Genomic in situ Hybridization between Persimmon (*Diospyros kaki*) and Several Wild Species of *Diospyros*

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

Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606–8502

Summary

To understand the relationships among the genomes of *Diospyros kaki* (persimmon) and its wild relatives, we performed genomic in situ hybridization (GISH) on somatic metaphase chromosomes of *D. kaki*, using digoxigenin (DIG)-11-UTP labeled DNA of eight *Diospyros* species. In a series of GISH, it appeared that the *D. glandulosa* probe hybridized to *D. kaki* chromosomes resulted with the strongest signal intensity. When comparable GISH was conducted, using unlabeled DNA of *D. kaki* as a blocking DNA, the highest concentration of blocking DNA was necessary to block hybridization of the *D. glandulosa* probe. These results suggest that the genomes of *D. kaki* and *D. glandulosa* share many common DNA sequences, and that the genome of *D. kaki* is most closely related to that of *D. glandulosa* among the species tested.

Key Words: *Diospyros*, genomic in situ hybridization (GISH), persimmon, repetitive sequences.

Introduction

Persimmon (*Diospyros kaki*) has been cultivated for over ten centuries in Japan. Since most wild species in the genus *Diospyros* are diploid (2n=2x=30) or tetraploid (2n=4x=60), a single or several diploid and/or tetraploid species could be involved in the speciation of the cultivated hexaploid (2n=6x=90) and/or nonaploid (2n=9x=135) *D. kaki*. Little information, however, is available on the speciation of *D. kaki* and phylogenetic relationships among *Diospyros* species.

Recently, genomic in situ hybridization (GISH), using genomic probes from parental plants or putative progenitor species, has been used to identify parental chromosomes and genomic compositions in many plant species. GISH successfully identified progenitor species in horticultural plants such as *Allium wakegi* (Hizume, 1994) and *Coffeea arabica* (Raina et al., 1998). Furthermore, GISH has been used to investigate genomic compositions in somatic hybrids between *Diospyros* species (Choi et al., 2002).

In this study, GISH, using genomic DNA probes from various *Diospyros* species, was conducted at the metaphase stage of chromosomes of *D. kaki*. The potential of GISH as a method to investigate the genomic composition and phylogenetic relationship between *D. kaki* and its wild relatives is discussed.

Materials and Methods

Chromosome preparation

*In vitro* shoots of *D. kaki* cv. Jiro were rooted as described by Choi et al. (2002). Root tips of 1–2 cm long were pretreated with 2 mM 8-hydroxyquinolin solution for 5 hr at 4 °C and fixed in methanol–acetic acid (3:1, v/v). Chromosome samples were prepared by an enzymatic maceration and air–drying method (Fukui, 1996). The composition of the enzyme solution was similar to that described by Tamura et al. (1998).

Plant materials and probe preparation

Nine *Diospyros* species, used for this study including *D. kaki*, are shown in Table 1 with their ploidy levels and regional distributions. *Diospyros glandulosa* was suggested to be a candidate progenitor species of hexaploid *D. kaki* because of their proximity of origin and their similar morphological character (Ng, 1978). *Diospyros oleifera*, *D. lotus*, *D. ehetroides* and *D. virginiana* were reported to be closely related to *D. kaki* on the basis of data from mtDNA or cpDNA analyses (Nakamura and Kobayashi, 1994; Yonemori et al., 1998). *Diospyros rhodocalyx* and *D. rhombifolia* were reported to be distantly related to *D. kaki* (Yonemori et al., 1998). The other species from Southern Africa, *D. mespiliformis* with unknown phylogenetic relationship with *D. kaki*, was also used in this study. For GISH, total DNA of nine *Diospyros* species was isolated by the CTAB method (Doyle and Doyle, 1987). The isolated DNA was labeled with digoxigenin (DIG)–11-UTP by
the High Prime DNA labeling system, according to the supplier’s instructions (Roche, Germany).

*Genomic in situ hybridization (GISH)*

Prior to GISH, chromosome samples on the slides were pretreated as described by Choi et al. (2002). The hybridization mixture consisted of 200 ng of DIG-labeled probe per slide that was dissolved in 15 μl of 50% formamide in 2 × SSC. For comparable GISH, we used unlabeled DNA of *D. kaki* as a blocking DNA and mixed it with a labeled probe in the hybridization mixture. The concentration of the tested blocking DNA was five to fifteen times greater than that of a labeled probe. The chromosome with its probe was denatured for 10 min at 80 °C and then kept at 37 °C for 7 days for hybridization. After hybridization, the chromosome samples were washed in 2 × SSC, 50% formamide in 2 × SSC, and 4 × SSC at 42 °C for 10 min. The chromosome samples on the slides were blocked with 5% (w/v) bovine serum albumin (BSA) in BT buffer (0.1% sodium hydrogen carbonate, 0.05% Tween 20, pH 8.3) at 37 °C for 5 min. Anti-DIG-FITC (10%, w/v, Roche, Germany) in 1% BSA in BT buffer was dropped onto the chromosome samples to detect DIG-labeled probe. Subsequent steps were the same as those described by Choi et al. (2002). The chromosome samples were placed under a fluorescent microscope (Axiohot, Zeiss, Oberkochen, Germany) and photographed with a high-sensitivity cooled CCD camera (PXL 1400, Photometrics, Ariz., U.S.A.). The B light excitation filter was used to detect signals from FITC. The signal images were analyzed by imaging software (IPLab Spectrum 3.1, Signal Analytics, CA, U.S.A.).

**Results and discussion**

More than forty cells from ten metaphase slides of chromosome samples were observed for each GISH using genomic probes from various species. In GISH, the signal intensity obtained with the *D. glandulosa* probe was almost as strong as that obtained with the *D. kaki* probe (Fig. 1A and I). Hybridization signals from the *D. glandulosa* probe were emitted from numerous regions of the chromosomes of *D. kaki* with the strongest signals detected at the telomeric and centromeric zones on several chromosomes. Among the probes of the other species, *D. oleifera* gave the strongest signal followed by weaker ones from *D. lotus* and *D. virginiana*. Again the strongest signals came from the telomeric and centromeric regions of several chromosomes (Fig. 1B, C and H). The hybridization signal from the *D. ehretioides* probe originated only at telomeric and centromeric parts of chromosomes of *D. kaki* (Fig. 1D). Probes of the remaining three species, *D. rhodocalyx*, *D. mespiliiformis* and *D. rhombifolia* gave very weak signals at the same regions (Fig. 1E-G). Comparable GISH, using blocking DNA, revealed that five times as much unlabeled blocking DNA of *D. kaki* was sufficient to block hybridization of labeled probes of *D. oleifera*, *D. lotus*, *D. virginiana* and *D. ehretioides* (Fig. 2A-D). However, when labeled DNA of *D. glandulosa* was used, at least fifteen times higher concentration of unlabeled blocking DNA of *D. kaki* was necessary to block its hybridization (Fig. 2E and F). It is known that chromosomes are strongly and uniformly labeled with the probes from the same species, whereas chromosomes are weakly and irregularly labeled with the probes from different species (Raina et al., 1998). This could be expected because phylogenetically close species have many DNA sequences in common. Hybridization patterns, observed in GISH with the *D. glandulosa* probe, suggest that *D. glandulosa* share more common DNA sequences with *D. kaki* than do the other species tested. In comparable GISH, a higher concentration of blocking DNA was also required to suppress hybridization of the *D. glandulosa* probe, which suggests that *D. kaki* and *D. glandulosa* share many common DNA sequences. Strong signals, detected at telomeric and centromeric parts of chromosomes of *D. kaki*, could be from highly conserved repetitive sequences such as rDNA or satellite sequences. Kitamura et al. (1997) suggested that the GISH signals, found at the telomeric regions of chromosomes in *Nicotiana* interspecific hybrids, were from repetitive sequences derived from parental plants. Recently, Choi

et al. (2003) suggested that Asian diploid species with as many rDNA sites as *D. lotus* (four sites) could be ancestral species of the hexaploid *D. kaki*, based on the number of rDNA sites of *D. kaki* and its wild relatives. Although the number of rDNA sites of *D. glandulosa* remains to be studied, preliminary results obtained with somatic hybrids of *D. kaki* and *D. glandulosa* indicated that they may be as many as four (data not shown).

These results obtained in this study support those previously reported on the phylogenetic relationship of *Diospyros* species based on mtDNA or cpDNA analyses with morphological characters (Nakayama and Kobayashi, 1994; Yonemori et al., 1998), except for *D. ehretioides*. In that report *D. ehretioides* was classified into the monophyletic group with *D. kaki* by cpDNA analysis (Yonemori et al., 1998). Large chromosomal

Fig. 2. Photomicrographs of fluorescent signals from comparable GISH at the metaphase stage of *D. kaki* chromosomes using unlabeled DNA of *D. kaki* as a blocking DNA. DIG-labeled genomic probes were derived from: A: *D. oleifera*, B: *D. lotus*, C: *D. ehretioides*, D: *D. virginiana*, E–F: *D. glandulosa*. The blocking DNA was five times (A–E) and fifteen times (F) more concentrated than the labeled probe. The genomic probe was detected with anti-DIG–FITC.
size and numerous DAPI bands of *D. ehrfetoides*, however, differed from other *Disopyros* species, including *D. kaki* (Choi et al., 2003). This might suggest that *D. ehrfetoides* has a different genomic evolution from other *Disopyros* species tested. The weak hybridization signal of the probe of *D. mespiliformis*, a native to Southern Africa, indicates that *D. mespiliformis* may not share common sequences with *D. kaki* as one might suspect because of the different morphological characters and distant origins.

*Disopyros kaki* may either be an allohexaploid or autoallohexaploid based on its homologous chromosomes that carry 45S rDNA sites (Choi et al., 2003) and the segregation of a molecular marker linked to natural astringency loss (Kanzaki et al., 2001). However, we could not establish a distinct subset of chromosomes of *D. kaki* by using genomic probes of other *Disopyros* species which implies that several species with very similar genomic composition were involved in the speciation of *D. kaki*. Based on the intensity and hybridization patterns of signals in GISH analysis, the most probable candidate for one of the progenitor species of *D. kaki* is *D. glandulosa*. This supports the argument of Ng (1978) who suggested that *D. glandulosa* could be involved in the speciation of *D. kaki* because they are endemic to Asia and possess similar morphological characteristics.

**Literature Cited**


Genomic *in situ* hybridization (GISH)による*Diospyros kaki*と数種のカキ属植物との類縁関係の推測

崔 永娥・田尾龍太郎・米森敏三・杉浦 明

京都大学大学院農学研究科 606-8502 京都市左京区

摘要

*Diospyros kaki*と近縁野生種との類縁関係を調べるため、Digoxigenin (DIG) - 11 - UTP によりラベルした 8 種のカキ属植物の全 DNAをプロープとした genomic in situ hybridization (GISH)を*D. kaki*の中期染色体を用いて行った。一定のGISH実験により、用いたサブノックプローブの中で、*D. glandulosa*のプロープが*D. kaki*の染色体に最も強く、かつ染色体全体にかけてハイブリダイズすることが示された。プローブのハイブリダイズを阻害するためには、他種より高い濃度のプローブ DNAが必要であることが示された。今回の実験により、*D. kaki*および*D. glandulosa*のゲノム内には、共通配列が数多く存在することが示唆された。