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Molecular evidence for the hybrid origin of *Paulownia Taiwaniana* based on RAPD markers and RFLP of chloroplast DNA

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Abstract Genomic DNA of *Paulownia fortunei*, *P. kawakamii* and *P. taiwaniana* were amplified with 10-base primers of arbitrary sequences using the polymerase chain reaction (PCR). A total of 351 DNA fragments were amplified from 23 primers and of these 265 fragments (75.5%) were polymorphic. Almost all of the PCR-amplified products of *P. taiwaniana* were shared by either *P. fortunei* or *P. kawakamii*, or both, and the number of polymorphic fragments shared by *P. taiwaniana* and *P. fortunei* was about equivalent to those shared by *P. taiwaniana* and *P. kawakamii*. Restriction fragments of chloroplast DNA (cpDNA) purified from *Paulownia* species and from reciprocal crosses between *P. fortunei* and *P. kawakamii* were analyzed. Restriction enzyme *Sal*I-digested cpDNA showed an identical pattern in both *P. kawakamii* and *P. taiwaniana*. These results further support the hypothesis that *P. taiwaniana* is the natural hybrid between *P. fortunei* and *P. kawakamii* and that the maternal parent of *P. taiwaniana* is *P. kawakamii*.

Key words RAPD · RFLP · Chloroplast DNA · Natural hybrid · *Paulownia taiwaniana*

Introduction

The genus *Paulownia* belonging to the family Scrophulariaceae has 12 species, the distribution of which is restricted to East Asia (Chen 1986; Xiong and Chen 1992). Three species, *P. fortunei* Hemsl., *P. kawakamii* Ito and *P. taiwaniana* Hu and Chang, are native to Taiwan (Hu and Chang 1975). *Paulownia taiwaniana* is a fast-growing timber species. It was planted extensively in eastern Taiwan during the 1960s and 1970s, reaching a maximum of 19,000 ha in 1977 (Rin 1979). The species was asexually propagated and maintained a high degree of genetic homogeneity. Finkeldey (1992) did not find any differences in 13 isozyme loci, including one locus for shikimate dehydrogenase (SKDH) and phosphoglucose isomerase (PGI), two loci for isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP) and glutamate oxaloacetate transaminase (GOT) and five loci for malate dehydrogenase (MDH), among 202 *P. taiwaniana* individuals collected in the field. However, both *P. fortunei* and *P. kawakamii* were very rare, and fewer than 8 individuals of *P. fortunei* and 20 individuals of *P. kawakamii* were found in Taiwan (Finkeldey 1992).

Since the introduction of the species in 1975 (Hu and Chang 1975), *P. taiwaniana* has been suspected to be the natural hybrid between *P. fortunei* and *P. kawakamii* based on the morphology of the inflorescence and trichomes on the petals and flowers (Hu and Lin 1975). The F_1 progeny of artificial reciprocal crosses between *P. fortunei* and *P. kawakamii* showed isozyme banding patterns for SKDH, GOT and IDH identical to those of *P. taiwaniana* (Lin and Wang 1991). In addition, the ratio of genotypic segregation among selfed progenies of *P. taiwaniana*, based on a single locus each of SKDH and PGI and two loci each of starch phosphorylase (SP) and GOT, were 1:2:1 and 3:6:3:1:2:1, respectively (Lin and Wang 1991). A high level of heterozygosity with 7 out of 13 gene loci on six isozyme systems found in *P. taiwaniana* could also be explained by the hybrid nature of the species (Finkeldey 1992).

Molecular markers such as restriction fragment length polymorphisms (RFLPs) are widely used in genetic studies. DNA markers can also be generated by the polymerase chain reaction (PCR) using short primers of arbitrary sequences, these are called random amplified polymorphic DNA (RAPD) (Williams et al. 1990). Carlson et al. (1991) confirmed the appropriate inheritance of RAPD markers in the F_1 progenies of Douglas fir and white spruce. Therefore, the use of RAPD markers

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provide a potential tool to study the phylogenetic relationships among *P. fortunei*, *P. kawakamii* and *P. taiwaniana*.

The plant chloroplast genome is maternally inherited with only a few exceptions (Gillham 1978; Neale and Sederoff 1989). Restriction analysis of chloroplast DNA (cpDNA) not only can provide molecular information on the phylogenetic relationships among plant species but also can be used to determine the paternal and maternal identity of a hybrid (Baum and Bailey 1989; Bremer and Jansen 1991; Philbrick and Jansen 1991; Stine et al. 1989). The purpose of the study presented here was to use RAPD markers and cpDNA RFLP to analyze the hybrid origin and maternal parent identity of *P. taiwaniana*.

Materials and methods

Plant material

Leaves of *P. fortunei* (PF) were collected from a tree planted in the garden of the Department of Botany, National Taiwan University in Taipei. Leaves of *P. kawakamii* (PK) were collected from native trees located along the roadside of the east-west transverse highway around Chia-Yang at an elevation of 1,800 m. Leaves of *P. taiwaniana* (PT) were collected from a tree planted in front of the Division of Silviculture, Taiwan Forestry Research Institute in Taipei. Hybrids of PF × PK and PK × PF were generated by reciprocal pollinations in the greenhouse with branches containing flowers of each of the two species being grafted onto PT seedlings (Lin and Wang 1991). Leaves harvested from the crossed hybrid seedlings were used for cpDNA isolation and restriction analysis.

Genomic and cpDNA isolation

Leaves collected from mature PF, PK and PT trees and artificially crossed hybrid seedlings were used for genomic and chloroplast DNA isolation. The method of cpDNA isolation was modified from Schuler and Zielinski (1989). Genomic DNA was co-purified using the same isolation procedure as that for cpDNA. Dark-treated leaves (50–100g) were ground in a blender with 300–500 ml grinding buffer (0.35 *M* sorbitol, 50 m*M* HEPES, 2 m*M* EDTA, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$ and 0.1% sodium ascorbate). After filtration through cheesecloth and miracloth, organelles, including the nuclei and chloroplasts, were pelleted by low-speed centrifugation. The crude organelle extract was layered on top of a 30–45–60% sucrose step-gradient and centrifuged in a Beckman L8-70M ultracentrifuge with SW28 swing bucket rotor at 112,700 *g* for 1 h. Purified chloroplasts collected from the 30–45% sucrose meniscus were subject to sakosyl lysis. Pellets collected from the sucrose gradient contained nuclei, and some broken chloroplasts were resuspended in the grinding buffer and lysed by sakosyl. The genomic and chloroplast DNA were further purified through CsCl ultracentrifugation in a 70.1 Ti rotor at 258,000 *g* for 20 h. The concentration of purified DNA was determined using a Hoeffer fluorometer, and the DNA was stored at 4 °C before use. Genomic DNA was adjusted to 10 ng/μl for use in the PCR reaction.

DNA amplification conditions

The PCR conditions reported by Williams et al. (1990) for generating RAPD markers were optimized for Coy Thermal Cycler with *Paulownia* genomic templates. Each 25 μl of PCR reaction mixture in 10 m*M* Tris-HCl buffer (pH 8.3) containing 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.001% gelatin, 100 μ*M* each of dATP, dCTP, dGTP and dTTP, 30 ng of primer, 20 ng of template DNA and 0.5 unit of Cetus AmpliTaq enzyme was placed in a 0.5 ml microfuge tube. The reaction mixtures were overlaid with 1 drop of autoclaved mineral oil. The amplification conditions included a total of 45 cycles with 2 min template denaturation at 94 °C, followed by 4 min 45 sec of primer annealing at 30 °C, and then 2 min of primer extension at 72 °C. The reactions were further incubated at 72 °C for 10 min and stored at 4 °C before the products were analyzed by gel electrophoresis.

Analysis of PCR products

The PCR products were separated in 1.4% agarose gel by electrophoresis in 1 × TBE buffer and stained with ethidium bromide (Williams et al. 1990). Because RAPDs are dominant markers, the character 1 was assigned to each of the identified DNA fragments and 0 was given to the absence of a DNA fragment at a relative position of the same mobility. Similarity coefficients (F) estimated from the proportions of shared amplified products by pairs of two species were calculated by the Nei's estimate of similarity, $F = 2Nxy/(Nx + Ny)$, where Nx and Ny were the numbers of amplified DNA fragments of the species X and Y, and Nxy was the number of DNA fragments shared by both species (Nei and Li 1979).

Restriction and southern analysis of cpDNA

Restriction analysis of cpDNA was done using commercially available 6-mer restriction enzymes, and the resulting DNA fragments were separated by electrophoresis in 1.0% agarose gel with 1 × TBE buffer. Southern hybridization was performed according to Sambrook et al. (1989). Either whole PT cpDNA or restriction fragment-purified cpDNA from the gel was labeled with dig-dUTP (Boehringer Mannheim Biochemica) by the random priming method according to the manufacturer's instructions.

Results and discussion

PCR amplification of genomic DNAs

Twenty-three primers with arbitrary sequences of 10 nucleotides were used in this study. The primers had G + C contents ranging from 50% to 100%. Primer sequences and the number of amplified DNA products of each primer for PF, PK and PT are listed in Table 1. A total of 351 PCR products were scored, and 265 (75.5%) of these were polymorphic among the three *Paulownia* species (Table 2). The number of amplified products ranged from 3 to 18 for any species/primer combination, and an average of 10 products per primer per species was obtained. The size of the amplified DNA products separated by electrophoresis in 1.4% agarose gels ranged from 0.3 to 3 kb (Figs. 1–3). More DNA products were amplified with PT genomic DNA than with genomic DNA from the other two species.

A total of 215, 225 and 253 DNA products were scored for PF, PK and PT, respectively. Of these, 86 DNA products were shared by all three species. With respect to unique DNA products that were amplified in only one species, 45, 48 and 2 were scored for PF, PK and PT, respectively. Five DNA products were shared by PF and PK but not PT. However, 86 DNA products were shared by PK and PT but not PF, and 79 DNA products were shared by PF and PT but not PK (Table 2). For those PCR-amplified fragments that were polymorphic, 36% of the fragments were unique and 62% were shared either by PF and PT or by PK and PT. Only 2% of the fragments were shared by PF and PK.

Table 1 Nucleotide sequences of the 10-mer random primer used in the study and the number of DNA fragments amplified from *Paulownia fortunei* (PF), *P. kawakamii* (PK) and *P. taiwaniana* (PT) genomic DNA with each primer

Primer ID	Nucleotide sequence (5′–3′)	Number of PCR products		
		PF	PK	PT
P1	CGGGATCCCG	8	8	11
P2	CGGAATTCCG	12	10	12
P4	CGAAGCTTCG	5	3	4
P6	CCGTCGACGA	11	13	14
P7	ACCGTAGCCG	9	12	14
P8	ACAGTCCTGC	11	16	14
P9	ACGGTACAGT	12	13	15
P10	ATTGCGTCCA	10	10	13
P11	ATGTCCTCGA	4	8	7
P12	TGGTCACTGC	10	13	12
P13	TCACGGTGCT	10	10	10
P15	TTGCGTCCAG	5	4	7
P16	TGACCCGGCG	9	9	12
P17	CGGACCCTGT	7	8	6
P18	CACGATGCAA	8	7	10
P19	CGGCCCCGGC	11	12	13
P20	CGTGCGCCAG	11	14	12
P21	CGTTGAGGCG	15	13	18
P22	GCTAGTTGCT	10	11	13
P23	GGATGTACGT	6	4	8
P24	GATGCGTGCA	14	10	10
P25	GGTACCGTGC	8	8	10
P26	GATCGTCGAG	9	9	8

Table 2 Summary on the results of PCR-amplified DNA fragments from *Paulownia fortunei* (PF), *P. kawakamii* (PK) and *P. taiwaniana* (PT) genomic DNA with 22 random primers

Description	Number of fragments			
	PF	PK	PT	Total
Number of scored fragments	215	225	253	351
Average fragments/primer	9.3	9.8	11	10
Number of polymorphic fragments	129	139	167	265
Number of unique fragments	45	48	2	95
Number of shared fragments	+	+		5
		+	+	86
	+		+	79
	+	+	+	86

Two species-specific fragments were amplified from PT genomic DNA. One was amplified by primer P16, the other by primer P19 (Fig. 3). If, as according to Lin and Wang (1991), PT is the natural hybrid between PF and PK, then these two fragments are non-parentally inherited. However, because of the extreme sensitivity of PCR amplification, non-parental bands might be observed (Riedy 1992). This phenomenon of non-parental fragments can be generated either by high rates of mutation, which did not seem likely in our experiment, or by PCR artifacts. Later, our PCR experiments included control reaction mixtures containing every ingredient but template DNA. The results from the no-template controls gave zero to more than 10 amplified products per reaction. It is obvious that these fragments were amplified from contaminated DNA template present in the reaction mixture. We have traced the source of template contamination to the *Taq* polymerase, dNTP mixtures and, occasionally, the primers. Careful manipulation during the PCR process helped reduce the contamination but could not totally eliminate the problem. Although traces of contaminated DNA may be out-competed by the add-in genomic DNAs in the PCR reaction and may not be visible in the agarose gels, fragments amplified from the contaminating template may still occasionally occur in some reactions.

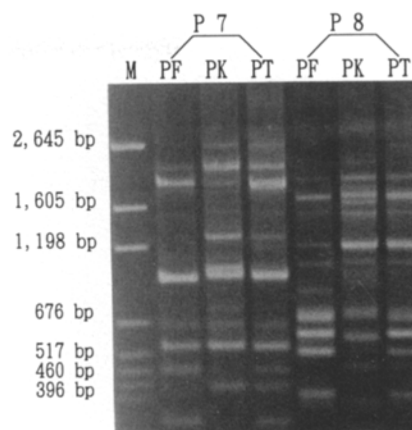

Fig. 1 Amplification DNA polymorphisms are shown in the ethidium bromide-stained agarose gel using random primers P7 and P8. *PF Paulownia fortunei*, *PK P. kawakamii*, *PT P. taiwaniana*, *M* pGEM DNA size markers

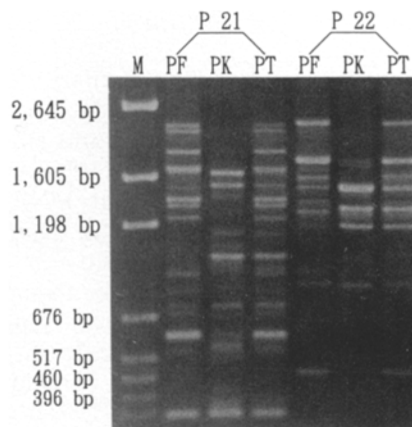

Fig. 2 Amplification DNA polymorphisms are shown in the ethidium bromide-stained agarose gel using random primers P21 and P22. *PF Paulownia fortunei*, *PK P. kawakamii*, *PT P. taiwaniana*, *M* pGEM DNA size markers

Similarity analysis

Similarity calculated from the shared fragments against the number of total amplified fragments between PF and PK, PF and PT and PK and PT was 41%, 70% and

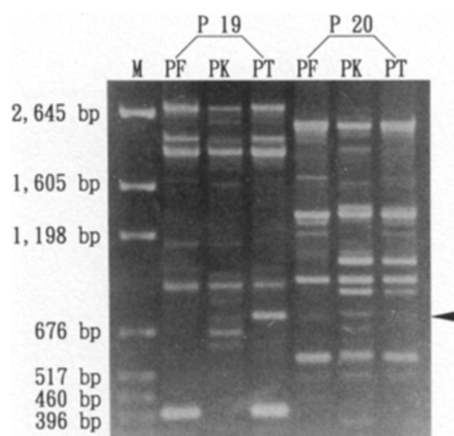

Fig. 3 Amplification DNA polymorphisms are shown in the ethidium bromide-stained agarose gel using random primers P19 and P20. *PF Paulownia fortunei*, *PK P. kawakamii*, *PT P. taiwaniana*, *M* pGEM DNA size markers. *Arrow* shows the non-parental DNA fragment amplified from *P. taiwaniana* template DNA with primer P19

72%, respectively. However, the similarity calculated from the shared polymorphic fragments against total polymorphic fragments between PF and PK, PF and PT and PK and PT was 4%, 53% and 56%, respectively (Table 3). These results suggest that DNA markers amplified from PT genomic DNA were inherited from the two supposed parents at a 1:1 ratio.

The RAPD technique has been used in such diverse applications as genetic mapping, phylogeny, population biology and epidemiology studies since its introduction in early 1990 (Rafalski et al. 1991). In comparison with the isozyme analysis of Lin and Wang (1991) and Finkeldey (1992), RAPD has the advantage of randomly representing a much larger proportion of the *Paulownia* genome. Our results show that almost all of the PCR-amplified PT genomic fragments (99.2%) were shared by PF and PK at roughly a 1:1 ratio. Two polymorphic DNA fragments were amplified only from PT, which could have been caused by PCR artifacts. Five polymorphic DNA fragments were shared by PF and PK but not by PT. This could be explained by the fact that they were heterozygous loci and that recessive alleles in terms of PCR amplification ability were inherited by PT. These RAPD data strongly support the hypothesis that *Paulownia taiwaniana* is the natural hybrid between *P. fortunei* and *P. kawakamii.*

Table 3 Similarity coefficients calculated from the percentage of shared amplified fragments against the number of total amplified fragments (treatment A) or only the shared polymorphic fragments against total polymorphic fragments (treatment B) of paired *Paulownia fortunei* (PF), *P. kawakamii* (PK) and *P. taiwaniana* (PT)

Treatment	Species	% Similarity		
		PF	PK	PT
	PF	100		
A	PK	41	100	
	PT	70	72	100
	PF	100		
B	PK	4	100	
	PT	53	56	100

RFLP analysis of cpDNA

Around 5–20 μg cpDNA could be purified from 50–100 g dark-treated leaves. Twenty-two restriction enzymes were used in the analysis, and the results are summarized in Table 4. Restriction enzyme digests produced 5–58 DNA fragments. The estimated size of *Paulownia* cpDNA is around 114 ± 7 kb. Of the 22 restriction enzymes, all having a recognition site of 6 bp, 21 enzymes did not produce any cpDNA RFLP. Only digestion by the enzyme *Sal*I produced a RFLP that could be used to differentiate PF and PK. These results indicate the close phylogenetic relationships among the *Paulownia* species. When cpDNAs were digested with *Sal*I, a 2.8-kb DNA fragment was observed in PF but not in PK, PT and the presumed F_2 progenies (selfed PT seedlings), whereas 2 DNA fragments with sizes of 1.9 and 0.9 kb were observed in the *Sal*I-restricted cpDNA of PK, PT and the presumed F_2 progenies but not in PF (Fig. 4).

The 2.8-kb *Sal*I restriction fragment of PF cpDNA was excised from the agarose gel and labeled with dig-dUTP. Southern hybridization with the 2.8-kb *Sal*I fragment as probe revealed that the 2.8-kb fragment was homologous to the 0.9- and 1.9-kb cpDNA fragments of

Table 4 Restriction enzyme analysis of the purified cpDNA of *Paulownia fortunei*, *P. kawakamii* and *P. taiwaniana* and the resulting RFLP among them

Restriction enzyme	Number of fragments	RFLP
*Apa*I	10	No
*Ava*I	19	No
*Bam*HI	34	No
*Bcl*I	19	No
*Bgl*I	10	No
*Bgl*II	22	No
*Bst*EII	11	No
*Dra*I	24	No
*Eco*RI	58	No
*Hpa*I	>20	No
*Hin*dIII	15	No
*Kpn*I	10	No
*Mlu*I	>5	No
*Nru*I	8	No
*Pst*I	10	No
*Pvu*I	>13	No
*Sal*I	8/9	Yes
*Sca*I	15	No
*Sma*I	>9	No
*Stu*I	11	No
*Xba*I	24	No
*Xho*I	17	No

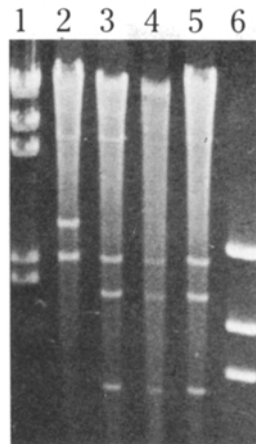

Fig. 4 Restriction enzyme *Sal*I-digested cpDNA of *Paulownia* sp. *Lane 1* Lambda/*Hin*dIII DNA size marker, *lane 6* pGEM DNA size marker, *lane 2 P. fortunei*, *lane 3 P. kawakamii*, *lane 4 P. taiwaniana*, *lane 5 P. taiwaniana* selfed seedlings

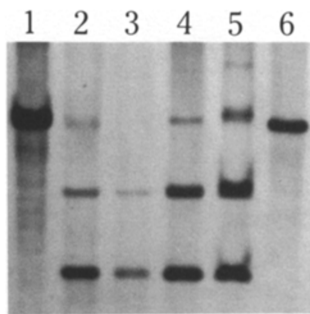

Fig. 5 Southern analysis of *Sal*I-digested *Paulownia* cpDNA using the 2.8-kb *Sal*I fragment of *P. fortunei* cpDNA labeled with dig-dUTP as probe. *Lane 1 P. fortunei* × *P. kawakamii* control-pollinated progeny (PF × PK), *lane 2 P. kawakamii* × *P. fortunei* control-pollinated progeny (PK × PF), *lane 3 P. taiwaniana* seedling, *lane 4 P. taiwaniana* (PT), *lane 5 P. kawakamii* (PK), *lane 6 P. fortunei* (PF)

PK and PT (Fig. 5). The *Sal*I restriction pattern of artificially crossed PF × PK cpDNA was identical to that of PF, and the reciprocal crossed PK × PF cpDNA was identical to that of PK. The ability of the *Sal*I enzyme to restrict the 2.8-kb cpDNA fragment into 0.9- and 1.9-kb fragments was probably due to a point mutation, but it needed to be clarified by sequencing the three *Sal*I fragments. Our results suggest that the chloroplast genomes of *Paulownia* sp. are maternally inherited as are the chloroplast genomes of most dicotyledon plants and that the maternal parent of *P. taiwaniana* is *P. kawakamii*.

Based on the above results, we recommend that *Paulownia taiwaniana* should be renamed as *P. kawakamii* × *P. fortunei*. Its fast growth rate, wide range of adaptability to different elevations and diverse growing sites make *P. taiwaniana* superior to its parents (Rin 1979). This vigor of growth is a typical heterosis expected for a hybrid. Natural hybrids between related species may occur in forests because of the interruption of natural boundaries, such as topographical or geographical barriers, between compatible species. We have provided an example using molecular markers to identify the origin of a natural hybrid and these techniques will be useful for studying other possible natural hybrids.

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