International Journal of Advanced Biotechnology and Research(IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 6, Issue2, 2015, pp144-151
http://www.bipublication.com

Research Article

Sex-Differentiation Based on Fluorescence *In Situ* Hybridization (FISH) with 5s and 45s rDNA of Egyptian Date Palm Trees

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[Received-22/03/2014, Published-01/04/2015]

ABSTRACT:

The date palm (*Phoenix dactylifera* L.), 2n=36, is a dioecious long-lived monocotyledonous plant, which belongs to the family Arecaceae. It is one of the excellent candidate crops in arid and semiarid regions of the world. Recently, fluorescence *in situ* hybridization (FISH) has become a powerful and useful tool for the direct detection of specific DNA fragments in the genome. In this technique, ribosomal DNA genes (45S and 5S rDNA) are commonly used as markers for the physical mapping of plant chromosomes to analyze genomic organization. To date, there is no report on rDNA loci in *Phoenix dactylifera* detected by FISH. The objective of this study was to develop a sex-specific fluorescence *in situ* hybridization (FISH) markers with 5S and 45S rDNA in Egyptian Date Palm Trees (cv. Zaghloul and Siwi). The results successfully revealed clear differences between males and females belonging to both cv. Zaghloul and Siwi by using 45S rDNA FISH. The fluorescence *in situ* hybridization (FISH) with 45S rDNA localized two clear telomeric intermediate-signals in female palm trees belonging to cv. Zaghloul and Siwi. While, it exhibited three clear telomeric intermediate-signals in male trees belonging to cv. Zaghloul and Siwi. On the other hand, results of fluorescence *in situ* hybridization (FISH) with 5S rDNA didn't reveal any clear differences between males and females belonging to cv. Siwi. Based on the above results, we hypothesize that the third signal (unpaired signal) of 45S rDNA probe characterized in both Siwi and Zaghloul males is located on a male chromosome (Y chromosome). This finding can be utilized and used as cytological marker to differentiate between male and female trees in Egyptian date palm at an early stage.

KEYWORDS: Date palm, Fluorescence in situ hybridization (FISH), 5S rDNA, 45S rDNA, Sex determination.

[I] NTRODUCTION

Date palm (*Phoenix dactylifera* L., 2n=36) is outstanding for the economic value of its different parts and its socio-economic significance in agrosystems of arid zones. In addition to its valuable fruit, the tree is cultivated for fuel, fiber and as shelter for ground crops. Annual world date production is about 3.7 million tons [1]. Egypt is the leading date production country in the

world (FAO, 2011). Egypt has very long history of date cultivation that dates back to 3200 BC. Yet the success of breeding programmes has been very low, due to the wide gap between pure science (genetics, physiology and plant pathology) and applied science concerning this species [2]. There is a general lack of understanding of the genetics of date palm or palm in general.

The identification of sex chromosomes in plants is problematic because most of them do not differ morphologically from autosomes or from one to another [3]. For example, in some species such as *Actinidia deliciosa* [4], X and Y chromosomes are too small to support observations of their distinguishing characteristics.

In date palm, a dioecious mode (separate male and female individuals) and the late initial reproductive age (5–10 years) are major practical constraints for genetic improvement. One male is used to hand pollinate about 90–100 females [5], and for centuries emphasis has been on clonal propagation of females. This reduces the genetic diversity of the cultivars, accelerating vulnerability to biotic and abiotic stresses. Early selection on young seedlings could enhance breeding programs and generate experimental male and female genetic stocks, but no cytogenetic protocol exists for sex determination in an immature date palm.

Date palm is cytogenetically recalcitrant material, having tiny and sticky chromosomes [6-8]. Classical Giemsa C-banding has not allowed plant cytogeneticists to differentiate between male and female individuals [9]. More recently, *in situ* hybridization (ISH) technique, developed more than 30 years ago [10-11], has proved to be a powerful technique in cytological biology studies. Jiang and Gill [12] stressed florescent *in situ* hybridization (FISH) as one of the most important techniques in plant molecular cytogenetics research, because it allows DNA sequences to be mapped directly on chromosomes [13-18].

Ribosomal DNA (rDNA) has highly conserved repetitive sequences in the plant genome, and the polymorphism or conservatism of their copy number and chromosomal localization are visual and comparative [19-21]. By comparing the number and distribution characteristics of rDNA sites on the chromosomes among species, interspecific phylogenetic relationships and the related mechanism of speciation and chromosomal evolution could be revealed.

Fluorescence *in situ* hybridization (FISH) of repetitive DNA sequences has been used as a tool for karyotype and genome analyses of a large number of plant species. The repetitive and tendemly organized ribosomal rDNA genes, 5S rDNA and 45S rDNA, are localized at one or more sites per chromosome set, and their characteristic positions provide useful markers for chromosome and genome identification. They have been used in several grasses such as *Triticum* and *Hordeum* [12], *Oryza* [22], *Sorghum* [23], *Thinopyrum* [24], *Festuca* [25], providing useful information about evolutionary and phylogenetic relationships between species.

Therefore, the present study aims to localize the chromosomal positions of 5S and 45S rDNA sites using fluorescence *in situ* hybridization (FISH) to develop cytological-based markers that can differentiate between male and female in Egyptian date palm trees.

[II] MATERIALS AND METHODS

2.1. Plant Material

Root tips from male and female of two Egyptian date palm cultivars (Siwi and Zaghloul) were used as a source for cytological preparation for fluorescence *in situ* hybridization (FISH) analysis.

2.2. Development of FISH Markers

2.2.1 DNA Probes

For sex chromosomal identification, two different universal probes (5 rDNA and 45 rDNA) were used.

2.2.2 Chromosome preparations

Chromosome preparations were carried out according to Jiang et al., [26] with some modification. Roots 1-2 cm long were cut, soaked in 2mM 8 hydroxy-quinoline for 4 hours and

directly fixed in ethanol/glacial acetic acid (3:1) for several hours. Then fixed root tips were stored in 70% ethanol. The root tips were enzymatically digested with 4% cellulase R10 and 1% pectinase y-23 for 40 min at 37°C. The enzymes were carefully washed from the softened material and replaced with distilled water for 10 min and finally fixed in 1:3 acetic acid and methanol in ice. For slide preparation one root tip was placed on the slide and one drop of fixing solution was added. The slide was dried for less than one sec. on a Bunsen burner flame and then left to completely dry at room temperature.

2.2.3 Fluorescence *in situ* hybridization

FISH protocol was adopted as described by Jiang et al [26] with slight modification. About 50ng of labeled DNA was used for each slide in a hybridization mixture containing 40% deionized formamide / 20% dextran sulfate / 2x SSC / 10ug of salmon sperm DNA / = 1ug of date palm genomic DNA. The mixture was denatured at 80 °C for 10min. Slide-bound chromosomal DNA was denatured in a solution of 70% formamide in 2x SSC for 2 min at 80 °C and dehydrated in -20°C ethanol series (70%, 90%, and 100% ethanol, 5 min each). Twenty microliters of hybridization mixture was applied to each slide and the cover sealed with rubber cement. The slides were then incubated over night at 37 °C, and then washed. Washing was performed in 4 steps: in 2x SSC for 5 min at room temperature, 2x SSC for 10 min at 42 °C, 2x SSC at room temperature for 5 min and 1x PBS (phosphate - buffered saline) at room temperature for 5 min. The biotinylated probes were detected with fluorescein isothiocyanate conjugated avidin (FITC - avidin) and the dig-labeled probes were detected with rhodamine - conjugated anti-dig antibody (Boehringer Mannheim). DAPI (4,6-Diamidino-2-phenylindole) was used as a chromosome counterstain in multicolor FISH.

2.2.4 Digital imaging

Slides were examined under an Olympus BX-60 fluorescence microscope. Chromosome and FISH signal images were captured using a SenSys CCD (charge-coupled device) camera connected to a Macintosh computer (Photometric, USA) and analyzed using IP-lab spectrum software (signal Analytics .USA). The three images were captured separately with SenSys camera and merged into single image using the IP-Lab and Photoshop (Adobe system) imaging software.

[III] RESULTS

3.1. Localization of the 5S and 45S rDNA Sites

3.1.1 FISH analysis of cv. Zaghloul

The fluorescence in situ hybridization analysis (FISH) revealed two sites for the 45S rDNA in the Zaghloul female. These two strong red large-sized signals were localized at the telomeric region of two separate and short chromosomes of date palm chromosomes (**Figure 1a**). While, for the Zaghloul male cytotype, a different organization was observed. Three red large-sized signals of 45S rDNA were detected and localized on the terminal end of three short date palm chromosomes (**Figure 1b**).

3.1.2 Bicolor FISH analysis of cv. Siwi

The bicolor fluorescence *in situ* hybridization analysis (45S rDNA: green signal and 5S rDNA: red signal) revealed two sites for both the 45S rDNA and 5S rDNA in the Siwi female. The two sites for 5S rDNA characterized as two red intermediate-sized signals localized at the sub-metacentric region of two chromosomes in Siwi female. Meanwhile, the two sites for 45S rDNA characterized as two clear green intermediate-sized signals localized at the telomeric region of two separate chromosomes in Siwi female (**Figure 1c**). Whereas, the Siwi male cytotype revealed a different organization comparing with Siwi female; especially for 45S rDNA sites. The two sites for 5S rDNA characterized as two green intermediate-sized signals localized at the sub-metacentric region of two separate chromosomes as in Siwi female cytotype. While, three sites for 45S rDNA were characterized as clear green intermediate-sized signals localized at the

telomeric region of three separate chromosomes there were only two sites characterized in Siwi female cytotype (**Figure 1d**).

Based on this observation, we hypothesize that the third unpaired signal of 45S probe characterized in both Siwi and Zaghloul males is located on a sexual chromosome, more specifically Y chromosome.

[IV] DISCUSSION

Heterochromatin, which at the molecular level is mainly composed of various types of repetitive DNA sequences, is frequently associated with telomeric, centromeric, and pericentromeric regions, as well as the nucleolar organizers in plant genomes [27-28]. The 5S ribosomal DNA (rDNA) genes of higher plants are usually organized into clusters of tandem repeats with thousands of copies at one or more positions in the genome. Each repeat consists of a highly conserved 5S rRNA coding region of approximately 120 base pairs in length and of non-transcribed spacer (NTS) regions that vary in size between 100 and 700 base pairs. Most repeats appear to be uniform in a species.

On the basis of the high degree of stability during the course of evolution, comparative studies of the nucleotide sequences of rDNA genes provide a means for analyzing phylogenetic relationship over a wide range of taxonomic levels [29]. The variation in sizes and sequences of the NTS of the 5S rRNA gene was found to be useful for the phylogenetic reconstruction of species [30], and to discover differences between cultivars in barley and wheat, and between the breeding lines in maize [31]. In addition, it is interesting to note that in all plant species in which 5S rDNA blocks have been observed the blocks within a chromosome complement seem to present the same reaction to the fluorochromes.

On the other side, 45S rDNA sites are the most widely documented chromosomal regions in eukaryotes. The analysis of the distribution of these sites along the chromosome in several genera has suggested some bias in their distribution. In order to evaluate if these loci are in fact non-randomly distributed and what is the

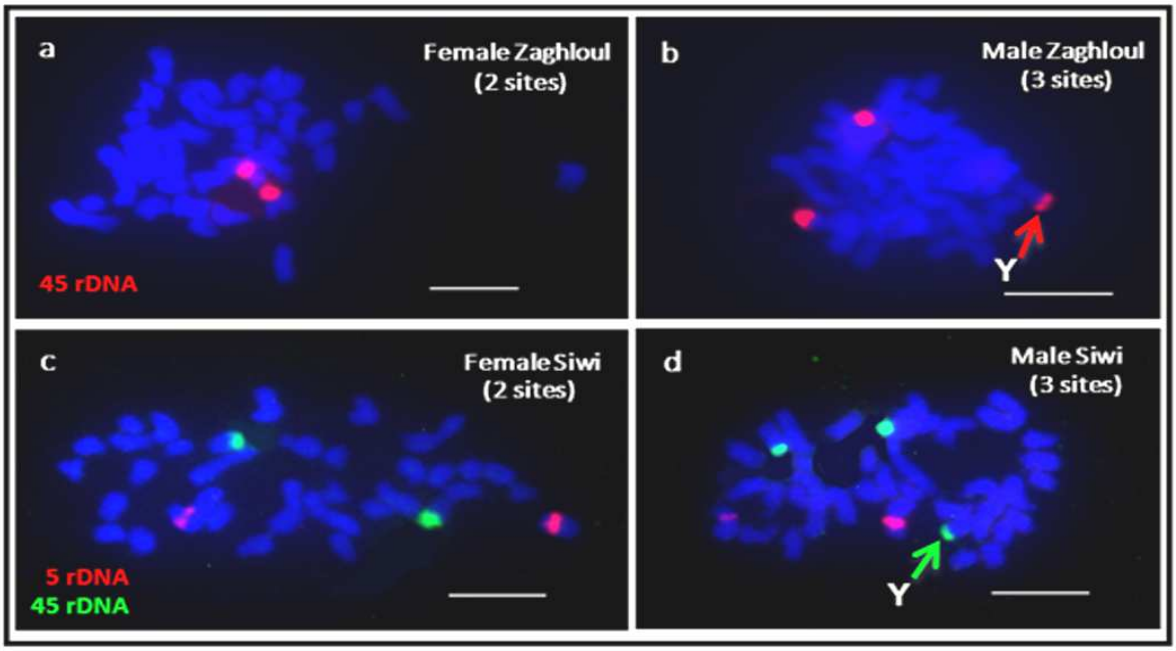

Figure 1: Fluorescence *in situ* hybridization of cv. Zaghloul (a & b : with green 45S rDNA probe) and cv. Siwi (c & d: with green 45S rDNA and red 5S rDNA probes), in metaphase chromosomes of date palm. Arrowheads in b & d indicate 45S rDNA sites suggested to be located on a Y chromosome. Scale bar=5µm.

influence of some chromosomal and karyotypic features on the distribution of these sites, a database was built with the position and number of 45S rDNA sites obtained by FISH together with other karyotypic data from 846 plant species. Moreover, Lima-de-Faria [32] showed that the secondary constrictions of mitotic chromosomes, which bear the 18S-5.8S-25S ribosomal RNA genes transcribed in the previous interphase, also called the nucleolus organizer regions (NORs) or 45S rDNA sites, were preferentially distributed on the short arms and in the subterminal region in most species of plants and animals. In agreement with above findings, Roa and Guerra [33] concluded that location of 45S rDNA sites do not vary randomly, occurring preferentially on the short arm and in the terminal region of chromosomes in angiosperms. The meaning of this preferential location is not known to date.

In this respect, at least two reports revealed association of such types of repeats (*ex.* 45S rDNA; 18S rDNA and 5S rDNA) signals with plant sex chromosomes, in liverwort [34] and spinach [35]. In other cases, 18S signals of female individuals occurred as pairs, while those of males were unpaired. Moreover, 5S rDNA has been reported in the intercalary region for wheat, and pea [36-37], while in tomato and sugar beet this site is in the region proximal to the centromere [38-39]. The tendemly array of 5S rDNA sites on the satellite has been also reported in *Vicia faba* and *Allium sativum* [40].

In contrast to the 45S rDNA site, the 5S rDNA site can only be located by FISH either in chromosomes or in interphase nuclei so that only after the popularization of this technique, in the last 2 decades, the position of the 5S rDNA sites has become widely known [41]. In most species, the loci for 5S and 45S rRNA genes are located in different chromosomes, whereas in other species the 2 loci are found in the same chromosome pair, sometimes closely linked to each other, as observed for example in different species of *Brassica* [42], *Aristolochia* [43] and *Rhynchospora* [44].

Meanwhile, Lee and Seo [45] constructed an accurate physical map showing the localization of the 5S and the 18S-26S rDNA genes in *Allium wakegi* by bicolor FISH. The signal of 5S rDNA was detected on the intercalary region of short arm of chromosome 15 (one region) and 9 (two region). while the signal of 18S-26S rDNA were detected on terminal region of short arm of chromosome 6, 10 and 14 including satellite and secondary constriction regions.

Whereas, Fabiane *et al.*, [46] reported that in *C. papaya*, just one major 18S rDNA site was observed, located in a medial position, close to the centromeric region of the largest chromosome. In contrast, two major sites and one minor site of 5S rDNA were observed, also near centromeres. Dual FISH revealed that 5S rDNA sites are non-syntenic to 18S rDNA sites. They results demonstrated that no differences were observed between cultivars or between male and female plants.

Indeed, Accumulation of transposable elements and satellite DNA has played an important role in the divergence and size enlargement of Y chromosomes [47-51].

[V] CONCLUSION

The fluorescence *in situ* hybridization (FISH) with 45S rDNA revealed a successful differentiation between males and females belonging to both cv. Zaghloul and Siwi in Egyptian date palm. Meanwhile, the fluorescence *in situ* hybridization (FISH) with 5S rDNA failed to differentiate between males and females belonging to cv. Siwi. Based on our findings, we hypothesize that unpaired signal of 45S rDNA probe characterized in both Siwi and Zaghloul males are located on Y chromosome. These findings can be consequently utilized and used as cytological marker to differentiate between male and female trees in Egyptian date palm at an early stage of development.

ACKNOWLEDGEMENTS

This research project was carried out in Agricultural Genetic Engineering Research Institute and funded by the Science and Technological Development Fund (STDF) under the program of U.S.- Egypt Joint Board on Scientific and Technological Cooperation, Contract No. 406. Moreover, Authors thanking Prof. Jiming Jiang (Professor of Cytogenetics, Horticulture Dept., University of Wisconsin-Madison, USA) for his valuable support to achieve FISH analysis in his lab.

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