DNA Restriction Fragment Length Variability in *Diospyros kaki* and Related *Diospyros* Species

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Additional index words. RFLPs, Japanese persimmon, mtDNA, cpDNA

Abstract. Restriction fragment length analyses of mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) were carried out on 11 cultivars of Japanese persimmon (*Diospyros kaki* Thunb.) and five other *Diospyros* species. Total genomic DNA was digested with seven restriction endonucleases, Southern blotted, and hybridized with five mtDNA probes (*Pst*I or *Sal*I fragments of *Brassica campestris* L. mtDNA) and one cpDNA probe [*pT*Bal, *Bam*HI fragment of *Nicotiana tabacum* (L.) cpDNA]. All Japanese persimmon cultivars displayed identical mtDNA and cpDNA fragment patterns, while polymorphisms among species were observed using both mtDNA and cpDNA probes. A low degree of polymorphism was observed between *D. kaki*, *D. oleifera* Cheng., *D. kuroi*wai Nakai, *D. virginiana* L., and *D. lotus* L., suggesting that these species are closely related. A high degree of polymorphism was observed between *D. rhombifolia* Hemsl. and the other five species, indicating that this species is more distantly related to the other five.

Restriction fragment length polymorphisms (RFLPs) have been used as genetic markers to identify species and cultivars, evaluate genetic diversity, and map genes of interest in numerous crops (Hubbard et al., 1992; Mauro et al., 1992; Menancio et al., 1990; Song et al., 1990; Watillon et al., 1991; Yamamoto et al., 1991). cpDNA and mtDNA are generally inherited maternally (Conde et al., 1979; Ichikawa and Hirai, 1983; Vedel et al., 1981). cpDNA and mtDNA RFLPs provide useful markers for studying phylogenetic and evolutionary relationships and for breeding studies (Baldwin et al., 1991; Haghighi and Hancock, 1992; Ishikawa et al., 1992). Japanese persimmon, native to East Asia, is one of the most important fruit-bearing trees in Japan. This species has been cultivated for hundreds of years, and there are more than 1000 cultivars. However, nothing is known about the degree of heterogeneity of mtDNA and cpDNA among *D. kaki* cultivars. We analyzed mtDNA and cpDNA in *D. kaki* and its closely related *Diospyros* species to evaluate cytoplasmic diversity among the cultivars of *D. kaki* and to provide new information on phylogenetic relationships of *D. kaki* and its closely related *Diospyros* species.

Materials and Methods

Total DNA was isolated from fully expanded fresh leaves of 11 typical cultivars of *D. kaki* and five of its related *Diospyros* species (Table 1). The trees were grown in the experimental orchard of the Akitsu Branch, Fruit Tree Research Station, Japan, according to the method of Honda and Hirai (1990) with the following modification: leaves were frozen in liquid N₂ and ground into powder that then was suspended in extraction buffer [100 mM Tris-HCl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 20 mg polyvinylpyrrolidone (PVP)/ml, 100 mM 2-mercaptoethanol] and pelleted by centrifugation (35,000×g for 5 min at 4°C) twice before DNA extraction. DNA (1 µg) was digested with one of seven restriction endonucleases (*Bam*HI, *Dra*I, *Eco*RI, *Hind*III, *Pst*I, *Pvu*II, *Xba*I; Nippon Gene Corp., Japan), and the resulting fragments were separated by electrophoresis through 0.8% agarose gels at a constant 0.8 V/cm for 13 to 14 h. After electrophoresis, the DNA was transferred to Tropilon 45 nylon membrane (Tropix Corp.) as described by Southern (1975). The DNA was bound with a UV crosslinker (Spectronics Corp.). DNA fragments used as probes were prepared from plasmid *pT*Bal, which con-

tains a 19.6-kb *Bam*HI fragment of *Nicotiana tabacum* cpDNA (Sugiura et al., 1986), or were *Pst*I or *Sal*I fragments of *Brassica campestris* mtDNA plasmids P4.8, P9.7, P5.7, P12.4, and S11.8 (Palmer and Shields, 1984). Probe DNA was labeled with digoxigenin (DIG) using a DIG labeling kit (Boehringer Mannheim). Membranes were prehybridized for 4 h at 68°C in a hybridization buffer [5× SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 5 mg blocking reagent/ml (Boehringer Mannheim Cat. no. 1096176), 1 mg N-lauroylsarcosine-Na/ml, 0.2 mg sodium dodecyl sulfate (SDS)/ml], then hybridized overnight at 68°C in the same buffer containing the labeled probe. The hybridized membrane was washed twice with 2× SSC, 1 mg SDS/ml for 5 min at room temperature, followed by two washes of 0.5× SSC, 1 mg SDS/ml for 15 min at 65°C. Probe-target DNA hybrids were visualized by chemiluminescent detection of DIG-labeled nucleic acids (Boehringer Mannheim). Genetic similarities were estimated based on the number of shared fragments between paired samples.

Results and Discussion

When blots of restriction-endonuclease-digested genomic DNA were hybridized with five mtDNA probes, RFLPs among species were observed in 32 out of 35 probe-enzyme combinations. However, mtDNA restriction patterns within species of *D. kaki* were monomorphic, regardless of the restriction endonucleases and mtDNA probes used. Polymorphisms were detected among species. For example, the P9.7 mtDNA probe did not detect polymorphisms in *Bam*HI-digested DNA of *D. kaki*, *D. oleifera*, *D. kuroi*wai, and *D. lotus*. However, RFLPs were detected among *D. virginiana*, *D. rhombifolia*, and these four species (data not shown). When the S11.8 mtDNA probe was hybridized to *Hind*III-digested DNA, *D. oleifera*, *D. kuroi*wai, *D. virginiana*, and *D. lotus* revealed a 17.5-kb fragment not present in *D. kaki*, and *D. rhombifolia* displayed instead a unique 14.5-kb fragment (Fig. 1A). The P5.7 mtDNA probe hybridized to *Bam*HI-digested DNA revealed a polymorphism unique to *D. kuroi*wai (Fig. 1B). By combining the mtDNA RFLP data, we were able to distinguish all species. When the frequencies of shared fragments between paired species (F value) were calculated, *D. kaki*, *D. oleifera*, *D. kuroi*wai, *D. virginiana*,

Table 1. *Diospyros* species examined for mtDNA and cpDNA polymorphisms.

Species	Cultivar	Origin
<i>D. kaki</i> ²	Shi-shan-shi, Da-mo-pan, Luo-tian-tian-shi	China
	Ban-si, Chong-do-si	Korea
	Fuyuu, Goshu, Jisha, Saijou	Japan
	Tai-wan-zheng-shi, Tai-wan-hong-shi	Taiwan
<i>D. oleifera</i>		China
<i>D. kuroi</i> wai		Japan
<i>D. virginiana</i>		USA
<i>D. lotus</i>		Japan ³
<i>D. rhombifolia</i>		China

²Eleven typical cultivars of *D. kaki*, distantly related to each other, were selected for analysis.

³*Diospyros lotus* is widely distributed in East, Central, and West Asia (Ng, 1979). The clones of *D. lotus* analyzed in this study originated in Japan.

Received for publication 16 Dec. 1992. Accepted for publication 25 Nov. 1993. Contribution no. E-164 of the Fruit Tree Research Station. We thank M. Sugiura and J.D. Palmer for their generosity in providing cpDNA or mtDNA clones. Thanks are also extended to M. Yamada for his valuable advice and suggestions and M.J. Clarkin for reading the manuscript. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

and *D. lotus* showed high relatedness to each other, with F values among them generally > 0.9. In contrast, *D. rhombrifolia* showed lower relatedness with the other species, illustrated by F values $\approx$ 0.6.

Similar results were obtained when digests were probed with the pTBa1 cpDNA probe. Intraspecific variation of *D. kaki* was not detected. RFLPs were detected among species, except *D. kaki* and *D. oleifera*, when *EcoRI*-, *DraI*-, *HindIII*-, and *XbaI*- digested DNA blots were hybridized with pTBa1. When pTBa1 was hybridized to *DraI*- digested DNA, the 11 cultivars of *D. kaki*, *D. oleifera*, and *D. kuroiway* were monomorphic. RFLPs were observed among *D. virginiana*, *D. lotus*, *D. rhombrifolia*, and the three other *Diospyros* species (Fig. 2). F values obtained by comparing frequency of shared fragments' relative degree of relatedness were similar to those detected from mtDNA RFLPs,

Diospyros kaki cultivars show high variation in morphological and physiological characteristics and have been distinguished by isozyme markers (Tao and Sugiura, 1987; Tao et al., 1989). When the mtDNA and cpDNA probes were used to detect RFLPs, however, intraspecific variation was not detected. These data suggest that the mtDNA and cpDNA of *D. kaki* are highly homogeneous. *Diospyros kaki*, *D. oleifera*, *D. kuroiway*, *D. virginiana*, and *D. lotus* showed high relatedness to each other; in contrast, *D. rhombrifolia* showed lower relatedness with the other species. This result agrees well with morphological taxonomy. Ng (1978) hypothesized that *D. kaki* originated from *D. roxburghii*. If his hypothesis is true, then the mtDNA and cpDNA fragment patterns of *D. roxburghii* should be the same as or more similar to *D. kaki* than to other closely related species. However, we were unable to obtain a sample of *D. roxburghii* for analysis. Additional RFLP analysis with more species, especially with *D. roxburghii*, is necessary to clarify the phylogenetic relationships among *D. kaki* and its closely related *Diospyros* species.

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Fig. 1. RFLPs of *Diospyros* species: (A) when mtDNA probe S11.8 was hybridized to *HindIII*-digested DNA, (B) when mtDNA probe P5.7 was hybridized to *BamHI*- digested DNA. Lanes 1-8, *D. kaki*; lane 9, *D. oleifera*; lane 10, *D. kuroiway*; lanes 11-12, *D. virginiana*; lanes 13-14, *D. lotus*; lanes 15-16, *D. rhombrifolia*.

Fig. 2. RFLPs of *Diospyros* species when the cpDNA probe pTBa1 was hybridized to *DraI*- digested DNA. Lanes 1-8, *D. kaki*; lane 9, *D. oleifera*; lane 10, *D. kuroiway*; lanes 11-12, *D. virginiana*; lanes 13-14, *D. lotus*; lanes 15-16, *D. rhombrifolia*.

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