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Scientia Horticulturae 117 (2008) 32–38
SCIENTIA HORTICULTURAE
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Phylogenetic analysis in some *Diospyros* spp. (Ebenaceae) and Japanese persimmon using chloroplast DNA PCR-RFLP markers

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Received 3 September 2007; received in revised form 16 January 2008; accepted 1 February 2008

Abstract

Ten universal primer pairs of chloroplast genome were used to amplify non-coding regions of chloroplast DNA (cpDNA) in 7 *Diospyros* L. species including 29 genotypes and approximately 20.4 kb, 12.6% of the chloroplast genome were analyzed. The amplified products were digested by seven restriction enzymes. The results showed that there were abundant polymorphisms in inter-specific cpDNA within the genus *Diospyros*. However, it was not observed intra-species variations in 22 tested genotypes of *Diospyros kaki* (Japanese persimmon), except for 'Male strain No. 9'. Persimmon had close relationship with *Diospyros lotus* and *Diospyros glaucifolia*, but distantly with *Diospyros virginiana* and *Diospyros rhombifolia* in diagram based on principal coordinates analysis and Wagner parsimony method. The discrepancy of digesting pattern in cpDNA suggested that the genotype Jinzaoshi was distinct with the remaining *Diospyros* spp., which revealed that Jinzaoshi may be a new species of the genus *Diospyros*.
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Keywords: *Diospyros* spp.; Chloroplast DNA; PCR-RFLP; Genetic relationships

1. Introduction

The genus *Diospyros* consists of approximately 400 species and its chromosome numbers were complex, which were widely distributed in tropical and subtropical regions and only a few native to temperate region. Japanese persimmon (*Diospyros kaki* Thunb.), as an important fruit tree, has been cultivated in China for more than 2000 years, which had astringent and non-astringent cultivars. Among them, non-astringent cultivars as fresh fruit need no post-harvest treatment, such as with carbon dioxide or ethanol to remove astringency. Then, each type was further classified into two sub-types: variant and constant type, depending on the relationship between presence of seeds and flesh color. The flesh color of variant type was influenced by pollination. Thus, cultivars were commonly classified into four groups: pollination variant non-astringent (PVNA), pollination constant non-astringent (PCNA), pollination variant astringent (PVA) and pollination constant astringent (PCA) (Yonemori et al., 2000). However, it was difficult to know where Japanese persimmon originated from and how it evolved.

Japanese persimmon was $2n = 6x = 90$ with a few exceptions of nonaploid cultivars (Zhuang et al., 1990). Ng (1978) suggested it was derived from *Diospyros roxburghii* based on the studies of morphologic similarities and geography distributions. So far, the relationship between wild and cultivated *Diospyros* spp. has been discussed based on series of molecular markers, such as RFLP (Nakamura and Kobayashi, 1994), RAPD (Yamagishi et al., 2005), AFLP (Kanzaki et al., 2000), SRAP (Guo and Luo, 2006), IRAP and REMAP (Guo et al., 2006). Chloroplast DNA (cpDNA) polymorphism has been used in the studies on the genus *Diospyros*. Yonemori et al. (1998) surveyed 2 cpDNA regions of 24 *Diospyros* spp. mainly in Thailand by 25 primer–enzyme combinations and considered that *D. kaki*, *Diospyros lotus* and *Diospyros virginiana* had an immediate common progenitor. However, Yonemori et al. (2008) confirmed that *D. kaki* was closely related to two diploid species based on the sequence variations in ITS and *mat*K regions. So far, it was not clear how *D. kaki* had happened from other related species.

Compared with the nuclear genome, the cpDNA has uniparental mode of inheritance and low mutation rate on

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0304-4238/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.scienta.2008.02.001

structure and sequence. So, it was considered to be an ideal system in phylogeny and population genetics (Palmer, 1987; Randall et al., 1998). Currently, sequence comparison or restriction analysis of fragments amplified with universal primers for cpDNA has been widely used in species identification, genetic diversity and phylogenetic studies in many plants (Badenes and Parfitt, 1995; Demesure et al., 1996; Parani et al., 2000; Xu et al., 2001; Wu et al., 2005). In this study, PCR-RFLP was performed on approximately 20.4 kb of the chloroplast genome using 10 universal primer pairs and seven restriction enzymes in 29 *Diospyros* L. genotypes including 1 Male strain, 5 pollination constant non-astringent cultivars and 5 related species native to China. The objective of this study was to test the variations between inter-specific and intra-specific chloroplast genome based on restriction site polymorphism and to learn phylogenetic relationships in the genus *Diospyros*.

2. Materials and methods

2.1. Plant materials

Seven *Diospyros* spp. including 29 genotypes were used in this study, which were sampled in Persimmon Repository of Huazhong Agriculture University, Wuhan, China. Ploidy level, astringent type and origin with collection sites were presented in Table 1. Jinzaoshi has not been identified in taxonomy among them.

2.2. DNA extraction

Total DNA was extracted from fully expanded fresh leaves using CTAB method with minor modifications according to Doyle and Doyle (1987).

2.3. PCR-RFLP analysis

Ten universal primer pairs were employed to amplify non-coding regions of cpDNA in *Diospyros* spp. The primer sequences were shown in Table 2 (Demesure et al., 1995; Dumolin-Lapegue et al., 1997; Parani et al., 2000). PCR amplifications were performed in a total volume of 25 μl containing 30 ng of template DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM Mg^{2+}, 0.2 mM dNTPs, 0.2 mM of each primer, and 1 U of *Taq* polymerase (TaKaRa Biotech Co. Ltd., Japan). PCR amplification was performed as the following profile: an initial 4 min denaturation at 94 °C, 35 cycles of 1 min at 94 °C, 45 s at 44–46 °C, 1–3 min at 72 °C corresponding to different primer combinations, and a 10 min final extension step at 72 °C. The PCR products were separated on 2% agarose gels and stained with ethidium bromide. Molecular weights were estimated using a DNA marker DL2000 (TaKaRa Biotech Co. Ltd., Japan).

Seven restriction enzymes, *AluI*, *HaeIII*, *HinfI*, *Hin6I*, *RsaI*, *MvaI* and *TaqI* were used for the digestion of the PCR-amplified cpDNA fragments, which were not same as the enzymes in

Table 1
List of *Diospyros* spp. used in this study with ploidy levels, collected locations and astringent types

No.	Genotype	Scientific name	Ploidy level	Astringent type	Distribution
1	Date plum	*D. lotus* L.	$2n = 2x = 30$	–	China
2	Chekiang persimmon	*D. glaucifolia* Metc.	$2n = 2x = 30$	–	China
3	Oily persimmon	*D. oleifera* Cheng.	$2n = 2x = 30$	–	China
4	Jinzaoshi	*D.* sp.	$2n = 2x = 30$	–	China
5	Diamond Leaf persimmon	*D. rhombifolia* Hemsl	$2n = 4x = 60$	–	China
6	Common persimmon	*D. virginiana* L.	$2n = 6x = 90$	–	USA
7	Mopanshi	*D. kaki* Thunb.	$2n = 6x = 90$	PCA	China
8	Tongpenshi	*D. kaki* Thunb.	$2n = 6x = 90$	PCA	China
9	Male strain No. 9	*D. kaki* Thunb.	$2n = 6x = 90$	–	China
10	Sifang-tianshi	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	China
11	Xiaoguo-tianshi	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	China
12	Luotiantianshi	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	China
13	Baogai-tianshi	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	China
14	Eshi No. 1	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	China
15	Taiwan-zhengshi	*D. kaki* Thunb.	$2n = 6x = 90$	PCA	China
16	Zenjimaru	*D. kaki* Thunb.	$2n = 6x = 90$	PVNA	Japan
17	Fuyuu	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	Japan
18	Youhou	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	Japan
19	Jirou	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	Japan
20	Maekawa-Jirou	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	Japan
21	Akagaki	*D. kaki* Thunb.	$2n = 6x = 90$	PVNA	Japan
22	Nishimura-wase	*D. kaki* Thunb.	$2n = 6x = 90$	PVNA	Japan
23	Hana-gosho	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	Japan
24	Suruga	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	Japan
25	Oku-gosho	*D. kaki* Thunb.	$2n = 6x = 90$	PCNA	Japan
26	Huashi No. 1	*D. kaki* Thunb.	$2n = 6x = 90$	PVNA	Japan
27	Sagoksi No. 2	*D. kaki* Thunb.	$2n = 6x = 90$	PCA	Korea
28	Hyakume	*D. kaki* Thunb.	$2n = 6x = 90$	PVNA	USA
29	Hiratanenashi	*D. kaki* Thunb.	$2n = 9x = 135$	PVA	Japan

Note: PCNA, pollination constant non-astringent; PVNA, pollination variant non-astringent; PVA, pollination variant astringent; PCA, pollination constant astringent.

Table 2
The sequence and amplification conditions of 10 cpDNA universal primer-pair combinations used in the 6 *Diospyros* species and 23 genotypes of *D. kaki* studied

No.	Region	Sequence forward	Sequence reverse	T_a (°C)	Size (bp)	Reference
1	*trn*H-*trn*K	5′-ACGGGAATTGAACCCGCGCA-3′	5′-CCGACTAGTTCCGGGTTCGA-3′	55	1650	Demesure et al. (1995)
2	*trn*S-*trnf*M	5′-GAGAGAGAGGGATTCGAACC-3′	5′-CATAACCTTGAGGTCACGGG-3′	55	1650	Demesure et al. (1995)
3	*rbc*L	5′-TGTCACCAAAAACAGAGACT-3′	5′-TTCCATACTTCACAAGCAGC-3′	55	1700	Parani et al. (2000)
4	*trnk*1-*trnk*2	5′-GGGTTGCCCGGGACTCGAAC-3′	5′-CAACGGTAGAGTACTCGGCTTTTA-3′	61.5	2500	Demesure et al. (1995)
5	*trn*C-*trn*D	5′-CCAGTTCAAATCTGGGTGTC-3′	5′-GGGATTGTAGTTCAATTGGT-3′	58	2950	Demesure et al. (1995)
6	*trn*D-*trn*T	5′-ACCAATTGAACTACAATCCC-3′	5′-CTACCACTGAGTTAAAAGGG-3′	55	1800	Demesure et al. (1995)
7	*trn*M-*rbc*L	5′-TGCTTTCATACGGCGGGACT-3′	5′-GCTTTAGTCTCTGTTTGTGG-3′	58	2850	Demesure et al. (1995)
8	*trn*F-*trn*Vr	5′-CTCGTGTCACCAGTTCAAAT-3′	5′-CCGAGAAGGTCTACGGTTCG-3′	58	2200	Dumolin-Lapegue et al. (1997)
9	*trn*S-*trn*T	5′-CGAGGGTTCGAATCCCTCTC-3′	5′-AGAGCATCGCATTTGTAATG-3′	55	1500	Demesure et al. (1995)
10	*psb*C-*trn*S	5′-GGTCGTGACCAAGAAACCAC-3′	5′-GGTTCGAATCCCTCTCTCTC-3′	55	1600	Demesure et al. (1995)

Yonemori's paper (1998), except of RsaI and TaqI. These restriction enzymes would recognize a four-nucleotide sequence and had perfect digested ability. They had been used to digest the cpDNA in other species, such as Prunus (Badenes and Parfitt, 1995), mangrove (Parani et al., 2000), houttuynia (Wu et al., 2005) and so on. Ten microliter digestions were carried out with 3 U of each restriction endonucleases, and incubated for 5 h at 37 °C (*Alu*I, *Hae*III, *Hinf*I, *Hin6*I, *Rsa*I, *Mva*I) or 65 °C (*Taq*I). Restriction fragment were electrophoresed on 2% agarose in 1× Tris–borate–EDTA buffer, and then stained by ethidium bromide, visualized under UV light and scoring bands.

2.4. Data analysis

The binary data matrix reflecting the presence (1) or absence (0) of each band was generated for each genotype. After Jaccard coefficient was calculated using the SIMINT programme, PCoA was performed based on genetic similarity matrices using the DCENTER and EIGEN algorithms of the NTSYS-pc software package (Rohlf, 2000). Furthermore, cluster analysis by Wagner parsimony method conducted with PHYLIP 3.62 (Felsenstein, 2004) after 500 bootstraps were done using SEQBOOT program.

3. Results

3.1. cpDNA PCR-RFLP polymorphism within the genus *Diospyros*

Ten corresponding cpDNA regions had successfully been amplified in all *Diospyros* genotypes examined in the present study. In this study, approximately 20.4 kb accounting for 12.6% of the chloroplast genome was amplified. However, it was found that each primer pair generated single monomorphic fragment and there was no difference in amplified fragment length among the 29 genotypes. It indicated that the sequence of chloroplast genome was conserved.

In the present study, 29 (41.4%) out of 70 primer enzyme combinations were detected interspecies and intra-species polymorphism in the chloroplast genomes. A total of 267 fragments were generated by digestion of the amplified products with *Alu*I, *Hae*III, *Hinf*I, *Hin6*I, *Rsa*I, *Mva*I and *Taq*I, of which 124 fragments were polymorphism. The digested patterns in chloroplast regions were listed in Table 3. Within all regions surveyed in this study, *trn*S-*trnf*M, *trnk*1-*trnk*2 and *trn*C-*trn*D had not detected the corresponding restriction sites of *Hae*III, *Hin6*I and *Mva*I, respectively. The productions amplified by *trn*H-*trn*K primer had not been digested by *Hin6*I and *Taq*I. However, the genetic information generated by RFLPs were aboundance in part combinations of primers and enzymes. Many variations in chloroplast genome sequence were found in surveyed regions of the genus *Diospyros*, such as *trn*H-*trn*K, *trn*S-*trnf*M, *trnk*1-*trnk*2 and *trn*C-*trn*D (Fig. 1). Interspecies polymorphism had been revealed in the digesting patterns, which indicated that base change had taken place on sequence of cpDNA during the evolution. On the other hand, variations in the bands length were visible by digesting the amplified products, for instance 600 bp in *trn*C-*trn*D/*Alu*I (Fig. 1A) and 480 and 280 bp in *trn*C-*trn*D/RsaI (Fig. 1B). It was observed that the insertions and/or deletions had occurred in chloroplast genome. In addition, the digested products of *D. virginiana* with *RsaI* had the high band intensity at 800 bp (Fig. 1B), which suggested that two fragments were superposition at this position.

Based on the sequence of *trn*C-*trn*D region, seven species could be divided into six groups by four restriction enzymes: (1) *D. rhombifolia*, (2) *D. virginiana* (3) *D. oleifera*, (4) Jinzaoshi, (5) *D. kaki*, (6) *D. lotus* and *D. glaucifolia*. Except for *trn*H-*trn*K/*Rsa*I and *rbc*L/*Alu*I, 27 primer/enzyme combinations showed monomorphic between *D. lotus* and *D. glaucifolia*, which indicated that both these sequences were homologous. The different genotype Jinzaoshi had the unique restriction site mutations with the other *Diospyros* spp. (Fig. 1C). *D. rhombifolia* and *D. virginiana* had obvious different patterns from that of the other related species, which suggested abundant variations in their chloroplast DNA (Fig. 1D).

Excepted single mutation site had been found existed in Male strain No. 9 by *trn*S-*trnf*M/RsaI combination, no other evident intra-specific polymorphism was examined among the 23 genotypes of *D. kaki*. The PCNA cultivars native to China, ‘Luotiantianshi’ and ‘Eshi No. 1’ were observed monophyletic of cpDNA sequence in 10 chloroplast genomic regions with the genotypes from Taiwan, Japan, Korea, and USA. In the case of 22 different types of lose astrigency genotypes, including the PCA (e.g. ‘Mopanshi’), PVA (e.g. ‘Hiratanenashi’), PVNA

Table 3
Presence (1) or absence (0) of mutation sites in the 10 chloroplast regions for the 6 *Diospyros* species and 23 genotypes of *D. kaki* in the study

No.	*trn*H-*trn*K					*trn*S-*trnf*M			*rbc*L			*trnk*1-*trnk*2			
	AluI	HaeIII	HinfI	MvaI	RsaI	HinfI	RsaI	TaqI	AluI	HaeIII	MvaI	AluI	HinfI	RsaI	TaqI
1	10111	010010	01110	01010	101000	0111	01001100	100	001011	011	100	1111010	10111	1101	1011010100
2	10111	010010	01110	01010	011110	0111	01001100	100	111111	011	100	1111010	10111	1101	1011010100
3	10111	010100	11010	01010	101000	0111	01001100	100	010111	011	100	1110110	01111	1011	0111000100
4	11111	010001	01110	00110	011001	1011	01001100	100	111111	011	011	1111010	10111	1101	1011000100
5	10111	010100	01101	01010	101000	0111	01001100	011	010111	011	100	1111010	10111	1101	1011000100
6	10111	101000	01110	10001	101000	0111	01001100	100	001011	100	100	1111001	10101	1101	0111001101
7	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
8	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
9	10111	010100	01110	01010	101000	0111	11110010	100	001011	011	100	1111010	10111	1101	1011100111
10	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
11	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
12	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
13	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
14	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
15	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
16	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
17	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
18	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
19	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
20	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
21	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
22	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
23	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
24	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
25	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
26	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
27	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
28	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111
29	10111	010100	01110	01010	101000	0111	01000111	100	001011	011	100	1111010	10111	1101	1011100111

No.	*trn*C-*trn*D				*trn*D-*trn*T	*trn*M-*rbc*L		*trn*F-*trn*Vr		*trn*S-*trn*T		*psb*C-*trn*S		
	AluI	HinfI	RsaI	TaqI	MvaI	Hin6I	HaeIII	Hin6I	RsaI	HinfI	RsaI	HaeIII	Hin6I	HinfI
1	10010100	101010	011011001	101010101	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
2	10010100	101010	011011001	101010101	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
3	10010100	101010	011011001	101011011	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
4	10011000	101010	011011001	101011001	1001	1001	1111	11001	0111111	1001	1001	100	0011010	101101000
5	11100011	101010	101100101	011100101	0111	0111	1101	10111	0111111	0110	0110	011	1010000	011110100
6	10011000	101010	001101011	001110001	1001	1001	1111	11001	1010111	1001	1001	100	0100101	101100011
7	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
8	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
9	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
10	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
11	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
12	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
13	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
14	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
15	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
16	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
17	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
18	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
19	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
20	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
21	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
22	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
23	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
24	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
25	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
26	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
27	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
28	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000
29	10010100	110101	011011001	101011001	1001	1001	1111	11001	0110111	1001	1001	100	0011010	101101000

Note: 1–29 refer to the numbers listed in Table 1.

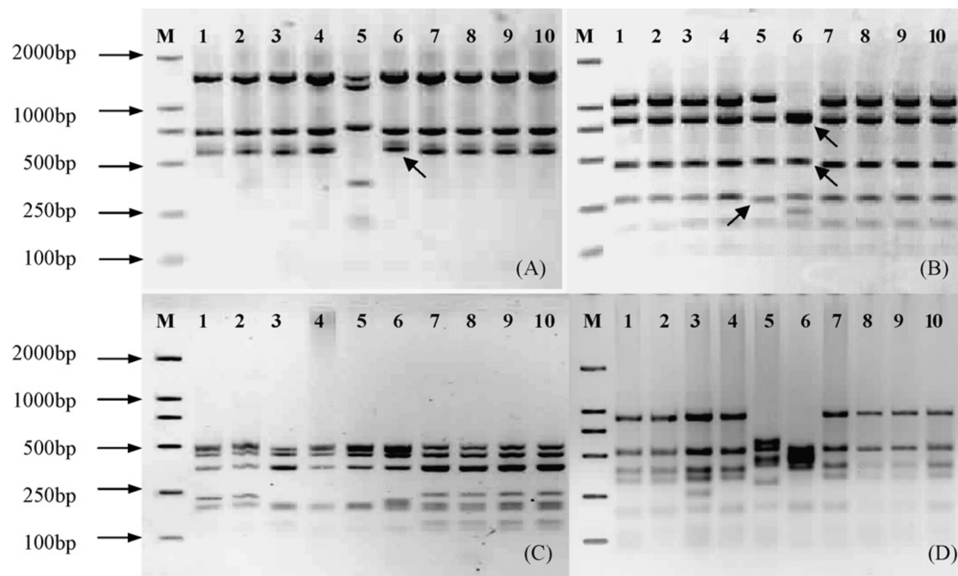

Fig. 1. Restriction fragments patterns of cpDNA from 10 *Diospyros* spp. by the fragments/enzyme combination *trn*C-*trn*D/AluI (A), *trn*C-*trn*D/RsaI (B), *trn*k1-*trn*k2/TaqI (C) and *trn*C-*trn*D/TaqI (D) of chloroplast genomic DNA from part of the 29 *Diospyros* spp. genotypes resolved on 2% agarose gel. Lane M correspond to DL2000 DNA ladder size marker. (1) *D. lotus*; (2) *D. glaucifolia*; (3) *D. oleifera*; (4) Jinzaoshi; (5) *D. rhombifolia*; (6) *D. virginiana*; (7) *D. kaki* Mopanshi; (8) *D. kaki* Luotiantianshi; (9) *D. kaki* Fuyuu; (10) *D. kaki* Hiratanenashi.

(e.g. 'Zenjimaru') and PCNA (e.g. 'Luotiantianshi' and 'Fuyuu'), no visual polymorphisms were found in the present study, which was incongruent with their clearly discrepancy in the morphological characterizations. In addition, nonaploid cultivar, 'Hiratannashi' presented the same restriction pattern with other hexaploid cultivars. This result revealed that sequences of the 10 chloroplast genomic regions examined were conserved in *D. kaki* in this study, although the persimmon cultivars had the different chromosome numbers, astringent types and economic traits.

3.2. Phylogenetic analysis in the genus Diospyros

According to the mutation data of restriction site, an unrooted phylogenetic tree among seven species of the genus *Diospyros* was generated based on Wagner parsimony method of PHYLIP 3.62 (Fig. 2). The polyploid species, *D. rhombifolia*, *D. virginiana* and *D. kaki* formed different groups in the tree and showed divergence from their furcation points, which suggested that *D. kaki* had relatively distant relationships with *D. rhombifolia* and *D. virginiana*, whereas close relationships between the diploid species, *D. lotus* and *D. glaucifolia* from China. That divergence showed in the diagram suggested that *D. kaki* and *D. virginiana* might have different progenitors, although both species were hexaploid species.

The PCoA demonstrated a clear divergence between *D. kaki* and its related species (Fig. 3). The hexaploid species *D. kaki* was far away from *D. rhombifolia* (tetraploid) and *D. virginiana* (hexaploid) in the diagram, whereas was close to the diploid species, *D. lotus*, *D. glaucifolia*, *D. oleifera* and Jinzaoshi. This result was consistent with that of Wagner parsimony analysis, which revealed that *D. lotus* and *D. glaucifolia* had very close relationship analyzed by principal coordinates analysis. Even Jinzaoshi was diploid (Yang et al., 1999), it has not yet been identified in taxonomy. The result in this study showed that Jinzaoshi was distinct from the other *Diospyros* spp. and had close relationship with *D. lotus* and *D. glaucifolia* (Fig. 3).

4. Discussion

High concentration of gels had commonly increased the ability of separation. One percent, 1.5%, 2% and 3% agarose gels were used in search of the length diversity of products,

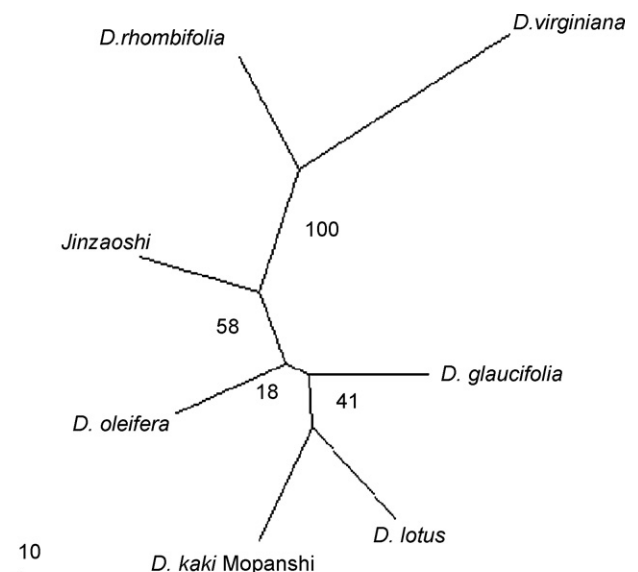

Fig. 2. Unrooted phylogenetic tree among seven species of the genus *Diospyros* generated based on Wagner parsimony method of PHYLIP 3.62 from cpDNA uncoding regions of PCR-RFLP markers. Numbers on the branches indicate the bootstrap values of 500 replicates.

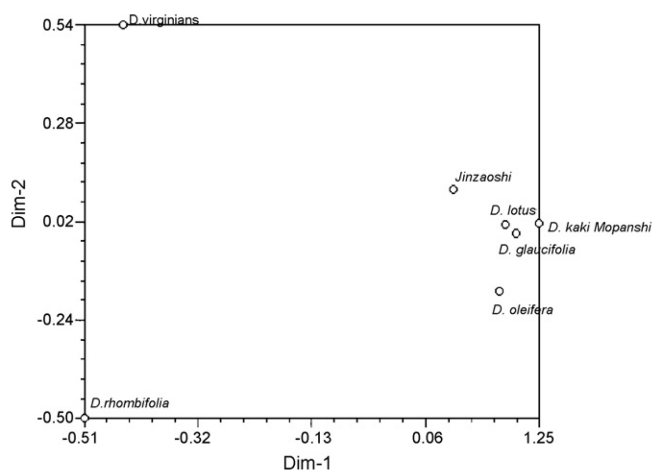

Fig. 3. Diagram based on principal coordinates analysis by NTSYS 2.10e programme showing the relationships among seven species of the genus *Diospyros* using cpDNA PCR-RFLP.

whereas it showed that no obvious difference was detected among different percentages gels. In this study, 2% agarose gels were used to separate the restriction fragments. In view of more investigated mutations, an increase in the number of enzymes would improve this analysis in some extent. At the same time, the more appropriate regions were detected, the more results reflected the actual event. If amplified fragments were a few and small, the method of clone and sequence was more economical and accurate than cpDNA PCR-RFLP molecular markers. In this study, although PCA, PCNA, PVA and PVNA persimmons were native from different regions, *D. kaki* cultivars were monotype as detected by cpDNA PCR-RFLP marker. It indicated that cpDNA sequences of same species were perfectly conserved. Therefore, the specimens in the same species would give identical cpDNA information despite of different origins.

In the previous studies, IRAP, REMAP and SRAP molecular markers were used to amplify the whole genome of the genus *Diospyros*. However it was difficult to determine whether the same size bands were homoplasy or not. Moreover, all results had not provided the direct evidence for the evolutions of *D. kaki* although the patterns showed the high interspecies and intra-species polymorphisms. PCR-RFLP markers assessing cpDNA variation had been proved to be a powerful tool for phylogenetic studies at the inter-specific level and higher (Badenes and Parfitt, 1995; Wu et al., 2005). The usefulness of the chloroplast genome for molecular evolutionary studies lies in not only its highly conserved in terms of genome size and structure, but also its predominant maternal inheritance in angiosperms (Droogenbroeck et al., 2004). Comparing with other markers, cpDNA PCR-RFLP was the most appropriate for phylogenetic relationship analysis among closely related species, though it generated low variability, even was unable to distinguish intra-specific variability.

Yonemori et al. (1998) had surveyed 3.2-kb and 2.1-kb cpDNA regions to investigate the phylogenetic relationships of 19 *Diospyros* species of Thailand and 5 temperate-zone species. Neighbor joining tree and parsimony analysis indicated that *D. virginiana* was classified as a monophyletic group with *D. kaki* and *D. lotus*. However, Nakatsuka et al. (2002) found that a subgroup of *Ty*1-*copia* retrotransposons was markedly increased just in *D. virginiana*. Choi et al. (2003) had confirmed that the genomic evolution of *D. virginiana* after speciation was different from that of *D. kaki* based on the lack of sequence homology in three different repetitive DNA. Guo and Luo (2006) and Guo et al. (2006) presumed that the phylogenetic relationship between *D. kaki* and *D. virginiana* was possibly more remote than other related species using SRAP, IRAP and REMAP. Hu and Luo (2006) examined the mitochondrial DNA polymorphism of seven *Diospyros* species and the cluster analysis demonstrated a clear divergence between *D. virginiana* and *D. kaki*. In this study, *D. kaki* was closely related to diploid species *D. lotus*, but it was distant from *D. virginiana*, which was in accordance with the results based on nuclear and mitochondrial genome analysis.

Presently, the sequences of the chloroplast genome were available in some horticultural plants, for example, *Citrus sinensis* (160,614 bp, Bausher et al., 2006) and *Vitis vinifera* (160,928 bp, Jansen et al., 2006). The complete chloroplast DNA of the genus *Diospyros* was estimated at the level of 161,000 bp approximately. The results in this study showed *D. kaki* and the related species had unique digesting patterns. Considering the maternal inheritance of chloroplast DNA, neither *D. rhombifolia* nor *D. virginiana* should be the progenitors of *D. kaki*. Phylogeny analysis suggested that three species, *D. rhombifolia*, *D. virginiana* and *D. kaki*, had different progenitors and distant evolutionary relationships whereas their karyotypes were polyploidized. This result did not support that an immediate common progenitor for *D. virginiana* and *D. kaki* revealed by cpDNA PCR-RFLP molecular marker in the previous research (Yonemori et al., 1998). The divergence occurred was probably due to the difference of mutation frequency in different regions of chloroplast genome.

The chromosome number of genotype Jinzaoshi was $2n = 2X = 30$, which was distributed in Songyang County and Jinyun County, Zhejiang province, China. It is a hardwood and its many morphological traits are different from the cultivated persimmon, for instance, the leaf is slippery without tentacle on the two sides and nervation is visible; tree skin is brown and lubricious without lenticel (Yang et al., 1999). The results based on SRAP, IRAP and REMAP in nuclear (Guo and Luo, 2006; Guo et al., 2006) and mitochondrial genome analysis (Hu and Luo, 2006) showed that Jinzaoshi had not been grouped with *D. kaki* and its related species. Because of high genetic variability in the interspecies and intra-species, Jinzaoshi had not been identified as a single species. In this study, all genotypes of *D. kaki* were monotypes, which indicated that cpDNA sequence were identical in the same species. By investigating the digesting pattern discrepancy in cpDNA, the Jinzaoshi was distinct from the remaining *Diospyros* spp. So, all above mentioned collectively suggested that it might be considered as a new species of *Diospyros* spp.

Acknowledgement

This research was supported by Natural Science Foundation of China (no. 30471203).

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