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NOTE

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Possibility of grouping of *Cyclobalanopsis* species (Fagaceae) grown in Japan based on an analysis of several regions of chloroplast DNA

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Abstract Four regions of chloroplast DNA were sequenced as a prospective genetic marker to identify Japanese representatives of *Cyclobalanopsis*: *Quercus acuta*, *Q. sessilifolia*, *Q. salicina*, *Q. myrsinaefolia*, *Q. glauca*, and *Q. gilva*. We found that *Q. gilva* was distinguished from other species based on both the *trn*L-*trn*F and *trn*T-*trn*L intergenic spacers. The evidence shows good coincidence with the fact that *Q. gilva* has several peculiar morphological features distinguishable from those of other species. There was no difference in *trn*L intron and *mat*K. Both *trn*T-*trn*L and *trn*L-*trn*F intergenic spacers are capable of being used as genetic markers to identify *Q. gilva* among *Cyclobalanopsis* species.

Key words Wood identification · *Cyclobalanopsis* · Chloroplast DNA

Introduction

Cyclobalanopsis is a subgenus of *Quercus*, and eight species are distributed throughout Japan: *Quercus acuta*, *Q. sessilifolia*, *Q. salicina*, *Q. myrsinaefolia*, *Q. glauca*, *Q. gilva*, *Q. miyagii*, and *Q. hondae*.[1] All these species, except *Q. miyagii* and *Q. hondae*, are widely distributed in the Japanese archipelago and grow south of Miyagi Prefecture.[1]

Anatomically, *Cyclobalanopsis* species demonstrate major common characteristics.[2] Despite the utility and importance of their timber, it is difficult to identify each species of *Cyclobalanopsis* by microscopic observation of wood anatomy.[2,3] Therefore, another method of identifying wood from each *Cyclobalanopsis* species is needed. Recently, several studies have utilized DNA polymorphism for wood identification.[4–6] To apply this method to identification in this subgenus, it was necessary to collect molecular data from the *Cyclobalanopsis* species. An individual organism has the same genetic information in all tissues. Therefore, we planned to obtain DNA from the leaves because extraction would be easy.

According to sequence comparison of a region of *rbc*L, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) in *Quercus*, there were one or two site mutations among subgen. *Cyclobalanopsis*, sect. *Prinus*, sect. *Cerris*, and sect. *Ilex*.[6] The prospective genetic marker is a region that evolves more rapidly than the *rbc*L gene to identify *Cyclobalanopsis* species. We selected three noncoding regions and one coding region of chloroplast DNA to examine whether they serve as genetic markers to identify six representative species of *Cyclobalanopsis* (Fig. 1). The three noncoding regions were the intergenic spacer between plastid $tRNA^{Thr}$ gene *trn*T and $tRNA^{Leu}$ gene *trn*L, *trn*L intron, and the intergenic spacer between *trn*L and $tRNA^{Phe}$ gene *trn*F.[7] The coding region that encodes a maturase for splicing the precursor of the plastid $tRNA^{Lys}$ gene $trnK^{8}$ is *mat*K. These regions have been used to estimate the phylogenetic relationship of woody plants.[9–12] Their mutation rates are about 2–5 times higher than that for *rbc*L.[13,14]

Accordingly, this study was conducted to search for the genetic markers necessary to identify six representative tree species of the subgenus *Cyclobalanopsis*: *Quercus acuta*, *Q. sessilifolia*, *Q. salicina*, *Q. myrsinaefolia*, *Q. glauca*, and *Q. gilva*. We excluded *Q. miyagii* and *Q. hondae* from this study because they are distributed only in restricted areas and their utilization as a wood resource has been limited.

Materials and methods

Fresh leaves of six species of the subgenus *Cyclobalanopsis* were collected in the Botanical Gardens of the Faculty of Science, Osaka City University, Kisaichi and the Kamigamo

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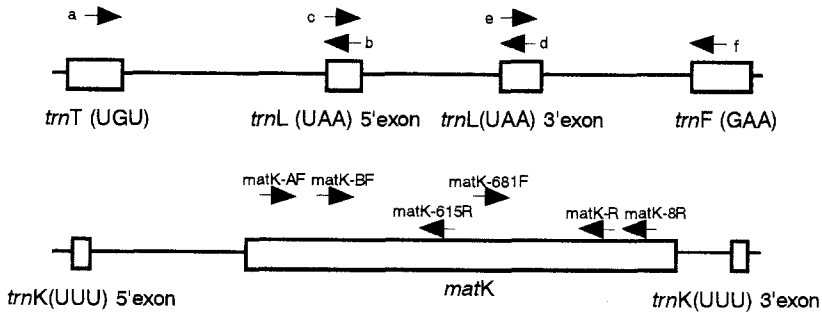

Fig. 1. Three noncoding regions and one coding region sequenced in this study. *Boxes* represent the coding regions. The positions and directions of primers are indicated by *arrows*

Table 1. Amplified regions and primers used in this study

Amplified regions	Primers	5'sequence3'	Length (bp)	Reference
*trn*T(UGU)-*trn*L(UAA) 5'exon	a	CATTACAAATGCGATGCTCT	749	7
intergenic spacer	b	TCTACCGATTTCGCCATATC		7
*trn*L(UAA) intron	c	CGAAATCGGTAGACGCTACG	518	7
	d	GGGGATAGAGGGACTTGAAC		7
*trn*L(UAA)3'exon-*trn*F(GAA)	e	GGTTCAAGTCCCTCTATCCC	428–429	7
intergenic spacer	f	ATTTGAACTGGTGACACGAG		7
*mat*K	matK-AF	CTATATCCACTTATCTTTCAGGAGT	1214	16
	matK-BF	TCAGAGGGATTTGCGTTTATTGTGG		16
	matK-R	CTGCATATACGCCCAAATCGGTCAA		16
	matK-8R	AAAGTTCTAGCACAAGAAAGTCGA		16
	matK-681F	CCGAAGTCTTTGCTAATGAT		Newly designated
	matK-615R	AATGAGAAGATTGGTTACGG		Newly designated

MatK-R, matK-8R, matK-681F, and matK-615R were only used for sequencing. The length of the *trn*T-*trn*L intergenic spacer was a region used for sequence comparison

Experimental Forest, Kyoto University. Samples from one of each species were obtained from the Botanical Gardens and Kamigamo Experimental Forest. Samples from the Botanical Gardens were the same as those used in our previous study.[6] Voucher specimens are deposited at the Laboratory of Cell Structure and Function, Wood Research Institute, Kyoto University.

Total DNA was extracted from the fresh leaves by the cetyltrimethylammonium bromide (CTAB) method[15] as modified in our previous report.[6] Three noncoding regions and a coding region of chloroplast DNA were amplified by the polymerase chain reaction (PCR) using the universal primers described by Taberlet et al.[7] and Ooi et al.[16] (Table 1). *Trn*L-*trn*T and *trn*L-*trn*F intergenic spacers were amplified from template DNA extracted from the samples collected in the Botanical Gardens and Kamigamo Experimental Forest. The intron of *trn*L was amplified from the samples collected in the Botanical Gardens, whereas *mat*K was in the Kamigamo Experimental Forest. The reaction mixture (50 μl) was the same as that in a previous study except for containing DNA at about 1 ng/μl.[6] The amplification was conducted in a thermal cycler (Perkin Elmer Gene Amp PCR System 2400) following certain cycle profiles. For the three noncoding regions the sequence was 1 cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50°–55°C, and 2 min at 72°C; and 1 cycle of 3 min at 72°C. For *mat*K the sequence was 1 cycle of 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 45°C, 2 min at 72°C, and 1 cycle of 3 min at 72°C.

The three noncoding regions and *mat*K were sequenced for six species. The primers were the same as those used for amplification of the three noncoding regions. Six primers were used for sequencing *mat*K: *mat*K-AF, *mat*K-BF, *mat*K-R, *mat*K-8R, *mat*K-681F, and *mat*K-615R (Table 1). *Mat*K-681F and *mat*K-615R were newly designated. The PCR products were purified by ultrafiltration with Microcon 100 (TaKaRa). Sequencing was directly carried out using a dye terminator cycle sequencing ready reaction kit (Perkin Elmer) with ABI Prism 377 (Perkin Elmer) as the method recommended by the supplier.

Results and discussion

Successful DNA amplification of four regions was obtained for all species studied with the primers listed in Table 1. The migration of these amplified fragments did not show any significant variation among six species by 1.5% agarose gel electrophoresis. This fact indicates that there is no long insertion or deletion in these regions among the six species. The lengths of amplified fragments were approximately 850, 430, 520, and 1200 bp, respectively. We sequenced these four regions and determined the exact length of each fragment excluding the regions where primers anneal (Table 1). However, we employed the same primers (a and b) for amplifying and sequencing *trn*T-*trn*L intergenic spacer, so we were unable to determine the nucleotide sequences of

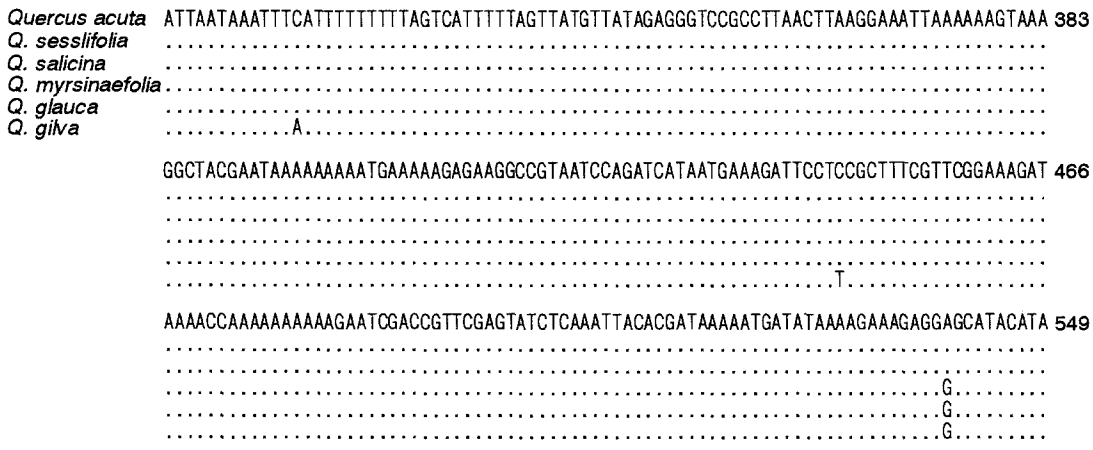

Fig. 2. Sequence comparison of a region of *trn*T-*trn*L intergenic spacer. The site number is counted from a nucleotide of the 5′ end in which the nucleotide sequence could be determined. A *dot* indicates that the same nucleotide given for *Quercus acuta* is present

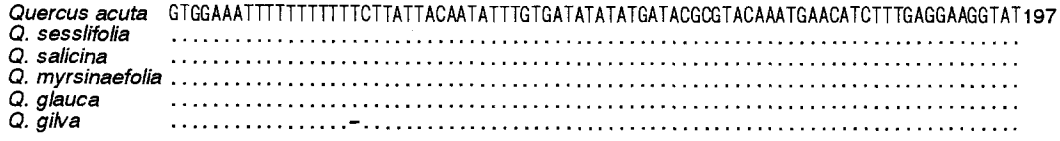

Fig. 3. Sequence comparison of a region of *trn*L-*trn*F intergenic spacer. The site number is counted from the nucleotide of the 5′ end excluding regions where primers e and f anneal. A *dot* indicates that the same nucleotide given for *Quercus acuta* is present. A *dash* indicates a deleted base

Table 2. Relation between chloroplast DNA types based on *trn*T-*trn*L intergenic spacer and morphological character in *Cyclobalanopsis*

cpDNA type	Species	Germination pattern[a]	Stigmatic surface	Male inflorescence	Fruit ripening	Position of aborted ovules
I	*Q. acuta, Q. sessilifolia, Q. salicina*	H-II	Spatulate	Dichasial	Biennial	Basal
II	*Q. myrsinaefolia, Q. glauca*	H-II	Spatulate	Dichasial	Annual	Lateral
III	*Q. gilva*	H-I	Capitate	Simple	Annual	Lateral[b]

[a] H-I, germination hypogeous, earlier leaves scaly, not differentiated even in the mature embryo, epicotyle scarcely developed; H-II, germination hypogeous, leaves normal from the first ones, epicotyle mound-like

[b] *Q. gilva* has acorns with aborted ovules at the lateral but is distinguishable from *Q. myrsinaefolia* and *Q. glauca* by a central axis elongating to the lateral middle part of the acorn

both ends. Thus, we dealt with the length of this region as 749 bp in this study. *Trn*T-*trn*L intergenic spacer and *trn*L-*trn*F intergenic spacer (428–429 bp long) had identical nucleotide sequences between two individuals of each six species. Other two regions, *trn*L intron (522 bp long) and *mat*K (1214 bp long), did not show any mutation among six species.

In the present study, we found that there is an insertion or deletion at position 135 in the *trn*F-*trn*L intergenic spacer, a transversion at position 313, and a transition at 447 in the *trn*T-*trn*L intergenic spacer between *Q. gilva* and other five *Cyclobalanopsis* species (Figs. 2, 3). In regard to wood anatomy, *Cyclobalanopsis* species have the following common characteristics: Vessels are in radial porous arrangements and exclusively solitary; perforation plates are simple; rays are homogeneous; broad rays and narrow rays exist.[2] It is unfortunately difficult to identify each species of *Cyclobalanopsis* by microscopic observation on wood anatomy.[2,3] Thus, this evidence indicates that both regions have the possibility of being genetic markers to distinguish between *Q. gilva* and the other five species, although each sequence was obtained from two individuals. Among Japanese *Cyclobalanopsis* species, it has been pointed out that *Q. gilva* has several peculiar morphological features compared with other species[17–19] (Table 2), which is in accord with our result.

Within the nucleotide sequence of a segment in the *trn*T-*trn*L intergenic spacer, two transitions were found at positions 447 and 540 and a transversion at 313 among six species (Fig. 2). The six species were grouped into three DNA types based on this region: I (*Q. acuta, Q. sesslifolia, Q. salicina*), II (*Q. myrsinaefolia, Q. glauca*), and III (*Q. gilva*). There was a nucleotide substitution at position 540 between DNA types I and II and three nucleotide substitutions (at positions 313, 447, and 540) between DNA types I and III (Fig. 2). This grouping is in good agreement with

differences in morphological features such as germination pattern, stigmatic surface, male inflorescence, fruit ripening, and the position of aborted ovules.[17–19]

The sequences of *trn*T-*trn*L and *trn*F-*trn*L intergenic spacers were obtained from two individuals. In a recent study, intraspecific variations, which are geographically structured, have been proven in *trn*F-*trn*L intergenic spacer and *trn*L intron of *Quercus* distributed in Europe.[20–22] Hence we need more molecular data about both *trn*T-*trn*L and *trn*F-*trn*L intergenic spacers of many individual trees in the populations spread throughout Japan to confirm that these sequences are species-specific. The genetic markers of both regions might be used to identify *Q. gilva* and classify *Cyclobalanopsis* species into three subgroups.

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