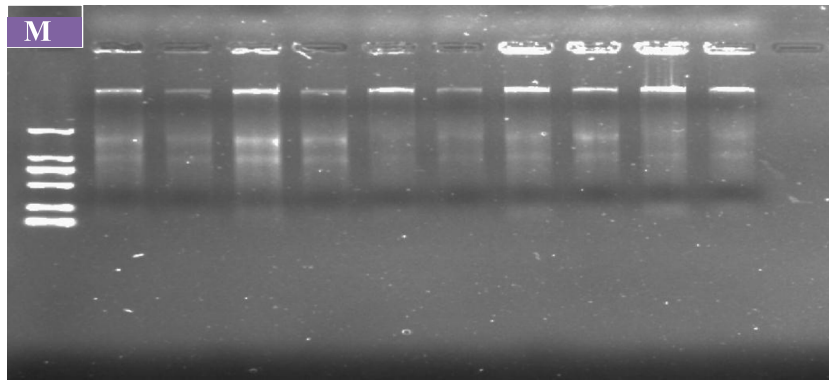


Fig. 1. A Photograph of a gel showing DNA isolated from female and male plants of different cultivars of date palms growing in Al-Ahsa Oasis, KSA (Burhi, Khalas and Sukkari). Legend: f = female plant and m = male plant.

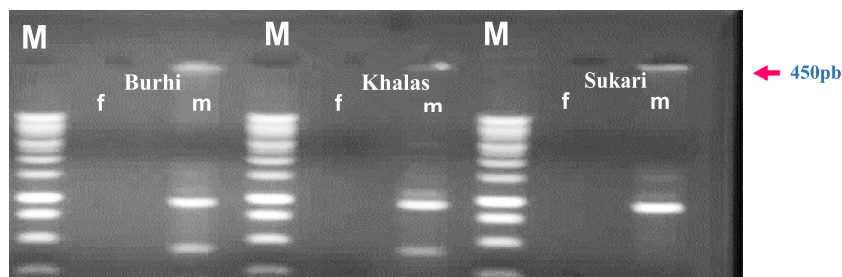


Fig. 2A. PCR amplification while screening of the presence of SRY gene from different varieties of Date palm (Burhi, Khalas and Sukkari) using specific primers Sry1 F + Sry1 R lane M DNA 1 KB Marker. Legend: f = female plant and m = male plant.

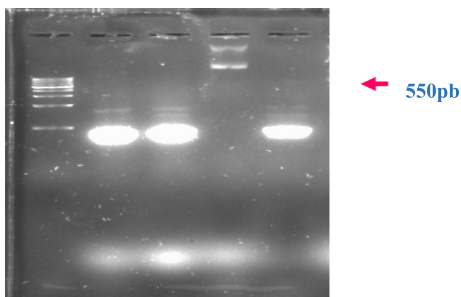


Fig. 2B. PCR Amplification products for clone of SRY full fragment after cloned in pGEMT vector (lane 2, 3 and 5 for Burhi, Khalas and Sukkari) and lane 4 for pGEMT vector only as control and lane M DNA 1 KB Marker.

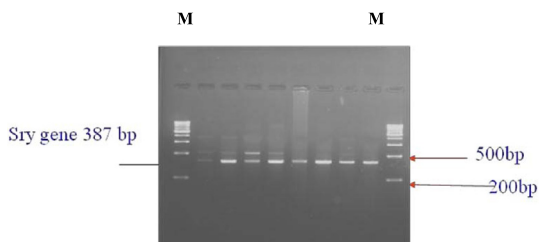


Fig. 3A. PCR amplification for screening of the presence of SRY (387 bp) gene in lane (2–9) date palm male plants with specific primer for gene only SRY-date-F and SRY-date-R primers lane (1 and 10) M DNA 1 KB Marker.

with papaya SRY (Fig. 5). The deduced DNA sequence also showed extensive homology with human SRY sequences (Fig. 6). The template is likely to be amplified in linear using this method, leading to a significant amount of noise. All the protocols of Reddy et al.

(2002) was used in this study involving restriction digestion of genomic DNA followed by partial filling to prevent self-ligation between fragments. In addition, the present protocol also employed biotinylated primers to enrich for the specific template prior to performing nested PCR. One of the adopter strands was also blocked at the 3' end by attaching an amine group (Reddy et al., 2002).

Computer-aided sequence to identify date-SRY. A partial sequence of the SRY gene is shown in Figs. 5 and 6. Computer-aided sequence analysis of Date-SRY revealed the ORF of SRY. Most of the sequence (DNA) analyses were performed with the CLC Vector program and Genbank database. Fig. 5 shows the homology searches with forward primers for SRY. Although there was a very low alignment, a very high degree of similarity was found when compared with *P. dactylifera L.*, complete genome Sequence ID: (Accession number: gb|JN375330.1). Homology searches were performed with BLAST. Multiple sequence alignments were performed with CLUSTALW.

3.5. - Deposition of isolated SRY DNA fragments into GenBank

Similar approach was adopted to isolate about 355-bp lengths DNA fragment of Date-SRY gene by walking the genomic DNA using primers SRY 1 R, SRY 2 R and T7 primer. The complete sequence of the DNA was presented in Fig. 3 and Fig. 4. The sequence information is also deposited in a public data base GenBank (BankIt1598036 DPSRY1 KC577225).

A PCR primer pair according to this experiment and invention consists of: a forward PCR primer consisting of 20 consecutive nucleotides of the sequence of nucleotides flanking, in 5', and a reverse PCR primer comprising, or consisting of, between 20 and 25 consecutive nucleotides of the sequence complementary to the sequence of at most nucleotides flanking, in 3'. The sequence of the SRY-F primer is SRY_F 5'- AAAGTGTAGTGTGTCACACTCAT-3'.

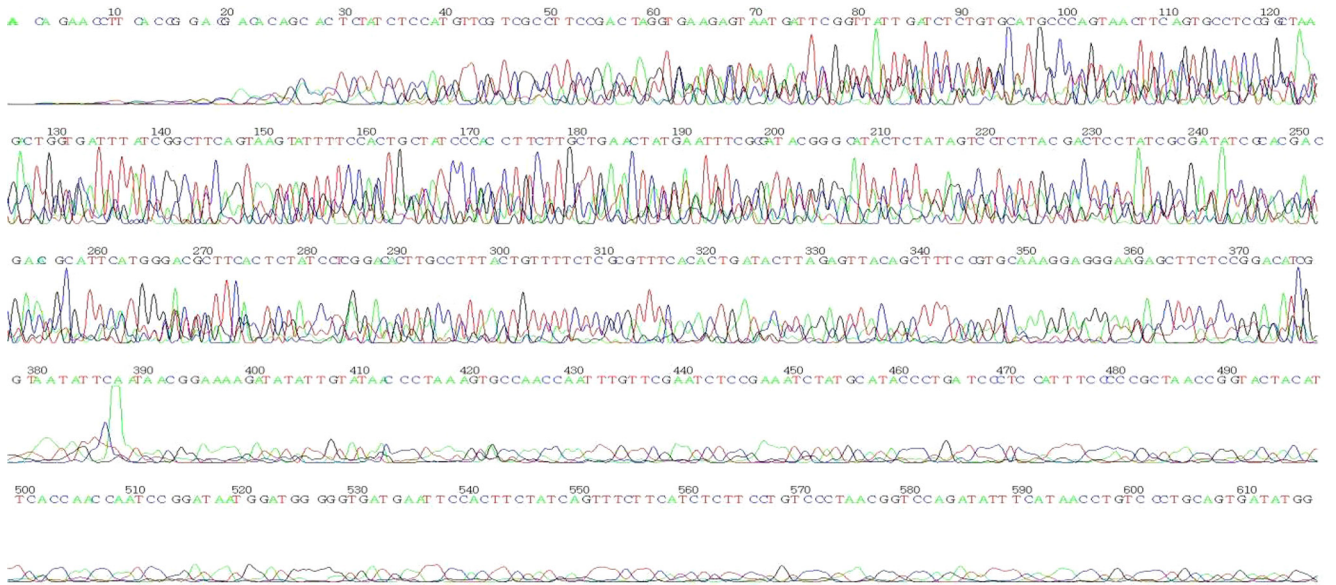


Fig. 3B. Reverse sequences of SRY gene, a sex determination gene from date palm (SRY-Date). The nucleotide sequence of the SRY full-length gene after isolated from Date palm and sequencing using reverse primer.

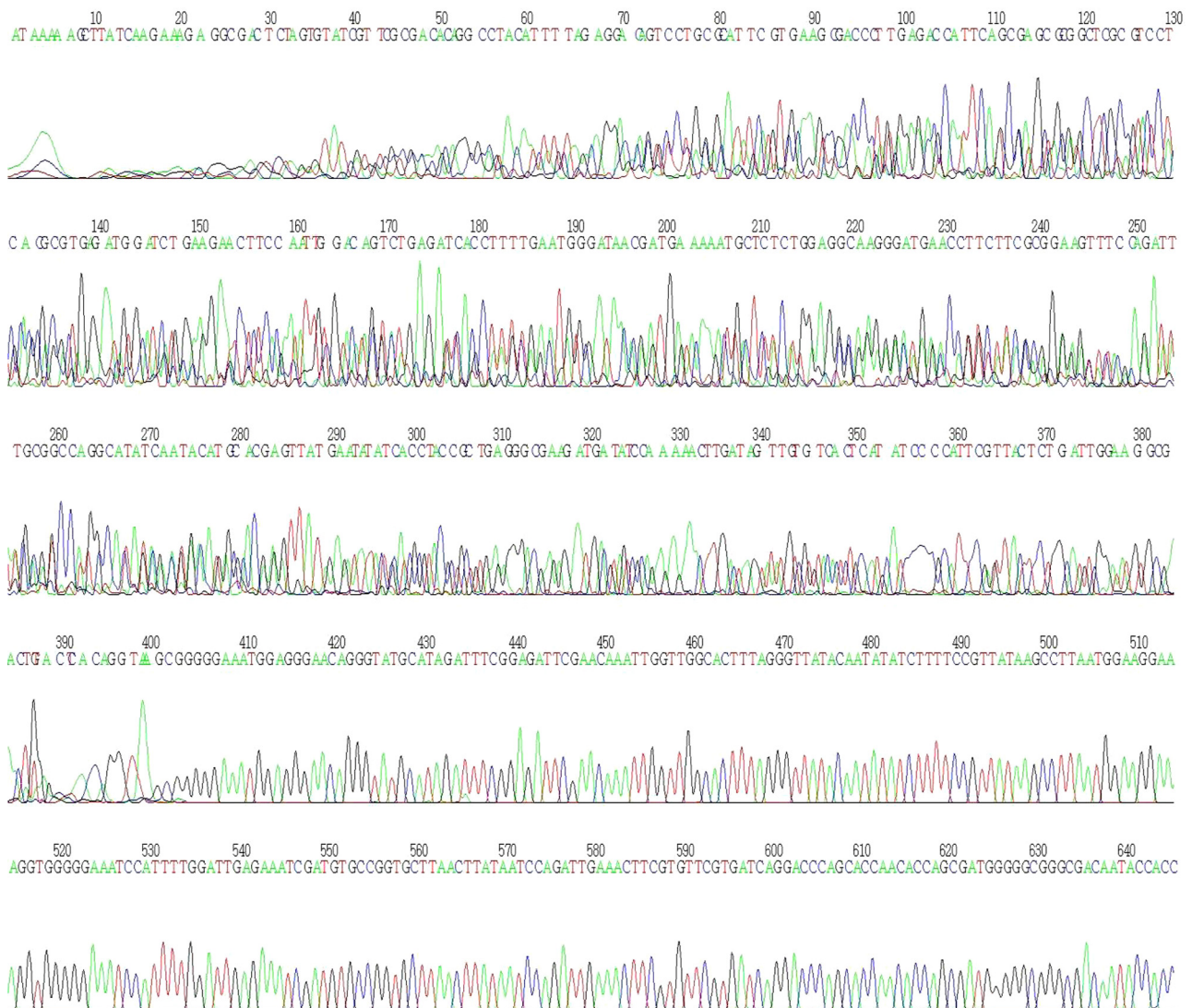


Fig. 4. Forward sequences of SRY gene, a sex determination gene from date palm (SRY-Date).

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Query 46 TCGCCTTCCGACTAGGTGAAGAGTAATGATTCGGTTATTGATCTCTGTGCATGCCAGTA 105
      |||
Sbjct 406 |||TCGCCTTCCGACGAGGTCGATACTT 347
      ATAATTCGGGTATTTCTCTCTGTGCATGGCCTGTA

Query 106 ACTTCAGTGCCTCCGGCTAAGACTGGTGATTTATCGGCTTCAGTAAGTATTTCCACTGC 165
      |
Sbjct 346 |||ATTTCTGTGCCTC 288
      CTGG-AAGAATGGCCATTTTCGGCTTCAGTAAGCATTTCCTACTGG

Query 166 TATCCCACCTTCTTGCTGAAGTATGAATTTTCGCGATACGGGCATACTCTATAGTCTCTT 225
      |||
Sbjct 287 TATCCCAGCTGCTTGCTGATCTCTGAGTTTCGC-ATTCTGGGATTCTCTAGAGCCATCTT 229

Query 226 ACGACTCCTATCGCGATATCGCACGACGAACGCATTTCATGGGACGCTTCACTCTATCCTC 285
      |||
Sbjct 228 |||GCGCCTCTGATCGCGAGACCACACGATGAATGCG 170
      TTCATGGGTCGCTTCACTCTATCCT-

Query 286 GGACACTTGCCTTTACTGTTTTCTCGCGTTTCACACTGATACTTAGAGTTACAGCTTTCC 345
      |||
Sbjct 169 |||GG 111
      AC-GTTGCCTTTACTGTTTTCTCCGTTTCACACTGATACTTAGAGTTACAGCTTTCA

Query 346 GTGCAAAGGAGGAAGAGCTTCTCCGGACATCGGTAATATTC 387
      |||
Sbjct 110 GTGCAAAGGAAGGAAGAGCTTCTCCGGAGAGCGGAATATTC 69

```

Fig. 5. Comparison of the DNA sequences of SRY-date with SRY genes from papaya (Accession number: **gb|AF000024.1|CPAF000024**). The nucleotide sequence of the cloned SRY Sequence Alignment Editor.

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Query 46 TCGCCTTCCGACTAGGTGAAGAGTAATGATTCGGTTATTGATCTCTGTGCATGCCAGTA 105
      |||
Sbjct 494 |||TCGCCTTCCGACGAGGTCGATACTT 435
      ATAATTCGGGTATTTCTCTCTGTGCATGGCCTGTA

Query 106 ACTTCAGTGCCTCCGGCTAAGACTGGTGATTTATCGGCTTCAGTAAGTATTTCCACTGC 165
      |
Sbjct 434 |||ATTTCTGTGCCTC 376
      CTGG-AAGAATGGCCATTTTCGGCTTCAGTAAGCATTTCCTACTGG

Query 166 TATCCCACCTTCTTGCTGAAGTATGAATTTTCGCGATACGGGCATACTCTATAGTCTCTT 225
      |||
Sbjct 375 TATCCCAGCTGCTTGCTGATCTCTGAGTTTCGC-ATTCTGGGATTCTCTAGAGCCATCTT 317

Query 226 ACGACTCCTATCGCGATATCGCACGACGAACGCATTTCATGGGACGCTTCACTCTATCCTC 285
      |||
Sbjct 316 |||GCGCCTCTGATCGCGAGACCACACGATGAATGCG 258
      TTCATGGGTCGCTTCACTCTATCCT-

Query 286 GGACACTTGCCTTTACTGTTTTCTCGCGTTTCACACTGATACTTAGAGTTACAGCTTTCC 345
      |||
Sbjct 257 |||GG 199
      AC-GTTGCCTTTACTGTTTTCTCCGTTTCACACTGATACTTAGAGTTACAGCTTTCA

Query 346 GTGCAAAGGAGGAAGAGCTTCTCCGGACATCGGTAATATTC 387
      |||
Sbjct 198 GTGCAAAGGAAGGAAGAGCTTCTCCGGAGAGCGGAATATTC 157

```

Fig. 6. Comparison of the DNA sequences of SRY-date with SRY genes from human (Homo sapiens clone 15 SRY (SRY) gene, complete cds, Accession number: **gb|JQ811918.1**).

The sequence of the SRY-1 R primer is 5'- GGGCTGTAAGTTATCG TAAAAGGAGC-3' and SRY_2 R is 5'- CTAGCTGGTACGTTGACCTT TTGTC-3.

The amplified DNA fragment was sequenced using reverse primer (Figure 3) and forward primer (Fig. 4). Using the vector-specific T7 and SP6 primers, the cloning of the SRY gene was further confirmed with subsequent sequencing (Figs. 3 and 4).

The sex was identified as male after additional PCR reactions using primers specific for the Y chromosome (SRY and universal primer). The SRY marker has been previously shown to yield, a 350-bp to 400-bp PCR products that overlaps in size with human (Drobnic 2006) and papaya (Yu et al., 2007, 2008) sequences. Amplification of samples from the male plants had only one band of 355-bp, (Fig. 7). The sequence of the SRY forward primer is determined as SRY-date-F 5'- **cgccctctaagtatctgtcgcaacg** - 3'.

The sequence of the SRY-date-R reverse primer is determined as SRY-date R 5'- **gtttgacttcgaagcagag** -3'.

A genomic fragment of approximately 355-bp was amplified from three date palm cultivars using specific primers identified from the human and papaya SRY sequence information.

The sequence was deposited in Genbank with the Accession number (BankIt1598036 DPSRY1 KC577225 and final released in GenBank: KJ873056.1).

/translation of the sry gene = "MALEIPQCDTLRSPIDWDTG
DKMLTGAKGWTFPFPEVQRFQAMHI
NKCTSYKYPPPLKAKILPKTCSLLPAHPASLLCFEVQTGQQVSGGNGG
NRVCIDFGDS
NKLVTGLGLYNISFPL"

plants. Moreover, the use of these techniques are simple and unique. Likewise, these are “universal” in the sense that it can be used to identify male plants regardless of the origin, variety or cultivar of the date palm.

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