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Fluorescent *in situ* hybridization of 18S and 5S rDNA in papaya (*Carica papaya* l.) and wild relatives

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Abstract — Papaya (*Carica papaya* L.) is cultivated widely as a fruit crop in the tropics. Related species as *Vasconcellea cundinamarcensis* and *V. goudotiana* are prospective sources of desirable genes. The requisite germplasm introgression will be facilitated by a better understanding of their cytogenetic relationships to papaya, but the chromosomes of Caricaceae species are very small and similar so conventional karyotyping is difficult and of limited utility. Here, we report on fluorescence in situ hybridization (FISH) of rDNA probes to create several karyological markers that enable chromosomes to be distinguished and to produce information about the genetic relationships among Caricaceae species. Based on the number and the position of rDNA sites, *V. cundinamarcensis* and *V. goudotiana* were the closest species while *C. papaya* was isolated from them. Both *Vasconcellea* species showed only one pair of 5S site whereas three pairs were found in *C. papaya*. On the other hand, one 18S site was observed in papaya, whereas four and five 18S sites were found in *V. goudotiana* and *V. cundinamarcensis*, respectively. It is possible that the unpaired signal of 18S probe in *V. cundinamarcensis* is located in a sexual chromosome, however, further studies are required to confirm this hypothesis of sex-chromosome linkage.

Keywords: *Carica papaya,* FISH, genetic relationships, rDNA probes, *Vasconcellea* spp.

INTRODUCTION

The Caricaceae family includes six genera that collectively encompass 35 species (Badillo 2000). All described species have 2n=2x=18 chromosomes and are regarded as diploids and dioecious, except *Vasconcellea monoica*, *V. cundinamarcensis* and *Carica papaya*, which are monoecious, monoecious-dioecious and polygamous, respectively (Badillo 1971). Papaya (*Carica papaya* L.) is the most economically important member of the family, but *Vasconcellea* is the largest genus of this family (Badillo 2001) and could be an important source of desirable traits for papaya improvement, such as resistance to PRSV-P (Horovitz and Jimenez 1967; Magdalita *et al.* 1988), cold tolerance and higher sugar content (Manshardt and Wenslaff 1989; Drew *et al.* 1998). The degree to which *Vasconcellea* germplasm can be used for papaya improvement, however, is ultimately most likely to depend on genomic relatedness, particularly chromosome structural similarities, meiotic homology, recombination and genetic compatibilities. Thus, comparative genetic and karyotypic characterization of these taxa would be an important step toward this prospective gene introgression.

Several studies have begun to define interspecific and intergeneric relationships in Caricaceae, based on the use of various molecular markers, including RAPD (Jobin-Decor *et al.* 1997), RFLP (Aradhya *et al.* 1999; Van Droogenbroeck *et al.* 2004), AFLP (Kim *et al.* 2002; Van Droogenbroeck *et al.* 2002; Kyndt *et al.* 2005) and SSR (Kyndt *et al.* 2006). They reveal a large genetic distance between *Carica* and *Vasconcellea*. At the genomic level, it is known that papaya has just 18 chromosomes (n=9), all of which are very short, between 1.0 and 4.25μm at mitotic metaphase (Datta 1971). The 1C DNA content level is estimated to be 368 Mbp by Bennett and Smith (1976), 372 Mbp (~3.8 pg) by Arumuganathan and Earle (1991) and 404 Mbp by Ohri *et al.* (2004). Although *C. papaya* can be dioecious,

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there have been no reports of heteromorphic or unpaired chromosomes, i.e., characteristics that might be expected of evolutionarily advanced sex chromosomes. Yet, an evolutionarily nascent sex-determining region has been defined (LIU *et al.* 2004). Overall, the small sizes of papaya chromosomes, absence of heteromorphic pairs and lack of cytomorphological markers have presented a difficult target for conventional karyotyping of papaya, thus additional approaches are needed.

A relatively new technique for exploring genomic and chromosomal relationships among species is fluorescent *in situ* hybridization (FISH). The fluorescent signals that demarcate FISH probe hybridization sites can be used as cytological markers for chromosome identification. In eukaryotes, tandemly repeated sequences such as 18-28S and 5S ribosomal genes (rDNA) are especially amenable to detection by FISH, and are localized at one or a few chromosome sites. In situ hybridization of rDNA can be used as convenient markers to distinguish chromosomes especially for species with small or similarly sized chromosomes (LEITCH and HESLOP-HARRISON 1992). Here, we examine application of this principle within the Caricaceae family. The main goals of this work were to localize the 18S and 5S rDNA sites in *C. papaya, V. goudotiana* and *V. cundinamarcensis* by dual-FISH and to determine the numbers and the positions of hybridization sites, with the goal of providing karyological markers and generating new information about their genome structures.

MATERIALS AND METHODS

Plant material and pretreatment - Seeds from two *C. papaya* cultivars, 'Solo' and 'Maradol', *V. goudotiana* and *V. cundinamarcensis* were germinated in pots and seedlings were grown under greenhouse conditions, between May and November, at Texas A&M University, College Station, TX (30.61N, 96.32W). Root tips with 1-2 cm lengths were collected from seedlings and/or plants growing in pots in a greenhouse and pretreated with 2 µM Trifluralin solution for 21h at 4° C. Subsequently, roots were fixed in ethanol: acetic acid (4:1) overnight at room temperature.

Metaphase preparation - Mitotic chromosome spreads were prepared from fixed root tips by enzymatic digestion and spread on microscope slides (JEWELL and ISLAM-FARIDI 1994). Slides without cover glasses were evaluated for further use by examination at 100X and 250X under phase-contrast optics.

Probe DNA isolation and labeling - Plasmids pAm033, containing a 470bp *Bam*H1 fragment of a 5S rDNA repeat of *Acacia melanoxylon* in pUC118 (kindly supplied by Rudi Appels) and pGmr3, containing a 4.5kb *Eco*R1 fragment of an 18S-28S rDNA repeat of *Glycine max* in pBR325 (kindly provided by Dr. E. Zimmer) were isolated by alkaline lysis and then further purified using Plant DNeasy spin columns (Qiagen, Valencia, CA). The DNA was then labeled with biotin-16-dUTP or digoxigenin-11-dUTP with the BioNick Labeling System (Roche Molecular Biochemicals, Indianapolis, IN).

In situ hybridization - Slide processing for denaturation, hybridization, detection and imaging were similar to those described for sorghum FISH-based mitotic karyotyping by KIM *et al.* (2005).

RESULTS

The diploid chromosome numbers observed in *V. goudotiana*, *V. cundinamarcensis* and *C. papaya* (Fig. 1) were 2n = 18 and the karyomorphology were regular and symmetric. In situ hybridization using 5S and 18S probes revealed signals of different intensities. In all species, chromosomes were metacentric or submetacentric. A differential DAPI staining was observed. Longitudinal variation in DAPI intensity suggests that AT base pair densities are distributed nonrandomly, levels are accentuated in segments near centromeres.

Dual-probe FISH analysis of *V. goudotiana* revealed 5S rDNA in just one pair of homologues and 18S rDNA in two other pairs, one major site and one medium-sized site, both near the respective centromeres (Fig. 1A). No chromosomes with 5S rDNA sites carried 18S rDNA sites. The larger 18S rDNA site was located centrally in one of the largest chromosome pairs; whereas the smaller 18S rDNA cluster and the 5S rDNA cluster occurred in mid-sized chromosomes. The 5S rDNA site was positioned sub-medially and the smaller 18S rDNA cluster was positioned subacrocentrically. No differences were observed between male and female plants.

In *V. cundinamarcensis*, five chromosome pairs seemed to include segments that were relatively DAPI-bright. The dual FISH revealed one pair of 5S rDNA signals and five 18S rDNA signals, all non-syntenic (Fig. 1B). The pair of 5S loci was located in a medium-sized pair of metacentric chromosomes, in or next to pericentromeric DAPI-bright heterochromatin, and very close to

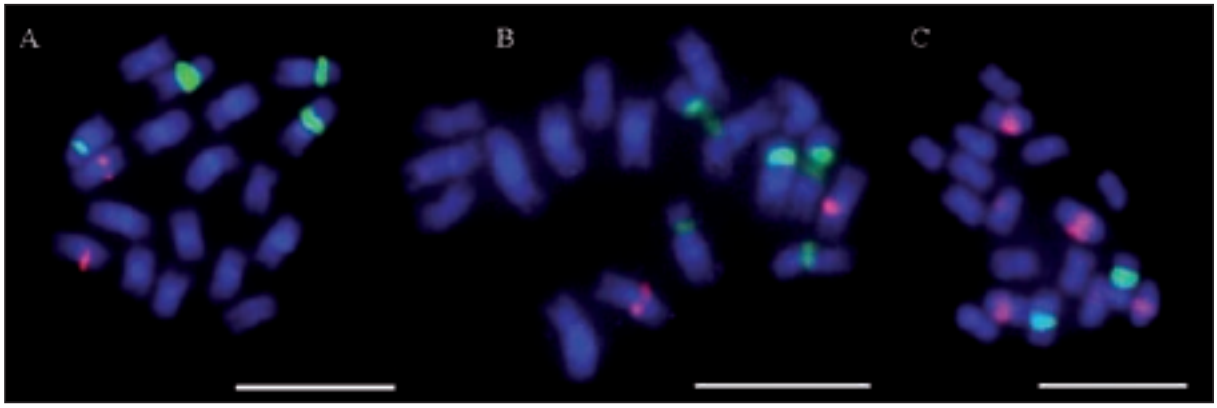

Fig. 1 — Dual-FISH of 18S and 5S probes to Caricaceae mitotic metaphase chromosome spreads. Each species was simultaneously hybridized with a biotin-labeled 5S rDNA probe and a digoxigenin-labeled 18S rDNA probe. The 18S signals were detected with FITC-conjugated anti-digoxigenin (*green*) and the 5S signals were detected with Cy3-conjugated streptoavidin (*red*). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI). **A)** *Vasconcellea goudotiana*; **B)** *V. cundinamarcensis*; **C)** *Carica papaya*. Bar = 5 µm.

the primary constriction. The five 18-28S rDNA sites included two pairs of sites, one of which occupied a submedial position of a chromosome for which no strong DAPI band was noted, and on the other pair a subterminal constriction indicated it is subacrocentric. The other pair of 18S sites was located subterminally in a metacentric chromosome with fairly intense pericentromeric DAPI banding. The 18S single was positioned more or less medially in a medium-sized submetacentric chromosome, distal to which there is a relatively strong short DAPI band. As shown in Figure 1B, one pair was differentiated not only by its position, but also by its relatively distended state.

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 (Fig. 1C). No differences were observed between cultivars or between male and female plants.

DISCUSSION

Previous cytological studies in papaya showed that chromosomes do not differ significantly in length or arm ratio and thus cannot be distinguished morphologically (DATTA 1971). Our findings indicate, however, that the locations of rDNA loci enable homologs to be recognized for four pairs of chromosomes. Other experiments will be required for scientific proof, but the observations suggest that in concert with chromosome morphology, DAPI banding, and relative size and positions of the rDNA clusters, we can reliably recognize four of the nine homologous chromosome pairs across cells. Although in some cases, DAPI staining alone will suffice for identification of all chromosomes of a given karyotype, and close relatives, e.g., to identify a derived primary trisomic, its longitudinal resolution limited conclusive inferences. Thus, more sophisticated FISH-based techniques are likely needed for robust analyses. The present results give us little basis for drawing inferences on the degree of portability among karyotypes and more complicated situations, such as segmental aneuploids, inversions and interchanges.

The number of both 5S and 18S rDNA sites observed suggest *V. cundinamarcensis* and *V. goudotiana* are related while *C. papaya* is apart from them. Both *Vasconcellea* species showed only one pair of 5S signals whereas in papaya three pairs were found. On the other hand, only one pair of 18S rDNA sites was found in papaya, whereas there were four and five sites in *V. goudotiana* and *V. cundinamarcensis*, respectively.

Until recently, *Vasconcellea* was considered a section, sister to the section *Carica*, within *Carica* genus; the main difference between sections was the ovary, pentalocular and unilocular respectively (BADILLO 1993). Afterward, based on genetic evidences (ARADHYA *et al.* 1999), *Vasconcellea* was rehabilitated to generic level and *Carica* genus is currently considered monospecific, containing on-

ly *C. papaya* (BADILLO 2000, 2001). Furthermore, other molecular analyses have confirmed the large genetic distance between *Carica* and *Vasconcellea* and support this taxonomic elevation (KIM *et al.* 2002; VAN DROOGENBROECK *et al.* 2004; KYNDT *et al.* 2006).

An additional factor reinforcing the distant relationship between these genera is the cross compatibility. Although many *Vasconcellea* species can be intercrossed, producing hybrids with different degrees of fertility (SAWANT 1958; JIMENEZ and HOROVITZ 1958; HOROVITZ and JIMENEZ 1967; MEKAKO and NAKASONE 1975) post-zygotic barriers were revealed in crosses between *C. papaya* and *Vasconcelllea* spp. (JIMENEZ and HOROVITZ 1958; MANSHARDT and WENSLAFF 1989; MAGDALITA *et al.* 1997; DREW *et al.* 1998) and some hybrids were obtained using embryo rescue (CHEN *et al.* 1991; MAGDALITA *et al.* 1996; DREW *et al.* 1998).

An interesting feature observed in this study was an unpaired 18S site in *V. cundinamarcensis*. A possible explanation can be related to sexual chromosomes in Caricaceae. Strict dioecism is prevalent in this family. Nearly all species have normal type of Y-chromosome and male individuals are heterogametic (XY) while females are homogametic (XX) (HOROVITZ and JIMENEZ 1972). Three exceptions are found: *V. monoica*, a strictly monoecious (BADILLO 1971) and homogametic species (ZZ) (HOROVITZ and JIMENEZ 1972), *C. papaya*, polygamous, with female (XX), male (XY_1) and hermaphrodite (XY_2) plants (STOREY 1941; HOFMEYR 1941), and *V. cundinamarcensis*, monoecious-dioecious, with female (XX), male (XY^{pm}) and hermaphrodite ($X^{pm}Y^{pm}$) individuals (HOROVITZ and JIMENEZ 1972). Specifically in *V. cundinamarcensis*, the hermaphrodite condition is due to an interaction between cytoplasm *pm* and sexual chromosome Y^{pm} (HOROVITZ and JIMENEZ 1972). In this species, pistillate and staminate specimens are stable and do not respond to seasonal climatic changes while the monoecious specimens form female, male and perfect (hermaphrodite) flowers in different proportions, depending on the environmental conditions (BADILLO 1971).

Meiotic analysis of *V. cundinamarcensis* showed three bivalents associated with the nucleolus. Nine bivalents were observed in metaphase I but frequently one of them was not on line at the equatorial plate, although no chromosomal heteromorphism was observed (DE ZERPA 1980). In *C. papaya*, one chromosome pair showed a delayed separation in anaphase (FRANKEL and GALUN 1977). Delayed anaphase separation is rather common in dioecious animal systems and is therefore considered to be an incipient XY system, but it has not been confirmed in plants. A recent molecular study in papaya revealed a short male specific region on the Y chromosome that does not recombine with X, suggesting that sex chromosomes could be evolving from a regular pair of autosomes (LIU *et al.* 2004), which could be an incipient sexual system.

Based on these facts and considering that all *V. cundinamarcensis* slides analyzed were provided from monoecious (and heterogametic) plants, we can hypothesize that the unpaired signal of 18S probe is located on a sexual chromosome, more specifically in X chromosome. In addition, there exist at least two reports that associate 18S signals with plant sex chromosomes, in liverwort (NAKAYAMA *et al.* 2001) and spinach (LAN *et al.* 2006). In both cases, 18S signals of female individuals occurred as pairs, while those of males were unpaired. In our study, none of the analyzed plants were female, so further FISH studies are required to confirm this hypothesis of sex-chromosome linkage, and, more specifically, X-chromosome linkage.

In addition to determining the physical position of 5S and 18S loci, it was demonstrated the FISH will be useful tool for karyotyping chromosomes and defining genomic relationships among these related taxa. Since the position of 5S and 18S rDNA gene loci are not yet defined in linkage maps in papaya, this might be the first report not only of these loci in *C. papaya* and the other Caricaceae species analyzed but also the first indication of synteny between rDNA and sex determination loci in Caricaceae.

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