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 Hincll-repetitive DNA in tandem. Fluorescent in situ hybridization showed that the EcoRV- and Hincll-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, minisatellite, and microsatellite DNAs and retrotransposons, constitute the major fraction of nuclear genomes in many higher plants (Bedbrook et al., 1980b; Schmidt and Metzlaff, 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...
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.

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

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 HinII and total DNA of D. erhetioides digested with DraI were fractionated electrophoretically. A basic repeat of 200 bp of D. oleifera and a band of 160 bp of D. erhetioides were cloned into the HinII 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, Amasham 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-hydroquinolinol 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 (w/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-
Rearrangements of Repeat DNAs in *Diospyros kaki* and its wild relatives

Some 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 BamHI-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.

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**Fig. 1.** Analysis of the EcoRV, HincII and Dral 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 Dral. Lower figures (D–F) show the Southern blots of the respective agarose gels probed with the EcoRV, HincII and Dral repeat probes. (A), (D) EcoRV repetitive DNA; (B), (E) HincII repetitive DNA; (C), (F) Dral 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.
Repetitive DNAs in \textit{Hin\textsubscript{c}II}-digests

After \textit{Hin\textsubscript{c}II} digestion and electrophoresis of genomic DNA, EtBr staining showed a prominent band in \textit{D. oleifera} and \textit{D. lotus} (Fig. 1B). This 200-bp band was cloned from \textit{D. oleifera}, and two of nine clones obtained were sequenced. The 206-bp repeat unit of the \textit{Hin\textsubscript{c}II}-fragment showed an AT content of 65\% (Fig. 2B). The presence of subrepeats was detected as in the \textit{EcoRV}-repeat, but no significant homology was found between the \textit{Hin\textsubscript{c}II}-repeat and the \textit{EcoRV}-repeat. Southern blot hybridization with the \textit{Hin\textsubscript{c}II}-repeat as a probe also showed ladder patterns in three species, \textit{D. oleifera}, \textit{D. glandulosa} and \textit{D. lotus} (Fig. 1E). This indicates that the \textit{Hin\textsubscript{c}II}-repeats are also arranged in tandem. In the digest of \textit{D. kaki}, a weak

Fig. 2. Nucleotide sequences of the cloned repeat fragment of each repetitive DNA. (A) the \textit{EcoRV}-repeat fragment from \textit{D. kaki}, (B) the \textit{Hin\textsubscript{c}II}-repeat fragment from \textit{D. oleifera}, and (C) the \textit{DraI}-repeat fragment from \textit{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.
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 Giesma bands in D. kaki and D. lotus (unpublished data). Giesma 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 BamHI-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. Nakatsu 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 Metzlaff, 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-repetitive 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 Dral-bands corresponding to smaller fragments in the D. ehretioides digest (Fig. 1F) suggest that the Dral-sequence family is dispersed in the D. ehretioides genome, like satellite DNA of Beta vulgaris (Schmidt and Metzlaff, 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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