Maternal Origin of ‘Tarokaja’ and Other Wabisuke Camellia Cultivars Indicated by Chloroplast DNA Variation

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The wabisuke camellia ‘Tarokaja’ is a very old cultivar of unknown origin. Based on morphological properties, it is thought to be an interspecific hybrid between *Camellia japonica* and an unidentified species from continental China. We analyzed chloroplast DNA from members of the genus *Camellia* to trace the maternal origin of ‘Tarokaja’. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the *atpI-atpH* spacer region using 57 progeny derived from 17 interspecific crosses confirmed that chloroplast DNA is inherited maternally in the genus *Camellia*. Based on PCR-RFLP and sequence analyses of the *atpI-atpH* region and referring to knowledge about morphological properties, we concluded that the maternal origin of ‘Tarokaja’ is a Chinese native species *C. pitardii* var. *pitardii*. Wabisuke camellia cultivars are classified into two groups. One group of wabisuke camellia cultivars is thought to be derived from crosses between ‘Tarokaja’ and *C. japonica*. Since most of them had a ‘Tarokaja’-type single-base in the *atpI-atpH* region, it was confirmed that these wabisuke cultivars are descended from ‘Tarokaja’. It is likely that ‘Hime-wabisuke’, ‘Kocho-wabisuke’, and ‘Sukiya’, which had a *C. japonica*-type single-base, are progeny descended from ‘Tarokaja’ with *C. japonica* as the maternal ancestor. Another group of wabisuke camellia cultivars is thought to be derived from *C. japonica* by mutation, and all but one of these had a *C. japonica*-type single-base. The exception, ‘Kibi’, had a ‘Tarokaja’-type single-base, so that ‘Kibi’ is inferred to be descended from ‘Tarokaja’.

Key Words: *Camellia pitardii* var. *pitardii*, *Camellia uraku* ‘Tarokaja’, chloroplast DNA, maternal origin, wabisuke camellia.

Introduction

There is a group of horticultural *Camellia* cultivars called the wabisuke camellias, which are distinguished by their atrophied and abortive androecia. They also have the characteristics of early flowering, small single flowers, sterile pistils, and no seed formation (Kirino, 1986). Written records of wabisuke camellia cultivars such as ‘Tarokaja’ (Fig. 1a) and ‘Kocho-wabisuke’ go back to the middle of the Edo period (1603–1867) (Tanaka et al., 2001). Kitamura (1952) reported that there are wabisuke camellias in temples in Kyoto, the old capital of Japan, which are thought to have been

planted 350–400 years ago. These observations suggest that the wabisuke camellias were generated more than 400 years ago. They have taken root in Japanese traditional culture and been loved for a long time in Japan.

Wabisuke camellias are classified into two groups (Kirino, 1986). One group is thought to be derived from mutations in the Japanese native species *Camellia japonica* L. These camellias generally have red flowers, a glabrous ovary, and a leaf shape that are similar to those of *C. japonica*. Members of the other group of wabisuke camellias have elliptical acuminate leaves. Most of these camellias have a pubescent ovary, which is not a characteristic of *C. japonica*. Members of this second group are thought to be derived from a cross between ‘Tarokaja’ and *C. japonica*, because ‘Tarokaja’ is a very old wabisuke camellia cultivar with a pubescent ovary and imperfect fertility, which can produce small numbers of pollen grains and seeds exceptionally among wabisuke camellias (Kirino, 1986; Tanaka et al., 2001).

Received; February 28, 2009. Accepted; June 30, 2009.

A part of this work was presented at the 2004 Spring Meeting of the Japanese Society for Horticultural Science.

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(a)

(b)

Fig. 1. Flowers of 'Tarokaja' (a) and *C. pitardii* var. *pitardii* (b).

It was verified by hybridization that the wabisuke camellias with atrophied androecia were derived from a cross between 'Tarokaja' and *C. japonica* (Kirino, 1986). The origin of 'Tarokaja' remains to be established, and an answer to this question is needed to shed light on the ancestry of the latter group of cultivars.

The origin of 'Tarokaja' has been of interest to researchers for a long time. Its flowering period is from December to April, and the flower color is purplish pink. The leaves of 'Tarokaja' are elliptical, acuminate, and serrulate. Camellia cultivars generally have little scent, but 'Tarokaja' is fragrant. Kitamura (1952) treated 'Tarokaja' as a species, *C. uraku* Kitamura. However, it is known only as a cultivated plant, and its geographical origins are unknown (Chang and Bartholomew, 1984; Gao et al., 2005; Sealy, 1958). Because the Japanese species *C. japonica* does not have a pubescent ovary, some researchers have speculated that 'Tarokaja' is derived from a species from continental China. Over the past 100 years, various Chinese species have been proposed as the origin of 'Tarokaja' and other wabisuke camellias. These include *C. reticulata* Lindl., *C. cuspidata* (Kochs) Wright, *C. rosae-flora* Hook., *C. sinensis* (L.) O. Kuntze, *C. saluenensis* Stapf ex Bean, *C. kissi* Wall., and *C. pitardii* Cohen Stuart var. *pitardii* (Kirino, 1986; Kitamura, 1970; Makino, 1910; Nakai, 1950; Tanaka et al., 2001). Based on morphological properties it seems most likely that 'Tarokaja' is an interspecific hybrid between *C. japonica* and *C. pitardii* var. *pitardii* (Fig. 1b) (Kirino, 1986; Tanaka et al., 2001).

Chloroplast DNA shows maternal inheritance in most flowering plants. It does not undergo recombination, and has a slow rate of evolution (Palmer, 1987). Therefore, chloroplast DNA is a useful tool for tracing maternal ancestors. We previously used the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to analyze chloroplast DNA in the genus *Camellia*. We found that 8 PCR-RFLP patterns (800a, 800b, 800c, 1200a, 1200b, 1200c, 1200d, and

1200e) were detected when the spacer region between *atpI* and *atpH* was amplified by PCR and digested using the restriction enzyme *TaqI* (Tanikawa et al., 2008). Sequence analysis of the *atpI-atpH* region showed variations that can be used to distinguish between the species. In this study, we conducted PCR-RFLP analysis of the *atpI-atpH* region from 57 progeny derived from 17 interspecific crosses of the genus *Camellia* to confirm that chloroplast DNA in the genus *Camellia* is maternally inherited. We sought to identify the maternal origin of 'Tarokaja' by comparing PCR-RFLP patterns and variations in the *atpI-atpH* region of 'Tarokaja' and a variety of *Camellia* species. We also tried to trace the origins of other wabisuke camellia cultivars and related cultivars using the chloroplast DNA variations.

Materials and Methods

Plant materials

We examined the parental lines and 57 progeny derived from 17 interspecific crosses of *Camellia* (Table 1). We also compared 'Tarokaja' with 23 accessions derived from 20 species of the genus *Camellia* that showed the 1200a PCR-RFLP pattern in our previous report (Tanikawa et al., 2008) (Table 2). Nine *C. japonica* cultivars, 26 wabisuke cultivars, and the related cultivars 'Funatsu', 'Kaga-wabisuke', and 'Seiobo' were also analyzed (Table 3). All camellia plants were grown at National Institute of Floricultural Science (NIFS).

DNA extraction

Young leaf samples were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total DNA, including chloroplast DNA, was extracted using the ISOPLANT II kit (Nippon Gene Co., Ltd., Tokyo, Japan). The extracted DNA samples were purified using CHROMA SPIN+TE-1000 columns (Clontech Laboratories, Inc., Mountain View, CA, USA).

Table 1. Inheritance of chloroplast DNA in interspecific hybrids of the genus *Camellia*. PCR-RFLP analysis was conducted by digestion of the PCR-amplified *atpI-atpH* region with *TaqI*. The PCR-RFLP patterns of *C. saluenensis*, *C. fraterna* Hance, *C. oleifera* Abel, *C. sasanqua* Thunb., *C. granthamiana* Sealy, *C. brevistyla* (Hay.) Cohen Stuart, *C. lutchuensis* Ito, *C. nokoensis* Hay., and *C. rosaeflora* were investigated in our previous study (Tanikawa et al., 2008).

Cross; Parent ♀ × ♂	PCR-RFLP pattern of parents		Numbers of progeny with PCR-RFLP pattern	
	Maternal	Paternal	Maternal	Paternal
<i>C. japonica</i> ‘Aki-no-yama’ × <i>C. saluenensis</i>	1200d	1200c	2	0
<i>C. japonica</i> ‘Arukawa’ × <i>C. saluenensis</i>	1200a	1200c	3	0
<i>C. japonica</i> ‘Kokuryu’ × <i>C. saluenensis</i>	1200a	1200c	2	0
<i>C. japonica</i> ‘Komomiji’ × <i>C. saluenensis</i>	1200a	1200c	1	0
<i>C. japonica</i> ‘Kon-wabisuke’ × <i>C. saluenensis</i>	1200a	1200c	3	0
<i>C. japonica</i> ‘Kuro-tsubaki’ × <i>C. saluenensis</i>	1200a	1200c	4	0
<i>C. japonica</i> ‘Miyakodori’ × <i>C. saluenensis</i>	1200a	1200c	3	0
<i>C. japonica</i> ‘Rasenzome’ × <i>C. saluenensis</i>	1200a	1200c	1	0
<i>C. japonica</i> ‘Suzukayama’ × <i>C. saluenensis</i>	1200a	1200c	1	0
<i>C. japonica</i> ‘Tokijiro’ × <i>C. saluenensis</i>	1200a	1200c	2	0
<i>C. japonica</i> ‘Kon-wabisuke’ × <i>C. fraterna</i>	1200a	1200c	4	0
<i>C. japonica</i> ‘Waka-no-ura’ × <i>C. fraterna</i>	1200a	1200c	2	0
<i>C. hiemalis</i> ‘Tachikantsubaki’ × <i>C. oleifera</i>	800b	1200a	13	0
<i>C. sasanqua</i> × <i>C. granthamiana</i>	800b	1200e	3	0
<i>C. brevistyla</i> × <i>C. oleifera</i>	1200b	1200a	3	0
<i>C. lutchuensis</i> × <i>C. nokoensis</i>	1200a	1200c	4	0
<i>C. rosaeflora</i> × <i>C. fraterna</i>	1200a	1200c	6	0

Table 2. Single nucleotide variations at the 88th position from the 3' end of the *atpI-atpH* region, in species and varieties of the genus *Camellia* possessing the 1200a PCR-RFLP pattern.

Species	NIFS No.	Nucleotide at the 88th position	DDBJ Accession No.
<i>C. uraku</i> ‘Tarokaja’	180	C	AB443610
<i>C. japonica</i> ‘Chinshin’	622	A	AB364653 ^z
<i>C. amplexicaulis</i>	1215	A	AB364664 ^z
<i>C. caudata</i>	913	A or deletion of 5 bp	AB443617
<i>C. chrysantha</i>	565	A	
<i>C. chrysantha</i> var. <i>microcarpa</i>	569	A	
<i>C. crapnelliana</i>	914	C	AB443611
<i>C. forrestii</i>	1207	A or deletion of 5 bp	
<i>C. furfuracea</i>	1219	A	AB443614
<i>C. gigantocarpa</i>	917	A	AB443612
<i>C. hongkongensis</i>	918	A	AB364665 ^z
<i>C. japonica</i> var. <i>hozanensis</i>	755	A	
<i>C. japonica</i> var. <i>hozanensis</i>	1097	A	
<i>C. japonica</i> var. <i>macrocarpa</i>	1069	A	
<i>C. lutchuensis</i>	951	A	AB364668 ^z
<i>C. lutchuensis</i>	1118	A	AB364669 ^z
<i>C. octopetala</i>	1193	A	AB443613
<i>C. oleifera</i>	760	A	AB364666 ^z
<i>C. oleifera</i>	1115	A	AB364667 ^z
<i>C. pitardii</i> var. <i>pitardii</i>	1218	C	AB443616
<i>C. polyodonta</i>	1004	C	AB443615
<i>C. rosaeflora</i>	1121	A	
<i>C. sinensis</i> ‘Toyoka’	1221	A	
<i>C. taliensis</i>	925	A	AB364663 ^z

^z Sequences analyzed in our previous report (Tanikawa et al., 2008).

Table 3. Single nucleotide variations at the 88th position from the 3' end of the *atpI-atpH* region in *C. japonica* cultivars, wabisuke cultivars of 'Tarokaja' origin, wabisuke cultivars derived from mutations in *C. japonica*, and in the related cultivars 'Funatsu', 'Kaga-wabisuke', and 'Seiobo'.

<i>C. japonica</i> cultivars		Wabisuke cultivars of 'Tarokaja' origin		Wabisuke cultivars derived from <i>C. japonica</i> mutations		Related cultivars	
'Fumon-in'	A	'Beni-wabisuke'	C	'Ichiko-wabisuke'	A	'Funatsu'	A
'Fusen-in-aka-yabu'	A	'Eishoji-wabisuke'	C	'Iyo-wabishin'	A	'Kaga-wabisuke'	C
'Inzen'	A	'Fukurin-wabisuke'	C	'Kibi'	C	'Seiobo'	C
'Mariasama'	A	'Gomangoku'	C	'Murage'	A		
'Mitsuhime'	A	'Hime-wabisuke'	A	'Sado-wabisuke'	A		
'Miyake-chidori'	A	'Hina-wabisuke'	C	'Sayo-wabisuke'	A		
'Miyakodori'	A	'Kanzaki-aka-wabisuke'	C	'Sumi-no-hikari'	A		
'Shirayuri'	A	'Kocho-wabisuke'	A	'Tenrinji-gakko'	A		
'Tomuro'	A	'Mikawa-sukiya'	C				
		'Otohime'	C				
		'Owari-wabisuke'	C				
		'Sagami-wabisuke'	C				
		'Shibenashi-wabisuke'	C				
		'Shibori-wabisuke'	C				
		'Shiro-wabisuke'	C				
		'Showa-wabisuke'	C				
		'Sukiya'	A				
		'Yutaka-wabisuke'	A				

PCR-RFLP analysis

The primer sequences used for PCR amplification of the chloroplast DNA *atpI-atpH* region of most accessions were 5'-CCGCAGCTTATATAGGCGAA-3' and 5'-TTGACCAACTCCAGGTCCAA-3' (Tsumura et al., 1996). The primers 5'-TGGAGGGCCATCATTGACTA-3' and 5'-TGATAAGTTCCTCGCACCAA-3' were used for *C. caudata* Wall. (NIFS No. 913) (Tanikawa et al., 2008). Each PCR reaction was carried out in a 20 µL volume containing 40 ng of template DNA, 3 pmol of each primer, 0.2 mM of each dNTP, 1 unit of *Ex Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan) and 2 µL of 10 × buffer for *Ex Taq*. The amplifications began with 94°C for 30 s for the initial denaturations, followed by 35 cycles of 94°C for 30 s, 60°C for 2 min, and 72°C for 3 min, with a final 7-min incubation at 72°C. To determine the sizes of the amplified fragments, 5 µL of the PCR products were electrophoresed on 2% agarose gels (SeaKem GTG Agarose, Lonza Walkersville, Inc., Walkersville, MD, USA) containing ethidium bromide. The PCR products were also digested with the *TaqI* restriction enzyme and the digest fragments were separated by electrophoresis in 4% agarose gels containing ethidium bromide. DNA fragments were observed in the gels under UV light.

Cloning and Sequencing of the *atpI-atpH* region

The PCR amplified *atpI-atpH* regions of 'Tarokaja' (NIFS No. 180), *C. crapanelliana* Tutch. (NIFS No. 914), *C. gigantocarpa* Hu et T. C. Huang (NIFS No. 917), *C. octopetala* Hu (NIFS No. 1193), *C. furfuracea* (Merr.) Cohen Stuart (NIFS No. 1219), *C. polyodonta* How ex

Hu (NIFS No. 1004), *C. pitardii* var. *pitardii* (NIFS No. 1218), and *C. caudata* (NIFS No. 913) were cloned and sequenced. The PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd., Buckinghamshire, UK). Purified DNA was ligated into the pT7Blue T-Vector (Novagen Inc., Madison, WI, USA) using a DNA Ligation Kit (Takara Bio Inc.) and then used to transform *Escherichia coli* strain JM109. The transformed cells were plated on LB agar plates containing X-gal, IPTG and ampicillin and incubated at 37°C overnight. White colonies were randomly selected and the presence of the desired insert was confirmed by PCR-RFLP. Three clones per transformation were selected. Plasmid DNA was isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany). Sequencing was performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). M13M4 and M13RV primers were used to sequence each clone from both directions. The sequences were analyzed using the DNASIS V3.7 program (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The determined sequence of the *atpI-atpH* region for each species was deposited in the DDBJ database (accession numbers AB443610–AB443617).

A single-base variation was found at the 88th position from the 3' end of the *atpI-atpH* region, excluding the primer region. In order to easily detect this nucleotide variation in other accessions, PCR-amplified products of *atpI-atpH* region were sequenced directly from the 3' end only, using the primer 5'-TTGACCAACTCCAGGTCCAA-3'. The PCR products were purified using the

GFX PCR DNA and Gel Band Purification Kit. Sequence analyses were performed as described above.

Results and Discussion

PCR-RFLP patterns were categorized according to our previous report; the PCR amplifications resulted in two major bands of approximately 800 bp and 1200 bp and then *TaqI* digestion of the PCR products resulted in 3 different band patterns derived from the 800 bp fragment (800a, 800b, and 800c) and 5 patterns derived from the 1200 bp fragment (1200a, 1200b, 1200c, 1200d, and 1200e) (Tanikawa et al., 2008). To investigate the inheritance of chloroplast DNA in the genus *Camellia*, the PCR-RFLP analysis was conducted using a total of 57 progeny from 17 crosses between parents showing different PCR-RFLP patterns. All the progeny exhibited the same PCR-RFLP patterns as the maternal parents, thus confirming that the chloroplast DNA is maternally inherited in the genus *Camellia*, as it is in most angiosperms (Table 1).

We next performed a PCR-RFLP analysis to trace the maternal lineage of ‘Tarokaja’. ‘Tarokaja’ is thought to have originated from a hybrid between *C. japonica* and a species from continental China. The *atpI-atpH* PCR-RFLP pattern of ‘Tarokaja’ was of the type 1200a. Our previous work indicated that *C. japonica* accessions are

of the types 1200a or 1200d (Tanikawa et al., 2008). Therefore, we sequenced the *atpI-atpH* region of ‘Tarokaja’ (AB443610) and compared it with that of a 1200a type of *C. japonica* (AB364653), in order to identify additional nucleotide variations that might be used to distinguish between ‘Tarokaja’ and *C. japonica*. Two sequences of multiple adenines in the *atpI-atpH* region were excluded from the comparison because we could not determine the exact numbers of adenines in these sequences (Tanikawa et al., 2008). A single nucleotide polymorphism was identified at the 88th position from the 3' end of *atpI-atpH* region (excluding primer sequence) (Fig. 2). This base was adenine in *C. japonica* and cytosine in ‘Tarokaja’. This clearly indicated that *C. japonica* is not a maternal ancestor of ‘Tarokaja’, and that another species showing the 1200a PCR-RFLP pattern must be its maternal ancestor.

In our previous study we found that 20 of the 49 *Camellia* species had the 1200a pattern, including *C. japonica* (Tanikawa et al., 2008). We therefore sequenced the 3' end of the *atpI-atpH* region in 22 accessions representing the other 19 species, and identified the base at the 88th position from the 3' end in each case (Table 2). Three species, *C. pitardii* var. *pitardii* (NIFS No. 1218), *C. crapnelliana* (NIFS No. 914), and *C. polyodonta* (NIFS No. 1004) had a cytosine

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1  TCCATGGAGGGCCATCATTGACTAGTTTTGCGAATAGTCTCTAGCCCAAGGTAATCTATTAGGGAATATAAATATACAAATACGTTATATACGATCTAG 100

101 AGTAATAACAAAAAGATTACGATATTAGAGAATTGTAAAAAAGGAAGAGATCTAAAGATCTTTTCTAAATTCCTGTTGGTCTACTT 200

201 ATATCATACCTCTCCTCAATGAATATTCTATTCTATTGTTGTCAGTCAATCCTTAATATTAGGAGTCATAATTGAATAAGAATTCTTGTTGGTATATGTT 300

301 TATGGCGTATGATTAAATAAAAAAAGTATGTTCTATAGAACGATTACCCCTTCAGTTGAATTTTATTCAATCAACGATTGACCAAAAGAAATT 400
                                     b
401 TTTAATAATAAATAATTGCATGAACCTTAGATCAATCAATAATGATATTTCTATGCAATGATTCGGCCTAATCAATTTGCCATTTCGATTCTATCCAT 500

501 GGGGGATAATGATAAGAAAAAGGGTGAAGAATTTACAAAAAAGATGAGATTCTATAAAGATGGGTTGGTTAGGAGAGGGAGAGCCGAACGGGT 600

601 CTATGTAATTTAATTGCTATAAGTCAACTCTTGATGTGGGTGTTTGTGAATGATTCTAGAATCCGATTGAATCTAAGATGGGAATAGTTGGTTTAC 700

701 GTTATGGAAGAAAGACATGTATATGTGATATTAGATTGACTTGACTAGTTAGATATATGAACATAAATTATATCTAATTTGCTCTACTATTGTGAATT 800

801 TCGATGGGGATTCCAATAGAAATCCTTCGGTCTCGTTTGTGACTGTGAGTTGAATGAATAACAAATGAAATCAAGAAAAAGATCGAAGAATTCAAAGAA 900

901 CGGTTGGGACAAAGAAATGGAAGAACGAAAGTCGTATGGATTACAAAGACTCTGTGGATAGAAGATATCGAAGTAGTTCTGATGATTCAATAATTT 1000

1001 TGACTCCAATTTGAAGTTCTTAGTTACTTCGACTGGATGAATTTTACGCGTGAATAATTACGTCATAATTCATTGGTTGATTGTATCATTAAACCATTTT 1100

1101 TATTTCTTTTTTGGTGGGAGGAACCTTATCATGAATCCACTGATTCTGCCGCTTCGTTATTGCTGCTGGATTGGCGTAGGACTTGCTTCTA
a

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Fig. 2. The nucleotide sequence of the *atpI-atpH* region of ‘Tarokaja’ (Accession number AB443610). Black box a: the 88th position from the 3' end, b: the 355th position from the 5' end where single-base substitution to thymine was detected in *C. crapnelliana*. Underlines show poly-A sequences where the exact numbers of adenines were not determined.

at the 88th position, as did 'Tarokaja'. We then compared the whole nucleotide sequences of the *atpI-atpH* regions in these 3 species and 'Tarokaja'. The sequences of *C. pitardii* var. *pitardii* (AB443616) and *C. polyodonta* (AB443615) were identical to that of 'Tarokaja', while a single nucleotide difference was found in the *C. crapnelliana* (AB443611) sequence when compared with that of 'Tarokaja'; the base of the 355th position from the 5' end was cytosine in 'Tarokaja', while the base was thymine in *C. crapnelliana* (Fig. 2).

Among the species that have been considered as candidates for the ancestor of 'Tarokaja', *C. reticulata*, *C. cuspidata*, *C. saluenensis*, and *C. kissi* showed the 1200c, 1200c, 1200c, and 1200e PCR-RFLP patterns, respectively (Tanikawa et al., 2008). *C. rosaeflora* and *C. sinensis* showed the 1200a pattern, but their 88th nucleotide bases were adenine (Table 2). Of the two species with nucleotide sequences in the *atpI-atpH* region that are identical with that of 'Tarokaja', *C. polyodonta* has never been considered previously to be the ancestor. *C. pitardii* var. *pitardii* has a pubescent ovary and elliptical, acuminate, and serrulate leaves, which are morphological properties of 'Tarokaja' as well (Chang and Bartholomew, 1984; Gao et al., 2005; Sealy, 1958). Both 'Tarokaja' and *C. pitardii* var. *pitardii* have chromosome number of $2n=30$ (Gao et al., 2005; Kirino, 1986). We therefore conclude that *C. pitardii* var. *pitardii* is the maternal ancestor of 'Tarokaja'.

In order to clarify the origins of other wabisuke camellias, we investigated the PCR-RFLP patterns and sequenced the 3' ends of the *atpI-atpH* regions of 38 cultivars. These included 18 wabisuke camellia cultivars that are thought to be progeny of 'Tarokaja', 8 wabisuke camellia cultivars that are thought to be generated from mutations of *C. japonica*, 9 *C. japonica* cultivars, and the related cultivars 'Funatsu', 'Kaga-wabisuke', and 'Seiobo'. All of them showed the 1200a PCR-RFLP pattern.

Of the 18 wabisuke camellia cultivars that are thought to be progeny of 'Tarokaja', 14 cultivars had a cytosine at the 88th position from the 3' end of the *atpI-atpH* region, as did 'Tarokaja' (Table 3). This result supports the hypothesis that these wabisuke cultivars were generated with 'Tarokaja' as their ancestor. The exceptions were 'Hime-wabisuke', 'Kocho-wabisuke', 'Sukiya', and 'Yutaka-wabisuke'. 'Funatsu' was bred artificially using *C. japonica* as the seed parent and 'Tarokaja' as the pollen parent (Kirino, 1986). Although 'Funatsu' is descended from 'Tarokaja', its androecium and pistil are normal and it can form seeds. However, some progeny of 'Funatsu' are wabisuke type camellias with atrophied androecia (Kirino, 1986; Yokoyama and Kirino, 2005). 'Yutaka-wabisuke' was produced by natural pollination of 'Funatsu' (Yokoyama and Kirino, 2005). Therefore, the 88th base from 3' end of the *atpI-atpH* region in both 'Funatsu' and 'Yutaka-wabisuke' was adenine, as it was in *C. japonica* (Table 3). It is

likely that 'Hime-wabisuke', 'Kocho-wabisuke', and 'Sukiya', which appear to be descended from 'Tarokaja' but have adenines at the 88th position (Table 3), are also progeny descended from 'Tarokaja' with *C. japonica* as the maternal ancestor, as it is for 'Yutaka-wabisuke'.

'Kaga-wabisuke' has the term 'wabisuke' in its cultivar name; however, it has a normal androecium and pistil, and it is fertile. Therefore 'Kaga-wabisuke' is not a wabisuke camellia in the strict sense of the word. However, based on its pubescent ovary, early flowering, flower color, and leaf shape, it is thought to be generated from 'Tarokaja' (Yokoyama and Kirino, 2005). The presence of a cytosine at the 88th position from the 3' end of the *atpI-atpH* region in 'Kaga-wabisuke' (Table 3) indicates that this cultivar was generated with 'Tarokaja' as a maternal ancestor. 'Seiobo' is known as an excellent camellia cultivar. It has very early-flowering and blooms from autumn onwards. Its androecium and pistil are normal. 'Kaga-wabisuke' is believed to be a parent of 'Seiobo' because they have some similar morphological features and they both came from Ishikawa Prefecture (Yokoyama and Kirino, 2005). We also found a cytosine at the 88th position in 'Seiobo' (Table 3), confirming that it is also of 'Tarokaja' lineage.

All of the *C. japonica* cultivars examined in this study had adenines at the 88th position from the 3' end of the *atpI-atpH* region (Table 3). All but one of the wabisuke camellia cultivars that are thought to be generated from mutations of *C. japonica* also had adenines at that position (Table 3). The exception was 'Kibi', which, like 'Tarokaja', has a cytosine in that position (Table 3). The origin of 'Kibi' has been in question because it has a pubescent ovary (Kirino, 1986; Yokoyama and Kirino, 2005). Our results show that it is highly probable that 'Kibi' is a wabisuke camellia of 'Tarokaja' origin.

'Tarokaja' has been loved as a horticultural cultivar for at least 400 years, and its origins have been discussed for a long time. In this study, we investigated variations in the *atpI-atpH* region of chloroplast DNA for a wide variety of *Camellia* species in our collection and found *C. pitardii* var. *pitardii* to be the maternal origin of 'Tarokaja'. Moreover, we verified the origins of a number of other wabisuke and the related cultivars. We will continue the genomic analysis with newly-obtained materials. Sakata et al. (1981) suggested a relationship between 'Tarokaja' and *C. saluenensis* based on their similarity of anthocyanin compositions. Now we are conducting chemotaxonomical analysis of anthocyanin and its related compounds between 'Tarokaja' and *C. pitardii* var. *pitardii*. We expect that these performances could support our conclusion or yield novel knowledge about 'Tarokaja' origin.

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