Simultaneous Visualization of 5S and 45S rDNAs in Persimmon (*Diospyros kaki*) and Several Wild Relatives (*Diospyros* spp.) by Fluorescent in situ Hybridization (FISH) and MultiColor FISH (MCFISH)

Young A Choi,¹ Ryutaro Tao, Keizo Yonemori, and Akira Sugiura

Lab of Pomology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

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ABSTRACT. 5S ribosomal DNA (rDNA) was visualized on the somatic metaphase chromosome of persimmon (*Diospyros kaki*) and ten wild *Diospyros* species by fluorescent in situ hybridization (FISH). The digoxigenin (DIG)-labeled 5S rDNA probe was hybridized onto the chromosomes and visualized by incubation with anti-DIG-fluorescein isothiocyanate (FITC). Strong signals of 5S rDNA probe were observed on several chromosomes of *Diospyros* species tested. Furthermore, multicolor FISH using 5S and 45S rDNA probes differently labeled with DIG and biotin, revealed separate localization of the two rDNA genes on different chromosomes of *Diospyros* species tested, suggesting that 5S and 45S rDNA sites can be used as chromosome markers in *Diospyros*. The number of 5S rDNA sites varied with the *Diospyros* species. More 5S rDNA sites were observed in four diploid species native to Southern Africa than in three Asian diploid species. The former had four or six 5S rDNA sites while the latter had two. Three Asian polyploid species had four to eight 5S rDNA sites. Among the Asian species, the number of 5S rDNA sites seemed to increase according to ploidy level of species. These features of 5S rDNA sites were very similar to those of 45S rDNA sites in *Diospyros*. Phylogenetic relationship between *D. kaki* and wild species tested are discussed based on the number and chromosomal distribution of 5S and 45S rDNA.

Persimmon (*Diospyros kaki* Thunb.) is one of the most important fruit trees cultivated for centuries in Japan (Sugiura and Subhadrabandhu, 1996). This species is also distributed in temperate regions of east Asia and at least 1000 cultivars have been developed. The genus *Diospyros*, to which persimmon belongs, consists of 400–500 species (Yonemori et al., 2000). Wild species of *Diospyros* are mostly diploid ($2n = 2x = 30$) with a small number of tetraploid species ($2n = 4x = 60$), while cultivated species, such as *D. kaki*, are hexaploid ($2n = 6x = 90$). Some of the seedless cultivars of *D. kaki* have been reported as nonaploid ($2n = 9x = 135$) (Tamura et al., 1998; Zhuang et al., 1990). Thus, it is speculated that a single or several diploid and/or tetraploid wild species were involved in the speciation of the cultivated polyploid *Diospyros* species.

So far, the relationship between wild and cultivated *Diospyros* species has been discussed based on the limited information obtained from isozyme, mitochondrial DNA and chloroplast DNA analyses (Nakamura and Kobayashi, 1994; Tao and Sugiura, 1987; Yonemori et al., 1998). Little information is available about the speciation of cultivated hexaploid species and the phylogenetic relationships among *Diospyros* species. Although chromosome numbers and nuclear DNA contents for *Diospyros* species have been reported (Tamura et al., 1998; Zhuang et al., 1990), only a few cytological studies have been conducted for the *Diospyros*. Small chromosomes of *Diospyros* (2–3 μ m long on the average at metaphase) make observation with a light microscope difficult (Choi et al., 2002; Tamura et al., 1998).

Fluorescent in situ hybridization (FISH), using labeled specific DNA fragments as probes was developed in the late 1980s as a most effective plant cytogenetic technique (Jiang and Gill, 1994). Direct visualization of defined DNA sequences such as ribosomal

RNA gene (rDNA) on the chromosomes has been applied to study the phylogenetic relationship in horticultural plants such as *Allium* and *Brassica* (Hasterok et al., 2001; Richroch et al., 1992). Furthermore, multicolor FISH (MCFISH) using 5S and 45S rDNA specific probes simultaneously have provided valuable information on the evolution of rDNA sites and the relationships between wild and cultivated polyploid species (Mishima et al., 2002; Raina and Mukai, 1999; Schrader et al., 2000; Taketa et al., 1999). Recently, FISH using a 45S rDNA probe was found useful to elucidate the chromosomal location and the variation in the number of sites of 45S rDNA in 10 *Diospyros* species (Choi et al., 2003).

In the present study, we performed FISH for physical mapping of 5S rDNA and MCFISH for simultaneous visualization of 5S and 45S rDNA genes on the somatic metaphase chromosomes of *D. kaki* and wild *Diospyros* species. The phylogeny of *Diospyros* species tested is discussed with special reference to variation in the numbers and chromosomal location of 5S rDNA sites compared with those of 45S rDNA.

Materials and Methods

PLANT MATERIALS AND CHROMOSOME PREPARATION. *Diospyros* species used in this study are shown in Table 1 with their ploidy levels and regional distributions. Young roots were collected from rooted shoots in vitro or seedlings as described before (Choi et al., 2002). Root tips (1–2 cm long) were pretreated with 2 mM 8-hydroquinoline solution for 5 h at 4 °C and fixed in a methanol-acetic acid (3:1) solution. Chromosome samples were prepared by an enzymatic maceration and air drying method (Fukui, 1996). The enzyme solution was composed of 4% (w/v) cellulase RS (Yakult Honsha, Tokyo), 1% (w/v) pectolyase Y-23 (Kikkoman Co., Tokyo), 0.07 M KCl and 7.5 mM Na₂EDTA (pH 4.0).

5S rDNA PROBE PREPARATION. For 5S rDNA detection, the 5S rDNA coding region was PCR amplified from genomic DNA of *Diospyros kaki* using oligonucleotide primers designed by Fukui et al. (1994a): a forward primer 5S-F (5'-GGATGCGATCATAC-

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¹Corresponding author; e-mail choi@kais.kyoto-u.ac.jp.

Table 1. Ploidy levels and regional distributions of eleven *Diospyros* species used in this study.

Species	Ploidy level	Distribution
<i>D. glabura</i>	2n = 2x = 30	Subtropical region of southern Africa
<i>D. austroafricana</i>	2n = 2x = 30	Subtropical region of southern Africa
<i>D. lycioides</i> ^a	2n = 2x = 30	Subtropical region of southern Africa
<i>D. simii</i>	2n = 2x = 30	Subtropical region of southern Africa
<i>D. ehretioides</i> ^a	2n = 2x = 30	Tropical and subtropical region of southeast Asia
<i>D. morrisiana</i>	2n = 2x = 30	Subtropical and temperate region of east Asia
<i>D. oldhami</i>	2n = 2x = 30	Subtropical and temperate region of east Asia
<i>D. lotus</i> 'Kunsenshi'	2n = 2x = 30	Temperate region of central and east Asia
<i>D. rhombifolia</i>	2n = 4x = 60	Temperate region of east Asia
<i>D. kaki</i> 'Jiro'	2n = 6x = 90	Temperate region of east Asia
<i>D. virginiana</i>	2n = 6x = 90	Temperate region of north America

^aSpecies used only in single mapping of 5S rDNA loci in this study.

CAGCAC -3') and a reverse primer 5S-R (5'-GGGAATGCAA-CACGAGGACT-3'). A standard PCR method was carried out using a thermal cycler (Perkin Elmer Cetus, Norwalk, Conn.). Total DNA of *D. kaki* isolated by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) was used as a template DNA. For PCR, a 50-μL aliquot reaction mixture containing 50 ng of template DNA, 0.2 mM of dNTP mixture, 0.4 μM of each primer and 1 unit of Taq DNA polymerase (Takara Shuzo Co., Ltd., Tokyo, Japan) was used. Samples were amplified by 35 thermal cycles of 94 °C for 30 s, 58 °C for 1.5 min, and 72 °C for 2 min. The PCR product was cloned into a TA cloning vector (Promega, Tokyo, Japan). Nucleotide sequences of the inserts of several clones were determined using an automatic DNA sequencer (ABI PRISM, Applied Biosystems, Calif.). The inserts of the cloned DNA were amplified and labeled with digoxigenin (DIG)-11-dUTP (Roche, Mannheim, Germany) simultaneously by PCR using 5S-F and 5S-R primers as described by Fukui et al. (1994a).

45S rDNA PROBE PREPARATION. 45S rDNA, consisting of a 18S-5.8S-25S rDNA gene cluster of rice (Sano and Sano, 1990) was labeled with biotin-16-dUTP by the High Prime DNA labeling system according to the supplier's instructions (Roche, Mannheim, Germany).

FISH/MCFISH. Before hybridization, the chromosome preparations were incubated with 100 mg·mL⁻¹ RNase A and washed with 2× SSC (0.3 M NaCl and 0.03 M sodium citrate), dehydrated with a 70%, 95%, and 99% ethanol series.

The FISH procedure using 5S rDNA specific probe was the same as that described by Fukui et al. (1994a). The MCFISH procedure using DIG-labeled 5S and biotinylated 45S rDNA probes was performed as described by Taketa et al. (1999) with a slight modification of probe concentration and its composition as follows. The probe mixture consisted of 20 ng of both labeled probes per slide that were dissolved in 15 μL mixture of 50% formamide in 2× SSC. The biotinylated probe was detected with avidin-rhodamine (1%, v/v, Roche, Mannheim, Germany) and biotinylated anti-avidin (1%, w/v, Vector Laboratories, Burlingame, Calif.) was applied as the secondary amplification of the fluorescent signals. The DIG labeled probe was detected with anti-DIG-fluorescein isothiocyanate (FITC) (10%, w/v, Roche, Mannheim, Germany) at the step of secondary amplification of biotinylated probe for simultaneous detection of both probes. The chromosomes on the slides were counterstained with 0.5 μg·mL⁻¹ 4, 6-diamidino-2-phenylindole (DAPI) and mounted with an antifadant solution as described by Choi et al. (2002). The chromosome samples were observed with a fluorescent microscope (Axiophot, Zeiss, Oberkochen, Germany) with a

high-sensitivity cooled CCD camera (PXL 1400, Photometrics, Ariz.). The B- and G- light excitation filters were used for the detection of FITC and rhodamine, respectively. The signal images were analyzed by imaging software (IPLab Spectrum 3.1, Signal Analytics, Calif.). More than 30 to 50 cells from at least ten metaphase slides in each species were observed for FISH and MCFISH.

Results

5S rDNA SITES IN ELEVEN DIOSPYROS SPECIES. Figure 1 shows the nucleotide sequence corresponding to the coding region of 5S rDNA cloned from *D. kaki*. The fragment of 5S rDNA from *D. kaki* showed higher than 97% DNA sequence identity to that from tomato, tobacco and *Arabidopsis thaliana*.

FISH using DIG-labeled 5S rDNA as a probe was performed on somatic chromosomes in 11 *Diospyros* species with different ploidy levels. The 5S rDNA specific probe hybridized to various numbers of sites ranging from two to six in the diploid species. *Diospyros glabura*, native to southern Africa, showed four 5S rDNA-specific signals on the chromosomes (Fig. 2A), and the other southern African species, *D. austroafricana*, *D. lycioides* and *D. simii*, appeared to have six 5S rDNA sites (Fig. 2B-D). Four Asian diploid species, *D. ehretioides*, *D. morrisiana*, *D. oldhami* and *D. lotus*, carried two 5S rDNA sites on the short arms of chromosomes (Fig. 2E-H). A variation in signal intensity was frequently observed in *D. lotus* (Fig. 2H and Fig. 3F, arrow). Three polyploid species carried four to eight rDNA sites on their chromosomes. Tetraploid *D. rhombifolia* had four chromosomes carrying 5S rDNA sites on the short arms of chromosomes (Fig. 2I). Hexaploid *D. kaki* bore eight 5S rDNA sites on its chromosomes (Fig. 2J). Three of the four pairs of 5S rDNA sites of *D. kaki* were visualized at the centromeric regions while the other pair was detected from proximal regions on the long arms (Fig. 2J, arrows) of chromosomes. Another hexaploid, *D. virginiana*, bore six 5S rDNA sites, which were visualized on the short arms

Fig. 1. The nucleotide sequences corresponding to the coding region of 5S rDNA cloned from *Diospyros kaki*. Arrows indicate the sequence of oligonucleotide primers used in PCR amplification of 5S rDNA sequence: F = the sequence of a forward primer; R = the reversed sequence of a reverse primer.

Fig. 2. Visualization of 5S rDNA loci on chromosomes stained with 4,6-diamidino-2-phenylindole (DAPI). Color images were obtained by combining the pseudo green fluorescein isothiocyanate (FITC) signals from 5S rDNA loci and the pseudo blue signals from chromosomes stained with DAPI. (A) *Diospyros glabura* ($2n = 2x = 30$), (B) *D. austroafricana* ($2n = 2x = 30$), (C) *D. lycioides* ($2n = 2x = 30$), (D) *D. simii* ($2n = 2x = 30$), (E) *D. ehretioides* ($2n = 2x = 30$), (F) *D. morrisiana* ($2n = 2x = 30$), (G) *D. oldhami* ($2n = 2x = 30$), (H) *D. lotus* ($2n = 2x = 30$), (I) *D. rhombifolia* ($2n = 4x = 60$), (J) *D. kaki* ($2n = 6x = 90$), (K) *D. virginiana* ($2n = 6x = 90$). Arrow in H indicates variation in signal intensity of 5S rDNA repeat unit observed in *D. lotus*. Arrows in J indicate proximal localization of 5S rDNA on chromosomes of *D. kaki*. Bar represents 10 μ m.

of chromosomes (Fig. 2K). All green signals from fluorescein isothiocyanate (FITC) were detected from centromeric or proximal regions on separate chromosomes of *Diospyros* species tested in this study.

SIMULTANEOUS VISUALIZATION OF 5S AND 45S rDNA GENES ON THE CHROMOSOMES OF *DIOSPYROS* SPECIES BY MCFISH. MCFISH, performed using DIG-labeled 5S rDNA and biotin-labeled 45S rDNA probes, visualized 5S rDNA (green signals detected with anti-DIG-FITC) and 45S rDNA (red signals detected with avidin-rhodamine) sites simultaneously on the chromosomes of the nine

Diospyros species (Fig. 3A–I). The 5S and 45S rDNA sites were separately localized on different chromosomes in all species used in this study.

Discussion

Probes specific to 45S rDNA and its subunits (5.8S, 18S or 25S) have been reported as effective chromosome markers in fruit trees (Roose et al., 1998; Schuster et al., 1997; Yamamoto et al., 1999) including *Diospyros* species (Choi et al., 2003). Since the 5S rDNA gene can be another useful chromosome marker (Cuadrado et al., 1995; Hasterok et al., 2001; Kamisugi et al., 1994; Mukai et al., 1990; Roose et al., 1998; Schuster et al., 1997), we determined if the 5S rDNA specific probe could be a chromosome marker for *Diospyros* species in this study. The signal intensity was strong enough to be detected and the probe hybridized to several chromosomes suggesting that the 5S rDNA specific probe is an effective chromosome marker in *Diospyros*. Hence, the 5S rDNA gene, an especially effective marker for diploid species, was clearly visualized by FISH even in the hexaploid species, such as *D. kaki* and *D. virginiana*.

Variations in the number of 5S rDNA sites of *Diospyros* species tested appeared to be quite similar to those of 45S rDNA sites (Table 2; Choi et al., 2003). The number of 5S rDNA sites in diploid species varied from two to six whereas that of 45S rDNA sites from two to eight. Four species, native to South Africa, had more 5S and 45S rDNA sites than those of Asian diploids. The geographical variability in the number of rDNA sites was observed in other plant species such as *Oryza* and *Zamiaceae* (Fukui et al., 1994b; Tagashira and Kondo, 2001). The recent study on the sequence analysis of the ITS and *matK* regions of *Diospyros* species revealed that African species were phylogenetically most distant species from *D. kaki* among the species tested (unpublished laboratory data). The difference in

the number of 5S rDNA sites between African and Asian species as observed in 45S rDNA sites suggests that the southern African species have evolved forming an independent phylogenetic group in *Diospyros*. This could suggest that southern African species were not involved directly in the speciation of cultivated hexaploid species. Furthermore, within the Asian species, the numbers of 5S rDNA sites seemed to increase depending on the ploidy levels of species as did the number of 45S rDNA sites (Table 2). This result was different from many other plant species that did not show any correlation between the number of 5S and 45S rDNAs sites (Castilho and Heslop-Harrison, 1995; Mishima et al., 2002; Schrader et al., 2000; Taketa et al., 1999).

Since the chromosomal distribution of rDNA genes could be an additionally important indicator in the phylogenetic study among related plant species (Castilho and Heslop-Harrison, 1995; Maluszynska and Heslop-Harrison, 1993; Zhang and Sang, 1999), we mapped 5S and 45S rDNA sites of nine *Diospyros* species simultaneously by MCFISH (Fig. 3). In all nine species tested, 45S rDNA was located at the nucleolus organizer region (NOR) or other chromosomal regions while 5S rDNA was detected at the proximal or centromeric parts of chromosomes

Fig. 3. Simultaneous visualization of 5S rDNA and 45S rDNA sites. Color images were obtained by combining the pseudo green FITC signals from 5S rDNA sites, the pseudo red rhodamine signals from 45S rDNA sites and the pseudo blue signals from chromosomes stained with DAPI. (A) *Diospyros glabura* ($2n = 2x = 30$), (B) *D. austroafricana* ($2n = 2x = 30$), (C) *D. simii* ($2n = 2x = 30$), (D) *D. morrisiana* ($2n = 2x = 30$), (E) *D. oldhami* ($2n = 2x = 30$), (F) *D. lotus* ($2n = 2x = 30$), (G) *D. rhombifolia* ($2n = 4x = 60$), (H) *D. kaki* ($2n = 6x = 90$), (I) *D. virginiana* ($2n = 6x = 90$). Arrow in F indicates variation in signal intensity of 5S rDNA repeat unit observed in *D. lotus*. Bar represents 10 μ m.

number of the target site in the chromosomes (Leitch and Heslop-Harrison, 1992). This length variation of 5S rDNA sites observed in *D. lotus* presumably originated from major deletion-insertion events in the region of the 5S rDNA repeat units during the evolution of species as reported in rye and tobacco (Cuadrado et al., 1995; Kitamura et al., 2000).

In conclusion, FISH and MCFISH using the 5S and 45S rDNA probes, successfully visualized their sites on the chromosomes of *Diospyros*. The pattern of localization of 5S and 45S rDNA genes on the chromosomes would serve as effective chromosome markers for *Diospyros* species. Furthermore, the similarity in pattern of variation in the number of 5S and 45S rDNA sites tested may have a significant implication for elucidating the phylogenetic relationship and the speciation

of polyploid species of *Diospyros*.

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other than those bearing the NOR. The different multiloci chromosomal distributions of the two rDNA genes showed that they are independent of each other as indicated in many other plants (Cuadrado et al., 1995; Kamisugi et al., 1994; Leitch and Heslop-Harrison, 1992; Rogers and Bendich, 1987). The localization of 5S and 45S rDNA genes on different chromosomes of *Diospyros* species tested suggests that reorganization has rarely occurred between the two rDNAs sites as indicated in *Brassicca* (Hasterok and Maluszynska, 2000).

As shown in Fig. 2H (arrow), a variation in signal intensity between 5S rDNA sites was frequently observed in *D. lotus*. Although *in situ* hybridization is not a quantitative method, differences in signal strength are correlated with variation in copy

Table 2. The number of 5S and 45S rDNA sites of eleven *Diospyros* species used in this study.

Species (ploidy level)	No. of 5S rDNA site	No. of 45S rDNA site ^z
<i>D. glabura</i> (2x)	4	6
<i>D. austroafricana</i> (2x)	6	6 ^y
<i>D. lycioides</i> (2x)	6	8
<i>D. simii</i> (2x)	6	8
<i>D. ehretioides</i> (2x)	2	2
<i>D. morrisiana</i> (2x)	2	2
<i>D. oldhami</i> (2x)	2	2
<i>D. lotus</i> 'Kunsenshi' (2x)	2	4
<i>D. rhombifolia</i> (4x)	4	8
<i>D. kaki</i> 'Jiro' (6x)	8	8
<i>D. virginiana</i> (6x)	6	8

^zReported previously by Choi et al., 2003.

^yThe number of 45S rDNA sites determined in this study.

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