Genomic distribution of three repetitive DNAs in cultivated hexaploid *Diospyros* spp. (*D. kaki* and *D. virginiana*) and their wild relatives

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To understand the genomic organization of *Diospyros* species with different ploidy levels, we cloned three different repetitive DNAs and compared their genomic distributions in ten *Diospyros* species, including hexaploid *D. kaki* and *D. virginiana*. Genomic Southern hybridization demonstrated that the *EcoRV*-repetitive DNA was present in tandem in the genomes of *D. glandulosa* ($2n=2x=30$), *D. oleifera* ($2n=2x=30$), *D. lotus* ($2n=2x=30$), *D. virginiana* ($2n=6x=90$) and *D. kaki* ($2n=6x=90$). All of these species except *D. virginiana* also contained the *HincII*-repetitive DNA in tandem. Fluorescent *in situ* hybridization showed that the *EcoRV*- and *HincII*-repetitive DNAs were predominantly located at the proximal or centromeric regions of chromosomes. The *DraI*-repetitive sequence cloned from *D. ehretioides* ($2n=2x=30$) was not found in the other *Diospyros* species tested. This suggests that *D. ehretioides* has a genomic organization different from that of the other *Diospyros* species. Speciation of hexaploid *Diospyros* species is also discussed with respect to the genomic distribution of the three repetitive DNAs cloned.

Key words: *Diospyros*, fluorescent *in situ* hybridization (FISH), persimmon, repetitive DNA, tandem repeat

INTRODUCTION

The genus *Diospyros* consists of 400–500 species and consists of species that fall into four ploidy levels, diploid ($2n=2x=30$), tetraploid ($2n=4x=60$), hexaploid ($2n=6x=90$) and nonaploid ($2n=9x=135$) (Yonemori et al., 2000). Most wild species of *Diospyros* are diploid, although some are polyploid, while cultivated *D. kaki* and *D. virginiana* are hexaploid. Some seedless cultivars of *D. kaki* are known to be nonaploid (Zhuang et al., 1990; Tamura et al., 1998). A single or several diploid and/or tetraploid wild species may have been involved in the speciation of the cultivated hexaploid and nonaploid species. So far, the phylogenetic relationships of *Diospyros* species have been discussed based on limited information from analyses of isozymes (Tao and Sugiura, 1987), RFLP of mitochondrial DNA (mtDNA) (Nakamura and Kobayashi, 1994) and PCR-RFLP of chloroplast DNA (cpDNA) (Yonemori et al., 1998).

During the last few decades, a number of studies on plant repetitive DNAs have been performed and detailed

information about the evolution of and differences among related species has been obtained (Gupta et al., 1990; Schmidt et al., 1991; Schmidt and Heslop-Harrison, 1998). Repetitive DNA elements, such as satellite, mini-satellite, and microsatellite DNAs and retrotransposons, constitute the major fraction of nuclear genomes in many higher plants (Bedbrook et al., 1980b; Schmidt and Metzloff, 1991; Kamm et al., 1994). These repetitive sequences show extensive differences in sequence motifs, abundance and distribution even between closely related species, indicating high species and/or genome specificity (Ingham et al., 1993; Charlesworth et al., 1994). Repetitive sequences are, therefore, useful as new tools for the genome analysis of polyploid species.

In this study, we isolated three repetitive DNAs from *Diospyros kaki* and two other species, *D. oleifera* and *D. ehretioides*, which were assumed to be closely related to *D. kaki* (Nakamura and Kobayashi, 1994; Yonemori et al., 1998). Genomic DNA blot analysis and fluorescent *in situ* hybridization (FISH) were performed to investigate the distribution and evolution of these repetitive sequences in the genomes of ten *Diospyros* species, including hexaploid *D. kaki* and *D. virginiana*. The speciation of hexaploid *Diospyros* species and genomic organization of

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the ten *Diospyros* species are discussed based on our results.

MATERIALS AND METHODS

The ten *Diospyros* species used in this study are listed in Table 1 with their ploidy levels and regional distributions. The diploid species *D. glandulosa* was suggested as a candidate progenitor species of hexaploid *D. kaki* based on the similarity of distribution area and morphological characters (Ng, 1978). Other diploids, *D. oleifera*, *D. lotus*, and *D. ehretioides*, and hexaploid *D. virginiana* were reported to be closely related to *D. kaki* as shown by mtDNA or cpDNA analysis (Nakamura and Kobayashi, 1994; Yonemori et al., 1998). These five species were used in this study to investigate genomic similarity among them and to *D. kaki*. Three species, *D. rhodocalyx*, *D. montana*, and *D. rhombifolia*, which were reported to be distantly related to *D. kaki* in spite of having a relatively similar distribution area to *D. kaki* (Yonemori et al., 1998), were used to understand the differences of genomic composition between *D. kaki* and its related species. A species from Southern Africa, *D. mespiliformis*, whose phylogenetic relationship with *D. kaki* remains unknown, was also used in this study. Total DNA of plant materials was extracted from mature leaves by the cetyltrimethylammonium bromide method (Doyle and Dolye, 1987).

Table 1. Ploidy, chromosome number and regional distribution of ten *Diospyros* species used in this study.

Species	Ploidy and chromosome number	Regional distribution (climate)
<i>D. glandulosa</i>	2n=2x=30	Southeast Asia (tropical to subtropical)
<i>D. oleifera</i>	2n=2x=30	East Asia (temperate)
<i>D. lotus</i>	2n=2x=30	Central and East Asia (temperate)
<i>D. ehretioides</i>	2n=2x=30	Southeast Asia (tropical to subtropical)
<i>D. mespiliformis</i>	2n=2x=30	Southern Africa (subtropical)
<i>D. rhodocalyx</i>	2n=2x=30	Southeast Asia (tropical)
<i>D. montana</i>	2n=2x=30	Southeast Asia (tropical to subtropical)
<i>D. rhombifolia</i>	2n=4x=60	East Asia (temperate)
<i>D. kaki</i>	2n=6x=90	East Asia (temperate)
<i>D. virginiana</i>	2n=6x=90	Northeast America (temperate)

Cloning and sequencing of repetitive DNAs Total DNA of *D. kaki* was digested with *EcoRV*, fractionated by electrophoresis on a 0.8% agarose gel, and stained with ethidium bromide (EtBr). The restriction profile showed a ladder of bands corresponding to multimeric DNA fragments of a basic repeat unit of approximately 200 bp in length. This basic repeat of 200 bp was eluted from the gel and cloned into the *EcoRV* site of pBluescriptII (-). Total DNA of *D. oleifera* digested with *HincII* and total

DNA of *D. ehretioides* digested with *DraI* were fractionated electrophoretically. A basic repeat of 200 bp of *D. oleifera* and a band of 160 bp of *D. ehretioides* were cloned into the *HincII* and *EcoRV* sites of pBluescriptII (-), respectively. Several clones each for the repetitive DNAs were sequenced with an automatic DNA sequencer (ABI PRISM, Applied Biosystems, CA, U.S.A.). The repetitive sequences obtained were labeled with digoxigenin (DIG)-11-dUTP by the direct PCR labeling system according to the supplier's instructions (Roche, Mannheim, Germany) and used as probes for genomic Southern blot analysis and FISH. The nucleotide sequences have been registered in the DDBJ/EMBL/GeneBank under the accession numbers AB107228 to AB107230.

Southern blot hybridization Total DNAs (1 µg) of *Diospyros* species digested with three kinds of restriction endonucleases previously used in cloning of repetitive DNAs were electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, NJ, U.S.A.). The membrane was probed with denatured DIG-labeled repetitive DNA at 60°C overnight. After washes [2 × 15 min at 60°C with 0.1 × SSC (20 × SSC consists of 3 M NaCl and 0.3 M sodium citrate) and 0.1% (w/v) sodium dodecylsulfate], colorimetric detection of the hybridization was carried out using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt according to the manufacturer's protocol (Roche, Mannheim, Germany).

Fluorescent *in situ* hybridization (FISH) Chromosome preparation, pretreatment with RNase and FISH were conducted as described before (Choi et al., 2003) with slight modifications. Briefly, root tips of *D. kaki* and *D. lotus* were excised and pretreated with 2 mM 8-hydroquinolin solution for 5 hrs at 4°C and fixed in a methanol-acetic acid solution (3:1, v/v). Chromosome spreads were prepared by an enzymatic maceration method (Fukui, 1996). The hybridization mixture contained 200 ng of the DIG-labeled probe per slide dissolved in 15 µl of 50% formamide in 2 × SSC. The probe mixture was denatured, applied to the slide and covered with a cover slip that was sealed with rubber bond. The chromosomes and probe mixture on the slides were denatured at 80°C for 10 min and the hybridization was performed at 37°C for 2 days. After washing with 2 × SSC, 50% formamide in 2 × SSC and 4 × SSC, each at 42°C for 10 min, the chromosome spreads were blocked with 5% (w/v) bovine serum albumin (BSA) in BT buffer (0.1% sodium hydrogen carbonate, pH 8.3) at 37°C for 5 min. Ten percent (v/v) anti-DIG-fluorescein isothiocyanate (FITC) (Roche, Mannheim, Germany) in 1% (w/v) BSA in BT buffer was dropped onto the chromosomes and incubated at 37°C for 60 min. Subsequent steps were the same as described by Choi et al. (2003). The chromo-

somes were observed with a fluorescence microscope (Axiophot, Zeiss, Oberkochen, Germany) with a high-sensitivity cooled CCD camera (PXL 1400, Photometrics, Ariz., U.S.A.). The FISH signals were analyzed with imaging software (IPLab Spectrum 3.1, Signal Analytics, CA, U.S.A.).

RESULTS

Repetitive DNAs in *EcoRV*-digests A strong band of about 200 bp (designated the *EcoRV*-repeat) was observed in the EtBr-stained gel of the *EcoRV*-digests of *D. kaki*, *D. glandulosa*, *D. oleifera*, *D. lotus* and *D. virginiana* genomic DNA (Fig. 1A). The band of *D. kaki* was cloned, and three of 12 clones obtained were sequenced. The 194-bp repeat unit was relatively AT-rich (67% AT) and contained a pair of inverted subrepeats within the sequence (Fig. 2A; arrows). No significant homology with this sequence was found in the DNA databases. Stop codons were present in all reading frames of the

monomer sequences, suggesting that the members of the *EcoRV*-repetitive DNA family do not encode any protein (data not shown). Southern hybridization of *EcoRV*-digests of the ten *Diospyros* species with the *EcoRV*-repeat as a probe revealed typical ladder band patterns of tandem repeats in the five species, although minor differences were found among them in the signal intensity and ladders of bands (Fig. 1D). Southern hybridization was also performed by hybridizing the same probe to the *Bam*HI-digests of the five species that had signals in the *EcoRV*-digests. Only *D. virginiana* showed a ladder pattern, while the other species had smear hybridization patterns (data not shown). FISH detected signals of the *EcoRV*-repeat probe on the metaphase chromosomes of *D. lotus* and *D. kaki* (Fig. 3). Signals appeared predominantly at the proximal or centromeric regions of the chromosomes, where strong DAPI bands were observed in the two species (Fig. 3A–C and D–F). Almost all chromosomes of *D. lotus* and approximately 70 of the 90 chromosomes of *D. kaki* showed the signals of the *EcoRV*-repeat

Fig. 1. Analysis of the *EcoRV*, *HincII* and *DraI* repetitive DNAs in ten *Diospyros* species. Upper figures (A–C) show ethidium bromide-stained gels of electrophoretically separated genomic DNA digested with *EcoRV*, *HincII* and *DraI*. Lower figures (D–F) show the Southern blots of the respective agarose gels probed with the *EcoRV*, *HincII* and *DraI* repeat probes. (A), (D) *EcoRV* repetitive DNA; (B), (E) *HincII* repetitive DNA; (C), (F) *DraI* repetitive DNA: lane 1, *D. kaki*; lane 2, *D. oleifera*; lane 3, *D. glandulosa*; lane 4, *D. lotus*; lane 5, *D. ehretioides*; lane 6, *D. mespiliformis*; lane 7, *D. rhodocalyx*; lane 8, *D. montana*; lane 9, *D. rhombifolia*; lane 10, *D. virginiana*; lane M, λ HindIII digest.

Fig. 2. Nucleotide sequences of the cloned repeat fragment of each repetitive DNA. (A) the *EcoRV*-repeat fragment from *D. kaki*, (B) the *HincII*-repeat fragment from *D. oleifera*, and (C) the *DraI*-repeat fragment from *D. ehretioides*. Arrows indicate direct (D) or inverted (I) subrepeats in the nucleotide sequence of each repetitive DNA. Nucleotide sequence data reported here are available in the DDBJ database under the accession numbers AB107228 to AB107230.

probe.

Repetitive DNAs in *HincII*-digests After *HincII* digestion and electrophoresis of genomic DNA, EtBr staining showed a prominent band in *D. oleifera* and *D. lotus* (Fig. 1B). This 200-bp band was cloned from *D. oleifera*, and two of nine clones obtained were sequenced. The 206-bp repeat unit of the *HincII*-fragment showed an AT content

of 65% (Fig. 2B). The presence of subrepeats was detected as in the *EcoRV*-repeat, but no significant homology was found between the *HincII*-repeat and the *EcoRV*-repeat. Southern blot hybridization with the *HincII*-repeat as a probe also showed ladder patterns in three species, *D. oleifera*, *D. glandulosa* and *D. lotus* (Fig. 1E). This indicates that the *HincII*-repeats are also arranged in tandem. In the digest of *D. kaki*, a weak

Fig. 3. Localization of the *EcoRV* (A–F) and *HincII* (G–I) repetitive DNAs on chromosomes of *D. lotus* ($2n=2x=30$) and *D. kaki* ($2n=6x=90$). (A) Colored and merged images of FITC signals (green) of the *EcoRV* repeat probe and the DAPI-stained chromosomes of *D. lotus* (blue). (B) FITC signals of the *EcoRV* repeat probe in the same metaphase spread after FISH. (C) DAPI-stained metaphase chromosomes in the same spread (D) Colored and merged images of FITC signals of the *EcoRV* repeat probe and the DAPI-stained chromosomes of *D. kaki*. (E) FITC signals of the *EcoRV* repeat probe in the same metaphase spread after FISH. (F) DAPI-stained metaphase chromosomes in the same spread. (G) Colored and merged images of FITC signals of the *HincII* repeat probe and the DAPI-stained chromosomes of *D. lotus*. (H) FITC signals of the *HincII* repeat probe in the same metaphase spread after FISH. (I) DAPI-stained metaphase chromosomes in the same spread. Bar represents 10 μm .

ladder pattern was observed, but monomeric and dimeric bands were missing and a 23.1-kb band was clear (Fig. 1E; arrowhead). The lack of hybridization signals in the *D. virginiana* digest suggested that the *HincII*-repeats do not exist in this genome. The FISH signals were

detected at the proximal or centromeric regions of *D. lotus* chromosomes, which corresponded to the DAPI bands (Fig. 3G–I). No FISH signals appeared on the chromosomes of *D. kaki* (data not shown).

Repetitive DNAs in *DraI*-digests EtBr-gel staining revealed the presence of *DraI*-bands in the digests of genomic DNA of six species, *D. kaki*, *D. glandulosa*, *D. oleifera*, *D. lotus*, *D. virginiana* and *D. ehretioides* (Fig. 1C). Among those bands, a 160-bp band from *D. ehretioides* was cloned, and two of 11 clones obtained were sequenced. The 164-bp repeat unit of the *DraI*-fragment had an AT content of 71% (Fig. 2C). This *DraI*-repeat also had a pair of subrepeats, but no significant homology to these repeat sequences was found in the DNA databases. Southern hybridization with the *DraI*-repeat as a probe revealed that this sequence exists only in the *D. ehretioides* genome (Fig. 1F). No clear ladder pattern was observed, although several bands appeared in the range of 160–300 bp. This suggests that the organization of the *DraI*-repetitive DNA is different from those of the other repetitive DNAs cloned here. No FISH signals were detected on the chromosomes of *D. ehretioides* (data not shown).

DISCUSSION

The typical ladder patterns observed in Southern blot hybridization with the *EcoRV*- and *HincII*- repeat as probes (Fig. 1D, E) suggested that these sequences are arrayed in tandem in the genomes of *Diospyros* species, as previously shown in other plant species (Iwabuchi et al., 1991; Kamm et al., 1994). The FISH signals from the *EcoRV*- and *HincII*- repeats coincided with the proximal and/or centromeric DAPI bands, which coincide with Giemsa bands in *D. kaki* and *D. lotus* (unpublished data). Giemsa bands reveal centromeric heterochromatin (Schwarzacher et al., 1980) and tandemly arrayed repetitive DNA is frequently localized in heterochromatin (Flavell, 1980). These sequences thus appeared to be components of proximal and/or centromeric heterochromatin in *D. kaki* and *D. lotus*, similar to other centromeric repetitive sequences (Murata et al., 1994; Kishii et al., 2001). Although the *DraI*-repetitive DNA in *D. ehretioides* showed a different hybridization pattern, three tandem repeats ranging from 160 to 200 bp similar to the 180-bp repeat in *Arabidopsis thaliana* (Murata et al., 1994) were detected.

No DNA sequences similar to the *EcoRV*- and *HincII*- repeats were detected by Southern hybridization in *D. rhodocalyx*, *D. mespiliformis*, *D. montana* and *D. rhombifolia* which are morphologically different from *D. kaki* (Utsunomiya et al., 1998; Yonemori et al., 1998). This suggests that both of these repetitive sequences are specific to *D. kaki* and its closely related species. The *EcoRV*-repeat was detected in five species: *D. kaki*, *D. glandulosa*, *D. oleifera*, *D. lotus* and *D. virginiana*. The *HincII*-repeat was also found in those species, except for in *D. virginiana*. This is noteworthy, because *D. virginiana* was classified as a monophyletic group with *D. kaki*

and *D. lotus* by cpDNA analysis (Yonemori et al., 1998).

The different genomic distributions of the *EcoRV*- and the *HincII*- repetitive DNAs suggests that the *EcoRV*-repetitive DNA may have evolved earlier and that the amplification of the *HincII*-repetitive DNA occurred after the divergence of *D. virginiana*, as suggested previously for two satellites isolated from *Beta vulgaris* (Schmidt et al., 1991). In Southern blots of *Bam*HI-digests with the *EcoRV*-repeat probe, a ladder pattern was observed only for *D. virginiana* (data not shown). These results suggest that the genomic evolution of *D. virginiana* after speciation was different from that of *D. kaki* and its related species. Nakatsuka et al. (2002) also suggested that *D. virginiana* was unlikely to be related to *D. kaki* in that a subgroup of *Ty1*-copia group retrotransposons was markedly increased in only *D. virginiana* of the species tested. The lack of significant sequence homology between the *EcoRV*- and *HincII*- tandem repeats also suggests that these two sequences evolved from different ancestral sequences, as shown previously in other plant species (Schmidt and Metzlaß, 1991). Tetraploid *Hordeum murinum* was suggested to be allopolyploid in that a cloned repetitive DNA was found in *H. murinum* (4x) but not in its identified diploid ancestor (Taketa et al., 2000). Taken together, these facts suggest that different ancestral species might have been involved in the speciation of *D. virginiana* and *D. kaki*.

The detection of the FISH signal of the *EcoRV*-repeat probe on the chromosomes of diploid *D. lotus* and hexaploid *D. kaki* suggest that a diploid with the *EcoRV*-repetitive DNA, such as *D. glandulosa*, *D. oleifera* and *D. lotus*, was involved in the speciation of *D. kaki*. Furthermore, the fact that almost all chromosomes of *D. lotus* and approximately 70 of the 90 chromosomes of *D. kaki* carry the *EcoRV*-repeat might imply that several species with very similar genomic composition were involved in the speciation of *D. kaki*. The observation of the segregation pattern of a molecular marker linked to natural astringency loss (Kanzaki et al., 2001) and four sets of chromosomes carrying 45S rDNA (Choi et al., 2003) suggested that *D. kaki* could be an autohexaploid or autoallohexaploid.

The presence of the *EcoRV*- and *HincII*- repetitive DNAs in *D. glandulosa* and *D. oleifera* reveals a certain degree of similarity of the genomic organization of these two diploids with that of *D. kaki* and *D. lotus*. The morphological features suggest that *D. glandulosa* could be one of the progenitor species of hexaploid *D. kaki* (Ng, 1978). The genomic background of *D. ehretioides*, a putative progenitor species of *D. kaki*, may be different from that of the other *Diospyros* species tested. The numerous satellite bands of *D. ehretioides* (Fig. 1A–C, lane 5) may contribute to the increased chromosomal length (Choi et al., 2003) and genome size (twice as much as that of hexaploid *D. kaki*; unpublished data of this

species). Amplification of satellite DNAs has been postulated to be one of the causes of the increase of genome size and chromosomal length in plant species (Uozu et al., 1997; Torrell and Valles, 2001; Valarik et al., 2002). In chloroplast DNA analysis, *D. glandulosa* and *D. oleifera* were classified in a monophyletic group with *D. rhombifolia* and *D. montana* while *D. ehretioides* was classified in the same clade as *D. kaki*, *D. lotus* and *D. virginiana* (Yonemori et al., 1998). Chloroplast DNA is inherited uniparentally in a strictly maternal fashion (Smith, 1989) and may not reflect the precise relationships of closely related species. The result of a recent study of the sequence of the ITS and *matK* regions were in agreement with our results here showing the closest relationship between *D. kaki* and three diploids, *D. glandulosa*, *D. oleifera* and *D. lotus* (Ino et al., 2002).

Rearrangement of the repetitive DNAs seems to have taken place in the genome of *Diospyros* species after speciation. *D. kaki* had a strong signal with the *HincII*-repeat probe in the range of 23.1kb (Fig. 1E). This may be the relic DNA (Bedbrook et al., 1980a) that was suggested to have diverged without sequence amplification and to have been rearranged in a large cluster by losing the restriction site and/or by C-methylation during the evolution of cereal plant species (Iwabuchi et al., 1991; Martinez-Zapater et al., 1986). The several *DraI*-bands corresponding to smaller fragments in the *D. ehretioides* digest (Fig. 1F) suggest that the *DraI*-sequence family is dispersed in the *D. ehretioides* genome, like satellite DNA of *Beta vulgaris* (Schmidt and Metzlauff, 1991). Different signal intensity of the repeat probe in FISH analysis, such as that seen in Fig. 3, has also been reported to be the result of rearrangement and/or amplification of sub-repeats (Kamm et al., 1995; Schmidt et al., 1991).

The mechanism of the deletion and diversification of these repetitive sequence families remains to be studied, along with the genome constitution of *Diospyros* species, and will be critical for elucidating the mechanism of speciation of *D. kaki* and its related species. In addition to providing information about the speciation of the hexaploid *D. kaki* and *D. virginiana*, the repetitive sequence analysis should be useful for the studying the genomic evolution of *Diospyros* species at the molecular level.

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