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ORIGINAL ARTICLE

Motonari Ohyama · Kei'ichi Baba · Takao Itoh

Wood identification of Japanese *Cyclobalanopsis* species (Fagaceae) based on DNA polymorphism of the intergenic spacer between *trn*T and *trn*L 5′exon

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Abstract DNA was extracted from wood samples of six representative *Cyclobalanopsis* species (Fagaceae) growing in Japan that cannot be distinguished from one another by conventional microscopy. A part of the intergenic spacer region between *trn*T and *trn*L 5′exon was amplified and sequenced. The sequences obtained from wood samples were grouped into three DNA types by a single nucleotide polymorphism as reported previously in leaf samples: I (*Quercus acuta*, *Q. sessilifolia*, *Q. salicina*), II (*Q. myrsinaefolia*, *Q. glauca*), and III (*Q. gilva*). Thus, *Q. gilva* can be distinguished from the other *Quercus* species, and the others are separated in two subgroups based on DNA polymorphism. The present findings support the possibility of wood identification based on DNA polymorphism.

Key words Wood identification · *Cyclobalanopsis* · Fagaceae · Chloroplast DNA

Introduction

Wood species have been identified by their macroscopic and microscopic features.[1,2] Although this method is valuable, isolated pieces of wood usually cannot be identified as to species and often cannot be assigned to a single genus[2] because they have the same anatomical features within each taxon. Identification of wood samples at the species level is often difficult in Japanese broadleaf trees.[3] Fagaceae is the family representative of Japanese broadleaf trees, but most of their wood species cannot be identified at the species level by their microscopic features alone.[3]

Genetic information can be analyzed from a small amount of DNA by the polymerase chain reaction (PCR) technique.[4,5] Using this technique, several investigators recently utilized DNA polymorphism to identify tree species. Species–specific molecular markers have been revealed and have proved useful as a new identification method,[6–9] but DNA has been extracted only from leaves so far.

Previously, we found several regions in chloroplast DNA useful as DNA markers to identify wood species of Fagaceae.[8,9] *Cyclobalanopsis*, which is a subgenus of *Quercus*, has eight species in Japan: *Quercus acuta*, *Q. sessilifolia*, *Q. salicina*, *Q. myrsinaefolia*, *Q. glauca*, *Q. gilva*, *Q. miyagii*, *Q. hondae*. Except for *Q. miyagii* and *Q. hondae*, these trees are widely distributed throughout Japan and grow south of Miyagi Prefecture.[10] Despite the high utility and importance of their timber in ancient to present times, it is absolutely difficult to identify each species of *Cyclobalanopsis* by microscopic observation of their anatomical features.[3,11]

In this study, we extracted DNA from wood samples of six species belonging to subgenus *Cyclobalanopsis* and identified them using a genetic marker. Previously,[9] we found that by using part of the *trn*T–*trn*L intergenic spacer on chloroplast DNA it was possible to group six representative species of *Cyclobalanopsis* into three DNA types using leaf samples (Fig. 1): I (*Q. acuta*, *Q. sessilifolia*, *Q. salicina*), II (*Q. myrsinaefolia*, *Q. glauca*), and III (*Q. gilva*). Plastids differentiate to chloroplasts in leaves and to amyloplasts or chromoplasts in other tissues. They retain exactly the same genetic information even after differentiation. Therefore, the above DNA marker may be obtained from any tissue of the plant.

Intergenic spacer regions of chloroplast DNA have been used to demonstrate intraspecific variations. Several studies indicated that the distribution of these variations shows geographical patterns caused by Quaternary climatic changes.[12] The sequences of the *trn*T–*trn*L intergenic spacer we reported previously were obtained from two individuals in each species. It is important to collect molecular data on *Cyclobalanopsis* species from various places to confirm that this *trn*T–*trn*L intergenic spacer is useful as a genetic marker.

In this study, we extracted DNA from wood samples of six representative species of the subgenus *Cyclobalanopsis* – *Q. acuta*, *Q. sessilifolia*, *Q. salicina*, *Q. myrsinaefolia*, *Q.*

M. Ohyama · K. Baba · T. Itoh
Wood Research Institute, Kyoto University, Uji, Kyoto 611-0011, Japan
Tel. +81-774-38-3634; Fax +81-774-38-3635
e-mail: motonari@kuwri.kyoto-u.ac.jp

glauca, *Q. gilva* – from various areas in Japan to identify their wood species using part of the *trn*T–*trn*L intergenic spacer as a genetic marker. 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.

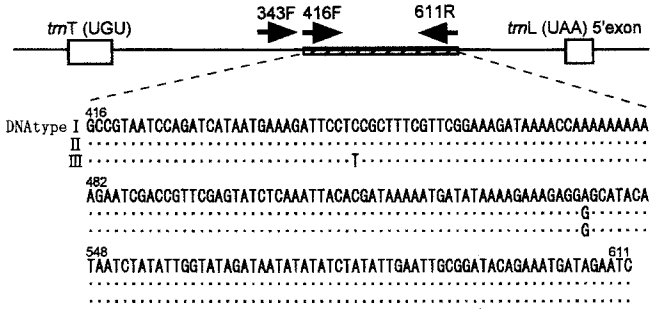

Fig. 1. Nucleotide sequence of a part of the intergenic spacer between *trn*T and *trn*L 5′exon. *Boxes* represent the coding regions. The positions and directions of primers are indicated by *arrows*. Each *dot* indicates that the same nucleotide given for DNA type I is present. The six representative species of Japanese *Cyclobalanopsis* are grouped into three DNA types as reported previously[9]: DNA type I (*Quercus acuta*, *Q. sessilifolia*, *Q. salicina*), II (*Q. myrsinaefolia*, *Q. glauca*), and III (*Q. gilva*)

Materials and methods

Total genomic DNAs were extracted from 26 wood samples as shown in Table 1. They met our requirements that they be authentic and from various areas in Japan. All samples except no. 16 had been stored in the Xylarium, Wood Research Institute, Kyoto University. Each sample has a KYOw registration number except samples 15 and 16 (Table 1). Voucher specimens are deposited at the Xylarium. Fourteen samples were sapwood, and the remaining twelve were unknown but were estimated to be heartwood. Three to five individuals per species from various areas were examined (Table 1; Fig. 2).

About 2g of each wood sample was ground with a ball mill. Then the wood powder was passed through a sieve with a hole diameter of 0.15mm. The ball mill and sieve were washed each time with an ultrasonic cleaner to avoid contamination with other samples. About 100mg of this

Table 1. Wood samples used in this study

Sample	KYOw no.[a]	Collection site	Amplified fragment[b]	Position in trunk[c]	Year[d]
Quercus acuta					
1	1615	Izumo Sub-branch Stat., Shimane Pref. Forest Exp. Stat.	A	S	1961
2	1722	Kyushu	A	S	1956
3	342	Univesity Forest, Wakayama, Kyoto Univ.	A	U	–
4	14410	University Forest, Chiba, Univ. Tokyo	B	S	1995
Q. salicina					
5	7494	Matsue Experimental Stat., Shimane Univ. Forest	A	S	1982
6	9281	Wakayama Experimental Forest, Hokkaido Univ.	A	S	1984
7	9538	University Forest, Ashiu, Kyoto Univ.	A	S	1984
8	5665	University Forest, Chiba, Univ. Tokyo	B	U	–
Q. sessilifolia					
9	4976	Kyushu	B	U	1924
10	9278	Wakayama Experimental Forest, Hokkaido Univ.	A	S	1984
11	68	Obi District Forest Office, Miyazaki Pref.	B	U	1931
Q. glauca					
12	4977	Kyushu	B	U	1924
13	12853	Minoo, Osaka Pref.	B	S	1982
14	9280	Wakayama Experimental Forest, Hokkaido Univ.	B	S	1984
15	–	Takatori, Nara Pref.	B	S	1997
16	–	Uji campus, Kyoto Univ.	B	S	1999
Q. myrsinaefolia					
17	1119	Nagano Pref.	A	S	1950
18	1616	Izumo Sub-branch Stat., Shimane Pref. Forest Exp. Stat.	A	S	1961
19	384	Univesity Forest, Wakayama, Kyoto Univ.	B	U	–
20	813	Yaku Island, Kagoshima Pref.	A	U	1956
21	13838	Mimata, Miyazaki Pref.	B	U	1962
Q. gilva					
22	5663	University Forest, Chiba, Univ. Tokyo	A	U	–
23	9279	Wakayama Experimental Forest, Hokkaido Univ.	B	S	1984
24	13839	Takashiro, Miyazaki Pref.	B	U	1962
25	4973	Kyushu	B	U	1924
26	64	Obi District Forest Office, Miyazaki Pref.	B	U	1931

[a] KYOw no. is the registration number of wood sample stored in the Xylarium, Wood Research Institute, Kyoto University
[b] See Table 2
[c] S, sapwood; U, unknown. Unknown samples were estimated to be heartwoods
[d] The year when the sample was collected or registered in the Xylarium

powder was extracted five times with 0.1 M Tris-HCl buffer (pH 8.0) containing 2% 2-mercaptoethanol, 0.05 M ascorbic acid, and 1% polyvinylpyrrolidone. The total DNA was extracted by a cetyltrimethylammonium bromide (CTAB) method,[13] as modified in our previous study.[8] After extraction, the DNA was purified with an ELU-QUIK DNA purification kit (Schleicher & Schuell).

Before amplifying the target region, we reconstructed template DNA to make amplification of the target region easy.[14] The reaction mixture (25 μl) contained DNA at less than 10 ng/μl, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs, and rTaq DNA polymerase 0.04 unit/μl (TOYOBO). The amplification was conducted in a thermal cycler (Gene Amp PCR System 2400, Perkin Elmer) using one cycle of 5 min at 94°C; 20 cycles of 0.5 min at 94°C, 0.5 min at 50°C, 0.5 min at 72°C; and one cycle of 3 min at 72°C.

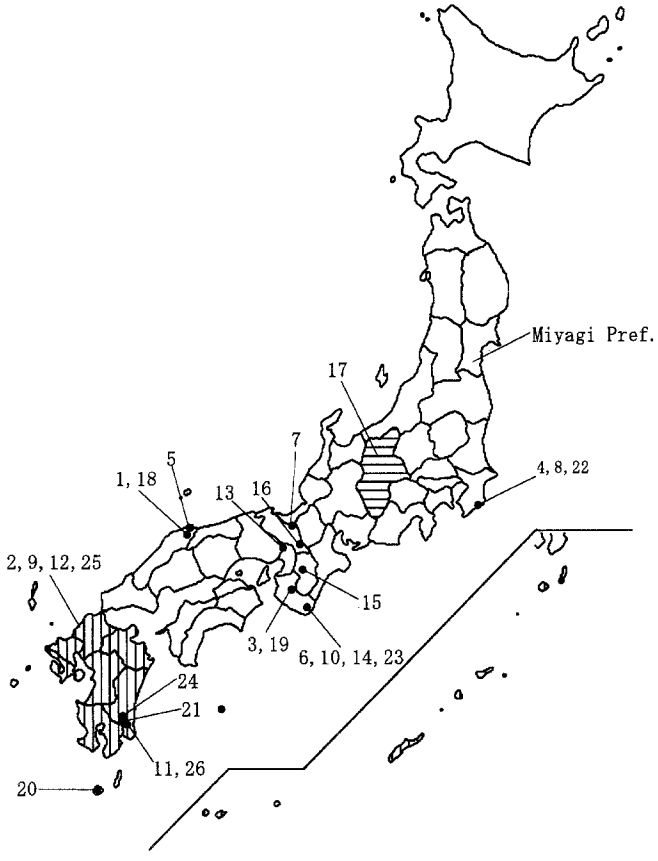

Fig. 2. Sites where the samples were collected. The numbers in the map correspond to the sample numbers in Table 1. The shaded area in lower left (*vertical hatching*) indicates Kyusyu (samples 2, 9, 12, 25). The other shaded area (*horizontal hatching*) indicates Nagano Prefecture (sample 17)

We designed two pairs of new primers for amplifying two segments in the *trn*L–*trn*T intergenic spacer that included site mutations among *Cyclobalanopsis* species (Fig. 1; Tables 1, 2). One of the amplified regions was about 200 bp (fragment A) and the other 270 bp (fragment B). The nucleotide sequences of primers are shown in Table 2. The reaction mixture (50 μl) of the first PCR contained reconstructed template DNA (5 μl of the above 25 μl), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs, 0.5 μM of each primer, and rTaq DNA polymerase 0.04 unit/μl (TOYOBO). The amplification was conducted in a thermal cycler (Gene Amp PCR System 2400) using one cycle of 5 min at 94°C; 40 cycles of 1 min at 94°C, 0.5 min at 52°C or 56°C, 0.5 min at 72°C; and one cycle of 3 min at 72°C. When the amount of amplified fragment was not enough, a second PCR was carried out under the same condition using the first reaction mixture as template DNA (1 μl).

Each amplified region (Table 1) was sequenced after purification by ultrafiltration with Microcon 100 (Takara). The primers were the same as those used for the amplification. Sequencing was directly carried out using a Thermo Sequenase II dye terminator cycle sequencing premix kit (Pharmacia) and ABI PRISM 377 (Perkin Elmer) according to the method recommended by the suppliers.

Results

The genomic DNA was obtained in amounts up to 400 ng/100 mg from sapwood samples; the amount of DNA from heartwood was less than that needed for the threshold of the fluorescent dye detection method. The gel electrophoresis of total DNA indicated that DNA in wood was highly degraded (Fig. 3). However, the quantity and quality were sufficient for PCR because the DNA was visualized by conventional staining with ethidium bromide; and the point at which the size was 270 bp, the longest target here, it stained slightly but positively, though the peak was at less than 160 bp. Indeed, the target region was amplified using all of the DNA sample.

The amplified fragments were approximately 200 bp (fragment A) and 270 bp (fragment B) on 3% agarose gel

Table 2. Primers used in this study

Primers	5′Sequence3′	Length (bp)	Annealing temperature (°C)
Fragment A			
416F	GCCGTAATCCAGATCATAAT	196	52
611R	GATTCTATCATTTCTGTATCCGCAA		
Fragment B			
343F	ATAGAGGGTCCGCCTTAACTTA	269	56
611R	GATTCTATCATTTCTGTATCCGCAA		

electrophoresis (Fig. 4). The primer pair for fragment A sometimes amplified several bands. We used the other primer pair for fragment B when nonspecific bands were observed. The amplified fragments showed no significant variation among the samples on the gel. The exact length of fragment A was 195–196 bp, and that of fragment B was 268–269 bp.

The nucleotide sequence from position 436 to position 586 (151 bp long)[9] of all samples of each species was compared. Within this region there were two transitions (at positions 447 and 540), a transversion at position 472, and an insertion/deletion at position 482 (Fig. 5). Note that the exact position at which an insertion/deletion of T occurred (described as position 482 in Fig. 5) was not determined because there are 10 Ts from position 473 to 482 (Fig. 5). According to these results, the 26 wood samples of six species were grouped into three DNA types (Figs. 5, 6): I (*Q. acuta*, *Q. sessilifolia*, *Q. salicina*), II (*Q. myrsinaefolia*, *Q. glauca*), and III (*Q. gilva*). There was a nucleotide substitution at positions 540 between DNA types I and II and two nucleotide substitutions at positions 447 and 540 between DNA types I and III (Fig. 5). The two transitions at positions 447 and 540 were different for the DNA types. On the other hand, the transversion at position 472 and insertion/deletion at position 482 were intraspecific mutations. These results are in accord with our previous report using leaf samples.[9]

Quercus salicina, *Q. myrsinaefolia*, and *Q. glauca* have several variants with intraspecific variations, whereas no variant was observed in *Q. acuta*, *Q. sessilifolia*, or *Q. gilva* (Figs. 5, 6). *Q. salicina* has one variant (sample 5), and its sequence differs from the sequence of DNA type I by an insertion/deletion at position 482. *Q. myrsinaefolia* has two variants (samples 17 and 19), and their sequences differ from the sequence of DNA type II by an insertion/deletion at position 482. *Q. glauca* has two variants (samples 12 and 14). The sequence of sample 14 differs from that of DNA type II by an insertion/deletion at position 482. On the other hand, there is a transversion at position 472 in the sequence of sample 12 compared with that of DNA type II.

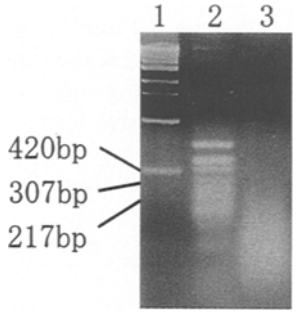

Fig. 3. Gel electrophoresis of total DNA extracted from a wood sample (sample 14). Lane *1*, λ DNA/*Sty* I digest DNA marker. Lane *2*, pBR322 DNA-*Msp* I digest DNA marker. Lane *3*, sample 14

Discussion

In wood anatomy, *Cyclobalanopsis* species has the following common characteristics in stem wood: Vessels are in radial porous arrangements and exclusively solitary; perforation plates are simple; rays are homogeneous; broad rays and narrow rays exist.[3] Shimaji investigated the anatomical features of stem wood and root wood and the ontogenetic development of broad rays on Fagaceae, but the description of all six *Cyclobalanopsis* species was identical.[11] It has not been possible to distinguish these species from

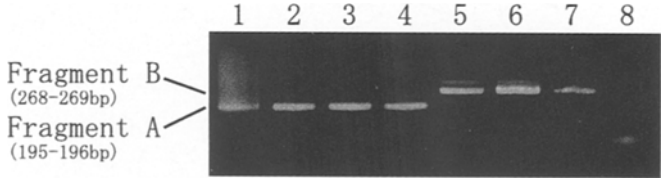

Fig. 4. Electrophoresis patterns of the amplified fragment in the intergenic spacer between *trn*T and *trn*L 5′exon. Lane *1*, sample 1 (fragment A). Lane *2*, sample 5 (fragment A). Lane *3*, sample 17 (fragment A). Lane *4*, leaf sample of *Q. glauca* collected in Kamigamo Experimental Forest, Kyoto University[9] (fragment A). Lane *5*, sample 4 (fragment B). Lane *6*, sample 24 (fragment B). Lane *7*, leaf sample of *Q. glauca* collected in Kamigamo Experimental Forest, Kyoto University[9] (fragment B). Lane *8*, negative control (fragment B)

Fig. 5. Nucleotide sequence of a part of intergenic spacer between *trn*T and *trn*L 5′exon (from position 436 to 586) from all samples. Each *dot* indicates the same nucleotide as that given for *Quercus acuta*. Each *dash* indicates an inserted/deleted base. The bases in parentheses indicates their variation type. An insertion/deletion may occur at any position from 473 to 482 but is described at position 482 for convenience

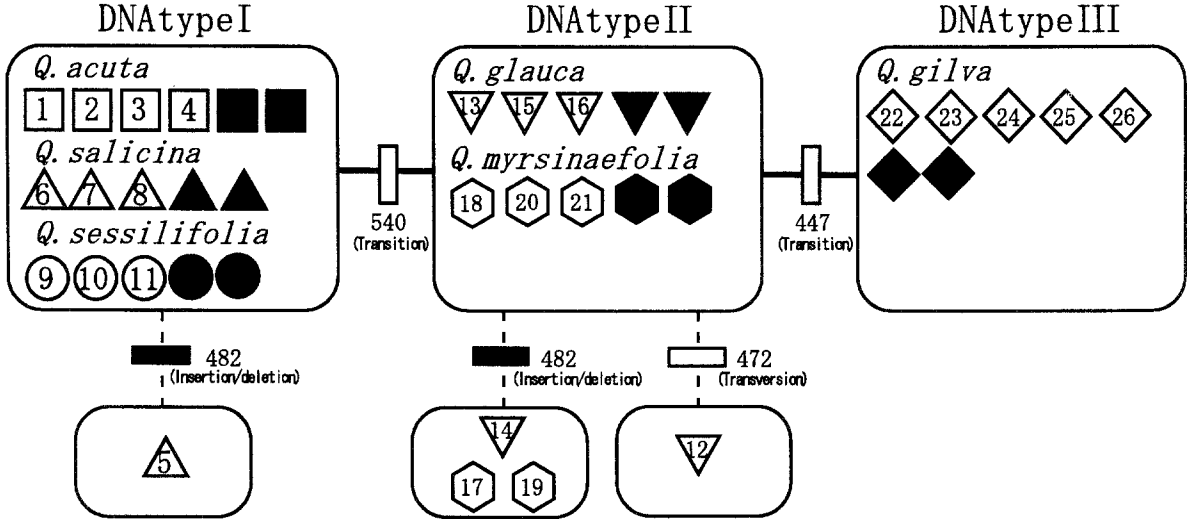

Fig. 6. Relation among *Q. acuta* (*squares*), *Q. salicina* (*triangles*), *Q. sessilifolia* (*circles*), *Q. myrsinaefolia* (*hexagons*), *Q. glauca* (*inverted triangles*), and *Q. gilva* (*diamonds*) based on the part of intergenic spacer between *trn*T and *trn*L 5′exon (from position 436 to 586). Numbers in the symbols correspond to the sample numbers in Table 1. Black symbols indicate individuals extracted from leaves in our previous report.[9] *Open cross-bars* with numbers show the transition/transversion, and the number indicates the position where the mutation occurred (see Fig. 5). *Filled cross-bars* with numbers show the insertion/deletion, and the number indicates the position where the mutation occurred (see Fig. 5). The *solid thick* lines indicate the position of mutations that characterized the difference between the two DNA types. The *dashed lines* indicate the intraspecific difference

each other by microscopy. However, in this study we showed that DNA polymorphism enables us to identify *Q. gilva* and to separate it into subgroups using wood samples. This indicates that DNA analysis is a powerful tool that can complement conventional methods of wood identification when only the wood sample is available. We were able to apply several species-specific genetic markers that have already been reported[6–8,15,16] to identify unknown wood samples.

We succeeded in amplifying the fragments of intergenic spacer between *trn*T and *trn*L 5′exon from the DNA of wood samples. Two substitutions at positions 447 and 540 separated the species into three DNA types (Figs. 5, 6): I (*Q. acuta*, *Q. sessilifolia*, *Q. salicina*), II (*Q. myrsinaefolia*, *Q. glauca*), and III (*Q. gilva*). This is consistent with our previous study using leaf samples.[9] These facts suggest that the DNA (approximately ≤250 bp) can be amplified and analyzed even if it is extracted from dead wood, and that it is not a problem to use samples other than wood, such as the leaf, to construct a database of species-specific DNA markers of such size.

It was reported that intraspecific variations in chloroplast DNA showed a geographic distribution.[12] The wood samples used in this study were collected from various regions in Japan (Fig. 2). Two single nucleotide substitutions at positions 447 and 540 were found in all the samples of every species that had such a polymorphism. Therefore, these single nucleotide polymorphisms would be peculiar to each DNA type. On the other hand, there were several intraspecific mutations of an insertion/deletion at position 482 and a transversion at position 472, as shown in Fig. 6. However, such insertion/deletion does not interfere with the analysis using the genetic markers noted above because it occurs much more frequently than the nucleotide substitution[17] that we used as a polymorphism between DNA types and that are predicted to be in a species. We can readily determine the position and type of mutation by sequencing. Such insertion/deletion may occur in the other three species (*Q. acuta*, *Q. sessilifolia*, *Q. gilva*) that showed no variant within the samples in this study, but it would not interfere with the analysis for the same reason mentioned above. We found that the intergenic spacer between *trn*T and *trn* L 5′exon is useful as a genetic marker to identify *Q. gilva.* Species-specific substitutions useful for identifying the other five species may exist in other regions of chloroplast DNA, mitochondrial DNA, and nuclear DNA.

The present findings support the possibility of wood identification based on DNA polymorphism. This new method has three advantages compared with the conventional microscopic identification of wood: First, it may be possible to identify all tree species at the species level in the future. Second, pieces of unknown wood can be identified using a small sample (less than 100 mg). Third, wood species can be identified without being influenced by the variability of wood structures depending on the individual, position in the tree (root, trunk, branch, sapwood or heartwood), or age of the wood material (juvenile or mature stem) because an individual organism has the same genetic information in all tissues.

Note

The report by Dumolin-Lapégue et al.[18] was published after we submitted this paper.

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