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Polymorphism analysis of Fagaceae and DNA-based identification of *Fagus* species grown in Japan based on the *rbc*L gene*

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Abstract Fagaceae species in Japan were identified by restriction fragment length polymorphism (RFLP) and sequence comparison of a region of *rbc*L. Of nine restriction endonucleases used for digestion, three (*Msp*I, *Rsa*I, *Hae*III) produced different restriction patterns in Fagaceae. Digestion by *Msp*I yielded four patterns: *Fagus* species, *Castanea crenata*, *Pasania glabra*, and others. Digestion by *Rsa*I and *Hae*III afforded two patterns: *Fagus* species and others. These facts indicate that *Castanea crenata* and *Pasania glabra* can be identified by *Msp*I restriction patterns of *rbc*L. Sequence comparison of a region of the *rbc*L gene among 20 species of Fagaceae showed that: (1) they could be divided into seven groups; (2) there is a site mutation between *Fagus crenata* and *F. japonica*. The latter indicates that the wood of both *Fagus* species are identifiable at the species level, which is not the case using conventional methods. This result indicates the possibility of wood identification based on DNA polymorphism in Fagaceae at the intrageneric level.

Key words Wood identification · Fagaceae · *rbc*L

Introduction

To date, wood species have been identified by their microscopic features.[1] Though this method is useful, it has limitations in the identification of Japanese hardwoods at the intergeneric level.[2] Molecular biological techniques such as the polymerase chain reaction (PCR)[3,4] enable one to analyze the genetic information of plants easily from a small amount of DNA. By using these techniques, DNA sequences of several regions of chloroplast DNA have been revealed and the phylogenetic relationships in some families of broadleaf trees estimated.[5–12] However, few studies have utilized DNA polymorphisms for wood identification. Shiraishi et al. distinguished *Pinus densiflora* SIEB. et ZUCC. from *P. thunbergii* PARL.[13,14] and *Larix kaempferi* from *L. gmelinii* var. *japonica*[15,16] based on polymorphisms in the *rbc*L gene, but no study on broadleaf trees has been reported to date.

Fagaceae is the family representative of Japanese broadleaf trees. These trees are much utilized by humans as a food source and wood resource. The wood species of Fagaceae grown in Japan cannot be identified at the species level by their microscopic anatomical features alone.[2] To facilitate the identification of hardwoods such as Fagaceae, it is important to develop new methods. The Fagaceae includes eight genera and about 600 species.[17] Five of the genera and 21 species are distributed in Japan.[18] Only *Castanea crenata*, *Castanopsis cuspidata*, and *Castanopsis cuspidata* var. *sieboldii* among the Fagaceae grown in Japan can be identified by microscopic observation at the species level. In the genera *Quercus* and *Pasania* it is possible to identify four taxa (section Cerris, section Prinus, section Ilex, and subgen. *Cyclobalanopsis*), but it is difficult to distinguish *Q. phillyraeodies*, *Pasania edulis*, *and P. glabra.* In the genus *Fagus*, *F. crenata* and *F. japonica* cannot be distinguished.

Sequences of *rbc*L have been widely used to estimate the phylogeny of woody plants including Fagaceae.[5,7,8,10,19] Because the *rbc*L gene is sometimes too conserved to clarify phylogenetic relationships at the intrageneric level, it seems reasonable to believe that there is no intraspecific variation in this region.

For the reasons outlined above, this study was conducted to collect the molecular data necessary to identify wood species of Fagaceae grown in Japan at the species level and

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to examine whether a region of *rbc*L that has been used for phylogenetic research can serve as a genetic marker.

Materials and methods

Fresh leaves of 17 species and one subspecies of Fagaceae were collected in the Botanical Gardens of the Faculty of Science, Osaka City University, Kisaichi and two species in Kamigamo Experimental Forest, Kyoto University (Table 1). The voucher specimens were deposited at the Laboratory of Cell Structure and Function, Wood Research Institute, Kyoto University.

Total DNA was extracted from the fresh leaves by a modified cetyltrimethylammonium bromide (CTAB) method.[20] Sephacryl S-300 (Pharmacia) was used to purify DNA.[21] About 100mg of the leaves was ground into fine powder in liquid nitrogen with a mortar and pestle. The powder of leaves was extracted two or three times with chloroform/methanol (3:1) containing threo-1,4-dimercapto-2,3-butanediol (DTT), 1mg/ml, and then was suspended in an extraction buffer containing 100mM Tris-HCl pH 9.0, 10mM ethylenediaminetetraacetic acid (EDTA), 2% sodium dodecyl sulfate (SDS), 1% CTAB, 1.4M NaCl, and DTT 1mg/ml before being incubated at 65°C for 10min. Next, extraction was carried out twice with an equal volume of chloroform/isoamyl alcohol (24:1). After centrifugation at 15000*g* for 10min, 0.6 volume of isopropanol was added to the supernatant, which was then kept at −80°C. The precipitates were dissolved in a buffer (100mM Tris-HCl pH 8.0, 1mM EDTA, 0.1M NaCl) and loaded on columns filled with Sephacryl S-300 (Pharmacia). The eluates were then extracted with 1 volume of phenol/chloroform (1:1) and centrifuged at 15000*g* for 3min. The DNA in the supernatant was precipitated by adding 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol at −80°C for 30min. After centrifugation at 15000*g* for 10min, the pellet was washed with 70% ethanol. The DNA was suspended in autoclaved water.

A region of the *rbc*L gene (positions 31–476) was amplified by the PCR using a pair of primers as follows. Primer 1: 5′-GTCGGATTCAAAGCTGGTGT-3′. Primer 2: 5′-CTTTCTACTTGGATACCATGAG-3′. The reaction mixture (50µl) contained 4ng/µl DNA, 10mM Tris-HCl pH 8.9, 1.5mM $MgCl_2$, 80mM KCl, BSA 0.5µg/µl, 0.1% sodium cholate, 0.1% Triton X-100, 0.25mM dNTPs, 2µM of each primer, and 0.05 unit/µl Tth DNA polymerase (TOYOBO). The amplification was conducted in a thermal cycler (Perkin Elmer Gene Amp PCR System 2400) using 1 cycle of 5min at 94°C; 30 cycles of 1min at 94°C, 1.5min at 55°C, 2min at 72°C; and 1 cycle of 3min at 72°C.

The amplified DNA region of 18 species obtained from the Botanical Gardens was digested with nine restriction endonucleases (*Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Nde*I, *Rsa*I, *Scr*FI, and *Taq*I) (Table 2). The PCR products were incubated under the reaction conditions presented in Table 2. Restriction fragments were electrophoresed in 4% Agarose X gel (Nippon Gene) at 100V for 2h.

The *rbc*L gene was sequenced partially for the 20 species shown in Table 1. The PCR products were purified by ultrafiltration with Microcon 100 (TaKaRa). The primers employed were the same as those used for amplification. Sequencing was directly carried out using the dye terminator cycle sequencing ready reaction kit (Perkin Elmer) and ABI PRISM 377 (Perkin Elmer).

Table 1. Tree species used in the study

Genus	Subgenus	Section	Species
Quercus	*Cyclobalanopsis*		*Q. acuta*
			Q. glauca
			Q. myrsinaefolia
			Q. sessilifolia
			Q. gilva
			Q. salicina
	Lepidobalanus	Cerris	*Q. acutissima*
			Q. variabilis
		Prinus	*Q. serrata*
			Q. mongolica
			Q. aliena
			Q. dentata
		Ilex	*Q. phillyraeoides*
Castanea			*C. crenata*
Castanopsis			*C. cuspidata*
			C. cuspidata var. *Sieboldii*
Pasania			*P. edulis*
			P. glabra
Fagus			*F. crenata*
			F. japonica

Q. dentata and *Q. phillyraeoides* were collected from Kamigamo Experimental Forest, Kyoto University. The others are from Botanical Gardens, Faculty of Science, Osaka City University, Kisaichi

Results

The amplified fragment of *rbc*L was about 450bp and showed no length variation among 18 species. Of the nine restriction endonucleases used for digestion, three (*Msp*I, *Hae*III, *Rsa*I) detected polymorphism in Fagaceae. Digestion by *Msp*I yielded four restriction patterns (M1–M4) resulting from four site mutations (Fig. 1): M1 occurred in *Fagus*, M2 in *Castanea crenara*, M3 in *Pasania glabra*, and M4 in other species (*Quercus, Castanopsis, Pasania edulis*). Digestion by *Hae*III and *Rsa*I each afforded two restriction patterns (H1, H2 and R1, R2) resulting from one site mutation (Fig. 2). H1 occurred in *Fagus* and H2 in other species (*Quercus, Castanea, Castanopsis, Pasania*). R1 occurred in *Fagus* and R2 in other species (*Quercus, Castanea, Castanopsis, Pasania*). With the other six restriction endonucleases, there were no differences in restriction fragment patterns among the Fagaceae.

We compared a segment of *rbc*L (406bp long) excluding regions where primers anneal among 20 species. The 20 species were classified based on this region into seven DNA types (Table 3). Four DNA types had only one species: IV (*Castanea crenata*), V (*Pasania glabra*), VI (*Fagus crenata*), VII (*F. japonica*). Figure 3 shows the nucleotide sequences

Table 2. Restriction endonucleases used for RFLP

Restriction endonuclease	Recognition sequence	Quantities of restriction endonucleas	Reaction buffere	Reaction temperature (°C)
*Alu*I	AGCT	0.3	M	37
*Hae*III	GGCC	1	M	37
*Hha*I	GCGC	1.2	B	37
*Hin*fI	GANTC	1.2	H	37
*Msp*I	CCGG	0.8	M	37
*Nde*I	GATC	0.5	H	37
*Rsa*I	GTAC	1	M	37
*Scr*FI	CCNGG	0.8	H	37
*Taq*I	TCGA	1	A	65

A buffer: 50 mM K acetate, 20 mM Tris acetate pH 7.9, 10 mM Mg acetate, 1 mM DTT. B buffer: 100 mM NaCl, 10 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol. H buffer: 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT. M buffer: 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT
RFLP, restriction fragment length polymorphism

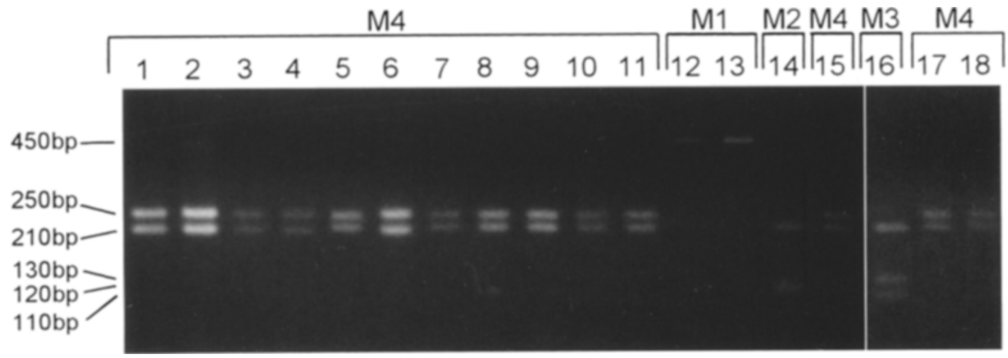

Fig. 1. *Msp*I restriction patterns (M1–M4) of a region of *rbc*L from 18 Fagaceae species. *M1*, *12*, *F. crenata*; *13*, *F. japonica*. *M2*, *14*, *C. crenata*. *M3*, *16*, *P. glabra*. *M4*, *1*, *Q. glauca*; *2*, *Q. acuta*; *3*, *Q. myrsinaefolia*; *4*, *Q. gilva*; *5*, *Q. sessilifolia*; *6*, *Q. salicina*; *7*, *Q. serrata*; *8*, *Q. mongolica*; *9*, *Q. aliena*; *10*, *Q. acutissima*; *11*, *Q. variabilis*; *15*, *P. edulis*; *17*, *C. cuspidata*; *18*, *C. cuspidata* var. *Sieboldii*

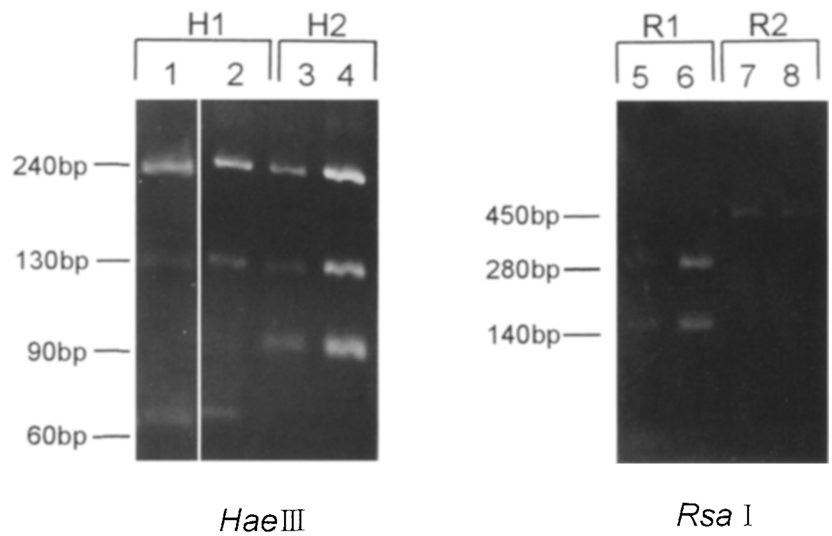

Fig. 2. Comparison of *Hae*III and *Rsa*I restriction patterns (H1, H2 and R1, R2) of a region of *rbc*L. Representative species are shown. *H1*, *1*, *F. japonica*; *2*, *F. crenata*. *H2*, *3*, *C. cuspidata* var. *Sieboldii*; *4*, *C. cuspidata*. *R1*, *5*, *F. crenata*; *6*, *F. japonica*. *R2*, *7*, *C. crenata*; *8*, *P. edulis*

representative of each DNA type. In this region, 19 sites were found to be variable. Synonymous substitutions were detected at 14 sites and nonsynonymous substitutions at 5 sites. RFLPs resulting from *Msp*I digestion were due to site mutation at position 150 in *Castanea crenata*, position 138 in *Pasania glabra*, position 267 in *Fagus* species, and position 273 in *Quercus glauca*, *Q. serrata*, and *Castanopsis cuspidata*. According to the sequence obtained from *Castanea crenata*, the 120-bp fragment detected in the restriction fragment pattern of *Castanea crenata* consists of

Table 3. CpDNA types of investigated species

DNA type	Species
I	*Q. glauca, Q. myrsinaefolia, Q. gilva, Q. salicina, Q. acuta, Q. sessilifolia*
II	*Q. acutissima, Q. variabilis, Q. phillyraeoides, P. edulis*
III	*Q. serrata, Q. mongolica, Q. aliena, Q. dentata, C. cuspidata, C. cuspidata* var. *Sieboldii*
IV	*C. crenata*
V	*P. glabra*
VI	*F. crenata*
VII	*F. japonica*

The nucleotide sequences of each DNA type are shown in Fig. 3

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Quercus glauca         TAAAGATTATAAATTGACTTATTATACTCCTGACTATCAAACCAAAGATACTGATATCTTGGCAGCCTTCCG 122
Q. acutissima          ........................................................................
Q. serrata             ........................................................................
Castanopsis cuspidata  ........................................................................
Castanea crenata       ........................................................................
Pasania glabra         ........................................................................
Fagus crenata          .....................................G....T.............................
F. japonica            .....................................G....T.............................
                       AGTAACTCCTCAACCTGGAGTTCCGCCCGAGGAAGCAGGGGCCGCGGTAGCTGCTGAATCTTCCACTGGGAC 194
                       ........................................................................
                       ........................................................................
                       ........................................................................
                       ...........................G............................................
                       ...............C........................................................
                       ..................G........T..A.....G..........................T.....T..
                       ..................G........T..A.....G..........................T.....T..
                       ATGGACAACTGTGTGGACTGACGGGCTTACCAGTCTTGATCGTTACAAAGGACGATGCTACCACATCGAGCC 266
                       ........................................................................
                       ........................................................................
                       ........................................................................
                       ..............................................T.........................
                       ........................................................................
                       .................T......................................................
                       .................T......................................................
                       GGTTGCCGGAGAAGAAAATCAATTTATTGCTTATGTAGCTTACCCCTTAGACCTCTTTGAAGAAGGTTCTGT 338
                       ......T.................................................................
                       .................G......................................................
                       .................G......................................................
                       ......T.................................................................
                       ......T.................................................................
                       A.....T..........G....................................T.................
                       A.....T..........G....................................T.................
                       TACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGATTCAAGGCCCTGCGCGCTCTACGTCTGGAGGA 410
                       ........................................................................
                       ........................................................................
                       ........................................................................
                       ........................................................................
                       ........................................................................
                       ........................................................................
                       ........................................................................
                       TTTGCGAATCCCTACTTCTTATTCTAAAACTTTCCAAGGTCCGCCT 456
                       ..............................................
                       ..............................................
                       ..............................................
                       ..............................................
                       ..............................................
                       .....................CG................C......
                       .............G.......CG................C......
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Fig. 3. Sequence comparison of a region of *rbc*L (51–456) for the eight Fagaceae. A *dot* indicates that the same nucleotide given for *Quercus glauca* is present. *Bold letters* show site mutations that caused length polymorphisms by *Msp*I, *Hae*III, and *Rsa*I

two fragments of approximately identical length. In the case of *Hae*III and *Rsa*I, length polymorphisms were caused by site mutations at positions 450 and 192 in *Fagus* species, respectively.

Discussion

In the present study, we partially sequenced the *rbc*L gene (51–456) and found a site mutation at position 424 between *Fagus crenata* and *F. japonica* (Fig. 3). It has been impossible to distinguish between these two species based on conventional methods of wood identification.[2] However, as we have already managed to extract DNA from wood, we can now identify *Fagus crenata* and *F. japonica*, respectively, using the simple method of single-strand conformation polymorphism (SSCP) and heteroduplex analysis. Athough there is a need to examine intraspecific variation in this region, it appears that each sequence is peculiar to that species. The rate of evolution of *rbc*L in Fagaceae is much slower than that of annual angiosperms.[22] Thus, this region should be useful as a genetic marker to distinguish the species *F. crenata* and *F. japonica*.

Of the 18 species examined, *Msp*I restriction patterns of *Castanea crenata* and *Pasania glabra* occurred in only one species each. These results were supported by the sequence comparison among 20 species. DNA type IV is seen only in *Castanea crenata* and DNA type V only in *Pasania glabra*. Although we need to examine intraspecific variation in this region, *Msp*I restriction patterns should be useful as a genetic marker to identify *Castanea crenata* and *Pasania glabra*. *Castanea crenata* is the only species that belongs to *Castanea* in Japan, and the wood of *C. crenata* can be identified by microscopic observation at the species level.[2] Therefore, though a genetic marker may be unnecessary to identify *Castanea crenata*, it aids in identification when a specimen is too small or shows abnormal shrinkage. *Pasania glabra* is difficult to distinguish from *Q. phillyraeodies* and *Pasania edulis* based merely on microscopic features. Therefore, a marker is useful for distinguishing *Pasania glabra* from *Q. phillyraeodies* and *Pasania edulis*.

More information is required to establish a method of DNA identification of wood in Fagaceae. We need to examine introns or intergenetic spacers where the substitution rate is faster than in the coding region to identify wood of the Fagaceae at the species level.

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