Genetic Relationships Among Hirado azalea Cultivars and Their Putative Parents Inferred from Flavonoid 3', 5' Hydroxylase Gene Sequences

Sunisa Meanchaipiboon¹, Nobuo Kobayashi² and Akira Nakatsuka^{2*}

¹The United Graduate School of Agricultural Sciences, Tottori University, Tottori 680-8853, Japan

²Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690-8504, Japan

The putative parents of the Hirado azalea (*Rhododendron* × *pulchrum*) are *R. scabrum*, *R. ripense*, *R. × mucronatum*, and other related cultivars. Hirado azalea shows a wide range of flower color variation, but the genetic basis for this color variation is not well understood. In this study, we investigated the anthocyanin pathway gene, flavonoid 3', 5' hydroxylase (F3'5'H), by genomic DNA analysis, cDNA sequence analysis, and deduced amino acid sequences to assess the genetic relationships between these taxa, as well as investigating the genetic basis of color variation in this group. In *R. scabrum* and red and pink flowered Hirado azalea cultivars, in which delphinidin (Dp) derivatives are absent, only the exon 2 region was amplified using specific primers in a coding region (CDS) of the F3'5'H gene, except for exon 1, suggesting that the DNA structure of exon 1 is defective in these plants that lack Dp derivatives. On the other hand, *R. ripense*, *R. macrosepalum*, and *R. yedoense* var. *poukhanense* and *R. × mucronatum* 'Shiro-ryūkyū', with Dp derivatives have normal F3'5'H DNA gene structure. The lengths of the F3'5'H cDNA nucleotide sequences of these wild species were 1533 bp (510 AA), whereas in *R. × mucronatum* 'Shiro-ryūkyū', two different sequence lengths were observed—1533 and 1551 bp (510 and 516 AA). *R. ripense*, *R. × mucronatum* 'Shiro-ryūkyū', and four purple and white flowered Hirado azalea cultivars were grouped in the same cluster in the F3'5'H gene phylogeny. Among the four Hirado azalea cultivars, the lengths of F3'5'H in CDS were 1551 bp, which included a 5 bp insertion adjacent to the stop codon in 'Ademurasaki' and 'Hakuhō'. However, 'Hirado-no-homare' and 'Shirokujyaku' lacked this insertion and had 1533 bp CDS. When PCR was performed to distinguish the 5 bp insertion, the amplified product was found in some *R. ripense* individuals and *R. × mucronatum* 'Shiro-ryūkyū', but not in *R. scabrum* or *R. macrosepalum*. These results suggest that the wide range of flower color in Hirado azalea cultivars is caused by variation in the F3'5'H genotype derived from hybridization between *R. scabrum* and either *R. ripense* or *R. × mucronatum* 'Shiro-ryūkyū'.

Key Words: delphinidin, expressed sequence, flower color, *Rhododendron scabrum*.

Introduction

Hirado azalea (*Rhododendron* × *pulchrum* Sweet) comprises a group of evergreen azaleas bred in Nagasaki Prefecture, Japan, especially in the Hirado region (Galle, 1987; Kobayashi, 2016). They are thought to have developed by natural hybridization between garden plants without artificial crossing (Nakao and Tamura, 1970). Hirado azalea is known for their large

flowers and various colors including white, pink, red, and purple forms. However, the origin of this color variation is not well understood. The red-flowered *R. scabrum* G. Don, purple-flowered *R. ripense* Makino, and white-flowered *R. × mucronatum* (Blume) G. Don 'Shiro-ryūkyū' are the putative parents of the Hirado azalea based on their morphologies including flower color, shape, and number of pistils and stamens and almost all cultivars are similar to *R. scabrum* or *R. × pulchrum* 'Ōmurasaki' (Tamura, 1962, 1963). Moreover, the genetic relationships among evergreen azaleas inferred from DNA markers, including amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), and expressed sequence tag (EST) markers, indicate that Hirado azalea is most closely related to *R. scabrum*, and cluster with *R. × mucronatum*,

Received; June 15, 2020. Accepted; September 18, 2020.

First Published Online in J-STAGE on November 27, 2020.

This research was supported by Grant-in-Aid for Scientific Research (KAKENHI No.18K05617) from the Japan Society for the Promotion of Science (JSPS).

* Corresponding author (E-mail: nakira@life.shimane-u.ac.jp).

R. ripense, and *R. macrosepalum* Maxim (Scariot et al., 2007a, b). As flavonoid 3', 5' hydroxylase (F3'5'H) plays a key role in flower color diversity in interspecific hybridization between purple flowered *R. kiusianum* and red flowered *R. kaempferi* (Mizuta et al., 2014), we investigated its presence and sequence variation to understand the flower color variation and genetic relationships among Hirado azalea and their putative parents.

The major pigments of evergreen azalea petals are anthocyanins and flavonols. Anthocyanins can be classified into two groups: cyanidin (Cy) derivatives and delphinidin (Dp) derivatives. The red-flowers of *R. scabrum* contain only Cy derivatives and the purple-flowers of *R. ripense* and *R. macrosepalum* contain both Cy and Dp derivatives as pigments (Mizuta et al., 2009; Meanchaipiboon et al., 2020). Previously, our group has reported correlations of flower color, the pigment composition pattern, and flavonoid biosynthesis-related gene expression among Hirado azalea and their related parents (Meanchaipiboon et al., 2020). The results suggested that the wide range of Hirado azalea flower colors is a result of hybridization, with *R. scabrum* as the base species and *R. ripense*, *R. macrosepalum* or *R. × mucronatum* 'Shiro-ryūkyū' as the other species. The latter three wild species and cultivars can produce Dp derivatives through the action of F3'5'H. However,

it remains unclear from which of these putative parent taxa the F3'5'H gene in Hirado azalea originates.

In this study, we investigated the genetic relationships among Hirado azalea and their putative parents, *R. scabrum*, *R. ripense*, and *R. × mucronatum* 'Shiro-ryūkyū', by analyzing the F3'5'H gene. Furthermore, we assessed the role of the F3'5'H gene in the origins of purple and white flowers in Hirado azalea.

Materials and Methods

Plant materials

Cutting clones of Hirado azalea cultivars were obtained from genuine collections in Hirado city, Nagasaki Prefecture. Evergreen azaleas, including 27 individuals of four wild species, and 19 cultivars from the subgenus *Tsutsusi* were obtained from the experimental field of Shimane University, Japan (Table 1; Meanchaipiboon et al., 2020). For genomic DNA extraction, 70 mg of young leaves were sampled from each plant. Closed flower buds were collected for RNA extraction and sequence analysis. Each sample was immediately frozen in liquid nitrogen and stored at -80°C until extraction for genomic DNA and total RNA.

DNA extraction and DNA analysis

DNA amplification was carried out for all samples to

Table 1. The samples used for sequencing and genotyping in this study.

		Anthocyanin composition ^y	
		Dp derivatives (–)	Dp derivatives (+)
Wild species	Ser. <i>Scabra</i>		<i>R. ripense</i> 1, 2 <i>R. macrosepalum</i> 1, 2 <i>R. yedoense</i> var. <i>poukhanense</i> 1
Cultivars	Ryūkyū azalea group Hirado azalea group		<i>R. × mucronatum</i> 'Shiro-ryūkyū' 'Ademurasaki' 'Hirado-no-homare' 'Hakuhō' 'Shiro-kujyaku'
Wild species ^z	Ser. <i>Scabra</i>	<i>R. scabrum</i> 1–8	<i>R. ripense</i> 3–13 <i>R. macrosepalum</i> 3, 4, 5
Cultivars	Hirado azalea group	'Hinomoto' 'Raijin' 'Heiwa-no-hikari' 'Hiōgi' 'Kumo-no-ue' 'Shinshō' 'Hinode' 'Banzairaku' 'Momoyama' 'Seibo' 'Saotome'	'Taihō' 'Hatsuyuki'
	Ōkirishima group	'Ōmurasaki'	

^z These wild species did not have their pigment composition confirmed, except for *R. scabrum* 1, 2.

^y Hirado azalea and 'Shiro-ryūkyū' have been investigated (Meanchaipiboon et al., 2020).

PCR amplification and gene cloning

PCR amplification

confirm the presence of the F3'5'H gene among wild species and cultivars. DNA extraction was conducted using the modified CTAB method following Kobayashi et al. (1998). The purified DNA was amplified using F3'5'H primers which include UTR regions in *R. × pulchrum* 'Ōmurasaki' F3'5'H (AB289598). The PCR mixture (10 µL) contained 1× Ex-taq buffer, 200 µM dNTPs, 0.2 µM of each primer (P1: 5'-CACATCTAAG GCAAACCAC-3' and P2: 5'-AGAGCTGCAAGAGG CACA-3'; FASMAC Co., Ltd., Atsugi, Japan), 0.25 U Ex-taq (TaKaRa Bio Inc., Shiga, Japan), and 5 ng template DNA. Amplification conditions were as follows: preheating at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 66°C for 30 s; extension at 68°C for 4 min; and final extension at 68°C for 5 min. The PCR products were run on a 1% agarose gel. ExcelBand 1KB DNA ladder (DM3100; SMOBIO Technology Inc., Hsinchu, Taiwan) was used as the DNA size marker. When the F3'5'H gene did not amplify with the P1 and P2 primer set, an actin gene (*R. × pulchrum*; AB610421) was amplified as a control to check the DNA template. The PCR mixture (10 µL) contained 1 × Ex-taq buffer, 200 µM dNTPs, 0.2 µM of each primer (forward; 5'-AGCAATGTATGTTGCT ATC-3' and reverse 5'-TGATCGAGTTGTAGGTA GT-3'; FASMAC), 0.25 U Ex-taq (TaKaRa Bio), and 5 ng template DNA. Amplification conditions were as follows: preheating at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 53°C for 30 s; extension at 72°C for 45 s; and final extension at 72°C for 2 min. The PCR products were run on a 1% agarose gel. ExcelBand 100 bp DNA ladder (DM2100; SMOBIO Technology) was used as the DNA size marker.

When the 5'-UTR to 3'-UTR region of the F3'5'H gene did not amplify with the P1 and P2 primer set, we attempted to amplify exon region 1 with P3 (5'-CTAAA AATGGGCACCCTTGA-3') and P4 (5'-CCAAAAGGA GTGCTTTAATGTT-3') and exon region 2 with P5 (5'-TGAACGGCTACTACATACCCAAGAAC-3') and P6 (5'-AGTTTCAGCCGTTGAGCCTA-3') (Fig. 2A; FASMAC). The PCR mixture (10 µL) contained 1× Ex-taq buffer, 200 µM dNTPs, 0.2 µM of each primer, 0.25 U Ex-taq (TaKaRa Bio), and 5 ng cDNA template. Amplification conditions were as follows: preheating at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 60°C for 30 s (for exon 1 region) or 55°C for 30 s (for exon 2); extension at 72°C for 1 min; and final extension at 72°C for 1.5 or 1.0 min. *R. × pulchrum* 'Ōmurasaki' was used as a positive control. ExcelBand 100 bp DNA ladder (DM2100; SMOBIO Technology) was used as the DNA size marker.

RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis were conducted to analyze the length of coding sequences and untranslated regions, and for amino acid sequence analysis.

Total RNA was extracted from the petals using the Hot-borate method (Wan and Wilkins, 1994). To avoid DNA contamination, DNA digestion was performed following Mizuta et al. (2010). The total RNA (5 µg) treated with DNase I was reverse-transcribed by oligo (dT) and ReverTra Ace reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's instructions.

Isolation of the F3'5'H gene and sequence analysis

cDNA sequence analysis was carried out to deduce the amino-acid sequences of F3'5'H in all sampled individuals for phylogenetic analysis. PCR amplification was performed using flower petal cDNA and a set of F3'5'H primers (P1 and P2; FASMAC). The PCR mixture (10 µL) contained 1× Ex-taq buffer, 200 µM dNTPs, 0.2 µM of each primer, 0.25 U Ex-taq (TaKaRa Bio), and 5 ng template cDNA. Amplification conditions were as follows: preheating at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 62°C for 30 s; extension at 72°C for 2 min; and final extension at 72°C for 5 min.

The amplified fragments were cloned into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA) and *E. coli* HST08 Premium Competent Cells (TaKaRa Bio). They were sequenced using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100 Genetic analyzer (Applied Biosystems), following plasmid DNA extraction by a FastGene Plasmid Mini kit (Nippon Genetics Co., Ltd., Tokyo, Japan). The nucleotide sequences were analyzed and translated to amino acid sequences using the program GENETYX-win, Version 13.0 (Genetyx Corp., Tokyo, Japan). The nucleotide sequences and protein sequences were aligned, and the phylogenetic tree was constructed using the neighbor-joining (NJ) method and bootstrap analysis (1000 replicates).

Detection of the F3'5'H genotype in wild species and cultivars

As we found polymorphism in the azalea F3'5'H gene, we attempted to use PCR to describe the variants of this gene. To confirm the presence of a 5 bp insertion at Exon 2 of the F3'5'H2 gene, samples from *R. ripense*, *R. macrosepalum*, and Hirado azalea were analyzed by PCR using an F3'5'H2-specific primer set (P7: 5'-CCA AGTGCTTATGCTATGTAGGC-3' and P8: 5'-GAAAC GACGGGAACATTCA-3'; FASMAC). The PCR mixture (10 µL) contained 1× Ex-taq buffer, 200 µM dNTPs, 0.2 µM of each primer, 0.25 U Ex-taq (TaKaRa Bio), and 5 ng gDNA template. Amplification conditions were as follows: preheating at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 60°C for 30 s; extension at 72°C for 15 s; and final extension at 72°C for 30 s. The PCR products were run on a 2% agarose gel.

Results

Amplification of the *F3'5'H* gene in genomic DNA

DNA amplification to confirm the presence of the *F3'5'H* gene among wild species and cultivars detected the full-length gene (approximately 4 kb) in genomic DNA of the taxa with Dp derivatives, i.e. *R. ripense*, *R. macrosepalum*, and *R. yedoense* var. *poukhanense* (Table 1; Fig. 1B). The band size of the *F3'5'H* gene was also approximately 4 kb in Hirado azalea cultivars with Dp derivatives ('Ademurasaki', 'Taihō', 'Hirado-no-homare', 'Hakuhō', 'Shiro-kujyaku', and 'Hatsuyuki'), and the white-flowered cultivar *R. × mucronatum* 'Shiro-ryūkyū'; 'Hirado-no-homare' also had a band size of approximately 1 kb, suggesting it is heterozygous (Fig. 1B). However, the *F3'5'H* gene could not be amplified with the P1 and P2 primer set in *R. scabrum*, which lacks Dp derivatives, as well as five red-flowered ('Hinomoto', 'Raijin', 'Hiōgi', 'Kumo-no-ue', and 'Heiwa-no-hikari'), and six red-purple flowered Hirado azalea cultivars ('Shinshō', 'Hinode', 'Banzairaku', 'Momoyama', 'Seibo', and 'Saotome') (Fig. 1C). These 19 DNA templates of samples without Dp could normally amplify the actin gene (approximately 550 bp) as a positive control gene (Fig. 1C). Furthermore, we investigated the CDS using specific primers for each exon in eight *R. scabrum* individuals (Fig. 2A). Exon 2 of the *F3'5'H* gene was amplified for all these plants, but exon 1 failed to amplify (Fig. 2B, C). Amplification of gDNA from the five red-flowered and six red-purple flowered Hirado azalea cultivars also

failed to amplify exon 1 (Fig. 2B), but did amplify exon 2, for which the band size was approximately 400 bp (Fig. 2C).

F3'5'H cDNA sequence analysis

Nearly full-length cDNA sequences of *F3'5'H* were amplified and sequenced from two *R. ripense* plants, two *R. macrosepalum* plants, one *R. yedoense* var. *poukhanense* plant, one *R. × mucronatum* 'Shiro-ryūkyū' plant, and one plant each of four Hirado azaleas; 'Ademurasaki', 'Hirado-no-homare', 'Hakuhō', and 'Shiro-kujyaku'. A band of approximately 1.5 kb was detected from 10 Hirado azalea cultivars with purple or white-flowers (data not shown). After cloning the amplified PCR products from wild species, we identified *F3'5'H* cDNA sequences in both *R. ripense* plants, both *R. macrosepalum* plants, and the *R. yedoense* var. *poukhanense* plant (DDBJ accession no. LC547905–LC547910).

The full-length sequences of the *F3'5'H* homologue genes contained 27 bp of 5' UTRs, 1533 bp of CDS, and 121 or 135 bp of 3' UTRs in *R. ripense* and *R. macrosepalum*, respectively (Table 2). These genes were named *RrF3'5'H1a* from *R. ripense* 1; *RrF3'5'H1b* from *R. ripense* 2; *RmF3'5'H1a* from *R. macrosepalum* 1; *RmF3'5'H1b1* and *RmF3'5'H1b2* from *R. macrosepalum* 2. In *R. yedoense* var. *poukhanense*, the *F3'5'H* homologue gene contained 27 bp of 5' UTRs, 1533 bp of CDS, and 135 bp of 3' UTRs. This gene was named *RyF3'5'H1* (Table 2). The *F3'5'H* gene had a length of 510 amino acid residues in the three wild

Fig. 1. gDNA analyses of the *F3'5'H* gene. (A) Diagram of the *F3'5'H* gene, indicating the primers used for each amplification. (B) and (C) Amplification to assess the presence of the *F3'5'H* gene among wild species and cultivars. Dp: Dp derivative production; M₁: 1 kb DNA marker (DM3100; SMOBIO Technology) and M₂: 100 bp DNA marker (DM2100). (B) Lanes 1–6: *R. macrosepalum* 1 and 2, *R. ripense* 1 and 2, *R. × mucronatum* 'Shiro-ryūkyū' and *R. yedoense* var. *poukhanense* 1; lanes 7–12: Hirado azalea cultivars 'Ademurasaki', 'Taihō', 'Hirado-no-homare', 'Hakuhō', 'Hatsuyuki', and 'Shiro-kujyaku'. (C) Lanes 1–8: eight individuals of *R. scabrum*; lanes 9–19: Hirado azalea cultivars 'Hinomoto', 'Raijin', 'Hiōgi', 'Kumo-no-ue', 'Heiwa-no-hikari', 'Shinshō', 'Hinode', 'Banzairaku', 'Momoyama', 'Seibo', and 'Saotome'; P: positive control ('Ōmurasaki').

Fig. 2. gDNA analyses of the F3'5'H gene exon 1 and exon 2. (A) Diagram of the F3'5'H gene indicating the primers used for each amplification. (B) Amplification of F3'5'H exon 1. (C) Amplification of F3'5'H exon 2. For (B) and (C) M: 100 bp DNA marker (DM2100; SMOBIO Technology); lanes 1–8: eight individuals of *R. scabrum*; lanes 9–19: Hirado azalea cultivars 'Hinomoto', 'Raijin', 'Hiōgi', 'Kumo-no-ue', 'Heiwa-no-hikari', 'Shinshō', 'Hinode', 'Banzairaku', 'Momoyama', 'Seibo', and 'Saotome'; P: positive control ('Ōmurasaki'). Black and white arrows indicate 685 bp and 405 bp, respectively.

Table 2. The accession numbers and lengths of each region of the F3'5'H gene in wild species and cultivars.

		Gene name	5'UTR	CDS	3'UTR	Accession no
<i>R. ripense</i> 1		RrF3'5'H1a	27	1533	121	LC547905
<i>R. ripense</i> 2		RrF3'5'H1b	27	1533	135	LC547906
<i>R. macrospalum</i> 1		RmF3'5'H1a	27	1533	121	LC547907
<i>R. macrospalum</i> 2		RmF3'5'H1b1	27	1533	135	LC547908
		RmF3'5'H1b2	27	1533	121	LC547909
<i>R. yedoense</i> var. <i>poukhanense</i>		RyF3'5'H1	27	1533	135	LC547910
<i>R. × mucronatum</i> 'Shiro-ryūkyū'		RmSRF3'5'H1	27	1533	135	LC547911
		RmSRF3'5'H2	27	1551	122	LC547912
Hirado azalea group	'Hirado-no-homare'	RpHHF3'5'H1	27	1533	135	LC547913
	'Shiro-kujyaku'	RpSKF3'5'H1	27	1533	135	LC547914
	'Ademurasaki'	RpF3'5'H2				In this study
	'Hakuho'	RpF3'5'H2	27	1551	108	In this study
Ōkirishima group	'Ōmurasaki'	RpF3'5'H2				AB488484 (Mizuta et al., 2010)

species.

Interestingly, the F3'5'H gene in *R. × mucronatum* 'Shiro-ryūkyū' displayed two different CDS lengths: 1533 bp (*RmSRF3'5'H1*; LC547911) and 1551 bp (*RmSRF3'5'H2*; LC547912), corresponding to 510 and 516 amino acid residues, respectively (Fig. 4C; Table 2). The F3'5'H gene in Hirado azalea cultivars also had two different CDS lengths: 1533 bp from 'Hirado-no-homare' and 'Shiro-kujyaku' (DDBJ accession no. LC547913–LC547914) as compared to 1551 bp from 'Ademurasaki' and 'Hakuho', corresponding to 510 and 516 amino acid residues, respectively (Fig. 4C; Table 2). The F3'5'H genes in 'Ademurasaki' and

'Hakuho' were identical to those of *R. × pulchrum* 'Ōmurasaki'. Moreover, Hirado-no-homare' and 'Hakuho' had different nucleotide sequences that were shorter as a result of deletion (data not shown).

Phylogenetic relationship of the F3'5'H gene among wild species, R. × mucronatum 'Shiro-ryūkyū', and Hirado azalea

The F3'5'H cDNA of wild species, *R. × mucronatum* 'Shiro-ryūkyū', and Hirado azalea cultivars was chosen for phylogenetic analysis due to similarities in their F3'5'H nucleotide sequences. The alignments of nucleotide sequences were compared between the wild

species, *R. × mucronatum* ‘Shiro-ryūkyū’, and four Hirado azalea cultivars: ‘Ademurasaki’, ‘Hirado-no-homare’, ‘Hakuhō’, and ‘Shiro-kujyaku’. Phylogenetic analysis indicated that there are two clusters. *R. ripense* and *R. × mucronatum* ‘Shiro-ryūkyū’ are closely related to Hirado azaleas and were assigned to the same cluster, while *R. yedoense* var. *poukhanense* and *R. macrosepalum* were assigned to another cluster (Fig. 3).

Detection of a 5 bp insertion in the F3'5'H gene of wild species and cultivars

In some Hirado azalea, there was a 5 bp (TTGTA) insertion in the F3'5'H cDNA gene (Fig. 4). PCR investigation with primers specific for this insertion did not amplify the F3'5'H gene in eight *R. scabrum*, eight *R. ripense* and five *R. macrosepalum* individuals or the cultivars ‘Taihō’, ‘Hirado-no-homare’, and ‘Shiro-kujyaku’, suggesting that these individuals did not have this insertion (Table 3). Amplification of the F3'5'H gene with these primers produced an approximately 300 bp PCR product in five azaleas *R. ripense*, *R. × mucronatum* ‘Shiro-ryūkyū’, *R. × pulchrum*

‘Ōmurasaki’, and three Hirado azalea cultivars, ‘Ademurasaki’, ‘Hakuhō’ and ‘Hatsuyuki’, suggesting that the 5 bp insertion is present in these cultivars (Table 3).

Discussion

Although recent genetic studies have found that Hirado azalea is closely related to the red-flowered

Fig. 3. Neighbor-joining tree of F3'5'H nucleotide sequences among wild species, *R. × mucronatum* ‘Shiro-ryūkyū’, and four Hirado azalea cultivars (underlined). The numbers at the nodes are bootstrap values. *Vaccinium corymbosum* was set as an out-group.

Fig. 4. cDNA sequence analysis. (A) Diagram showing the 5 bp insertion in exon 2 of the F3'5'H gene. Nucleotide sequence (B) and translated amino acid (C) alignments of the exon 2 region of F3'5'H near the insertion. Dots indicate the stop codon (TAG and TGA).

Table 3. PCR analysis of the F3'5'H exon 2 insertion in *R. scabrum*, *R. ripense*, *R. macrosepalum* and Hirado azalea with delphinidin derivatives.

		No insertion	5 bp insertion
Wild species ^z		<i>R. scabrum</i> (8)	
		<i>R. ripense</i> (8)	<i>R. ripense</i> (5)
		<i>R. macrosepalum</i> (5)	
Cultivars	Ryūkyū azalea group		<i>R. × mucronatum</i> ‘Shiro-ryūkyū’
	Hirado azalea group	‘Hirado-no-homare’	‘Ademurasaki’
		‘Shiro-kujyaku’	‘Hakuhō’
		‘Taihō’	‘Hatsuyuki’
	Ōkirishima group		‘Ōmurasaki’

^z The parentheses show individual numbers of wild species.

R. scabrum (Scariot et al., 2007a, b), this does not explain the wide flower color variation in this cultivar group. Our investigation of the F3'5'H gene, which plays a key role in flower color diversity in interspecific hybridization between purple and red azalea flowers (Mizuta et al., 2014; Meanchaipiboon et al., 2020), suggests that flower color variation in Hirado azalea may be due to variations in this gene arising from hybridization of *R. scabrum* with either *R. ripense* or *R. × mucronatum* 'Shiro-ryūkyū'.

PCR analysis of the F3'5'H gene from gDNA using P1 and P2 primers showed that taxa without Dp derivatives, including certain Hirado azalea, did not have about 4 kb of a F3'5'H nucleotide, similar to *R. ripense* or *R. × mucronatum* 'Shiro-ryūkyū' (Fig. 1). Eight plants of *R. scabrum* did not have an amplified exon 1 region after PCR using P3 and P4 primers (Fig. 2B). Similarly, exon 1 of red-and red-purple flowered Hirado azalea lacking Dp derivatives was not amplified but exon 2 was (Fig. 2B, C). We tried to amplify the exon 1 region using another primer set, but PCR products were not detected in almost all tested *R. scabrum* and Hirado azalea, except for *R. ripense* and *R. × pulchrum* 'Ōmurasaki' (data not shown). These results suggest that the exon 1 region of the F3'5'H gene is defective in certain Hirado azalea and *R. scabrum* as compared to *R. ripense* or *R. × pulchrum* 'Ōmurasaki'. However, the reason for the defective DNA sequence of the exon 1 region in Dp derivative-lacking cultivars is unclear, so to clarify why F3'5'H did not function for accumulation of Dp derivatives, we are going to gather upstream information, including promoter region.

Our results add to the evidence for a loss of F3'5'H activity as a source of color variation in plants. In delphinium, genomic PCR analysis indicated that the pale-pink garden cultivar 'SHP' lacked F3'5'H, suggesting that the F3'5'H gene in 'SHP' may either have a substantial alteration or deletion of the ORF sequence (Miyagawa et al., 2014). In a neutron beam-induced *Pisum sativum* mutant with pink flowers, it was reported that the deletion of a large part of the ORF region of F3'5'H gene caused the loss of F3'5'H activity (Moreau et al., 2012). Similarly, our results suggest that the red-flowered wild species *R. scabrum* may have an alteration or deletion of the F3'5'H ORF sequence.

Phylogenetic analysis of the F3'5'H sequences of Hirado azalea and their putative parents showed that *R. × mucronatum* 'Shiro-ryūkyū' and *R. ripense* are closely related to Hirado azalea (Fig. 3). In addition, previous SSR marker analyses showed that *R. ripense* and 14 cultivars of *R. × mucronatum* were clustered with 14 cultivars of *R. × pulchrum* (Yamamoto et al., 2019). Scariot et al. (2007b) investigated the genetic relationship among evergreen azaleas using AFLP, SSR, and EST markers. The consensus tree for these species and cultivars showed that Hirado azalea such as 'Ademurasaki' and *R. × pulchrum* 'Ōmurasaki' are

closely related to *R. scabrum*. Tamura (1962) reported that the flower color of a hybrid between *R. scabrum* and *R. ripense* was similar to *R. × pulchrum* 'Ōmurasaki'. Moreover, morphological analysis of *R. macrosepalum* and *R. yedoense* var *poukhanense* suggested that these wild species were unlikely to be putative parents of Hirado azalea (Tamura, 1962). Our genetic analysis based on the F3'5'H gene sequence support the idea that *R. macrosepalum* and *R. yedoense* var *poukhanense* are not closely related to Hirado azalea (Fig. 3). In combination, our results and those of previous studies suggest that purple-flowered Hirado azaleas 'Ademurasaki' and *R. × pulchrum* 'Ōmurasaki' developed from hybridization between *R. scabrum* and *R. ripense* or *R. × mucronatum*.

In lily hybrids, origin lily species were identified using nucleotide sequence alignments of the MYB12 gene that regulates anthocyanin accumulation in tepals. In lilies, Asiatic and Oriental inter-specific hybrid cultivar groups are differentiated with respect to polymorphisms of MYB12 (Yamagishi et al., 2014; Yamagishi and Nakatsuka, 2017). Similarly, we found that nucleotide sequence alignments of the F3'5'H gene of wild species and cultivars are different, resulting in F3'5'H gene polymorphisms. A 5 bp insertion in the F3'5'H gene is found in some cultivars. Therefore, we developed a specific marker to detect polymorphisms in wild species and other cultivars. Investigation of the 5 bp insertion in the F3'5'H gene in the wild species *R. scabrum*, *R. ripense* and *R. macrosepalum* showed that the insertion was absent from all *R. scabrum* and *R. macrosepalum* plants, whereas it was present in some *R. ripense* plants and *R. × mucronatum* 'Shiro-ryūkyū' (Table 3). This suggests that the wide range of flower color in Hirado azalea may be partially due to the introduction of a 5 bp insertion in the F3'5'H gene from *R. ripense* and *R. × mucronatum* 'Shiro-ryūkyū' to *R. scabrum*.

In this study, we investigated gDNA and cDNA sequences of the F3'5'H gene in wild species and cultivars. The results suggest that Hirado azalea lacking Dp derivatives has an F3'5'H gene derived from *R. scabrum*, whereas Hirado azalea with Dp derivatives, which shows a wide range of color variation, has F3'5'H genes derived from *R. ripense* or *R. × mucronatum* 'Shiro-ryūkyū', due to hybridization of these species with *R. scabrum*. To further clarify the genetic relationships among Hirado azalea and their putative parents—*R. scabrum*, *R. ripense*, and *R. × mucronatum*—we are investigating the F3'5'H gene in additional species and cultivars because the putative parents only make a limited genetic contribution in *R. scabrum* to Hirado azalea with Dp derivatives.

Acknowledgements

The authors thank the faculty of Life and Environmental Sciences of Shimane University for financial

support in publishing this report.

Literature Cited

- Galle, F. C. 1987. *Azaleas*. Revised and enlarged Ed. Timber Press Inc., Portland, Oregon.
- Kobayashi, N. 2016. Tsutsuji. p. 151–180. In: M. Shibata (ed.). *Hana no hinshu kairyou no nihonshi*, Bunkyo, Tokyo (In Japanese).
- Kobayashi, N., T. Horikoshi, H. Katsuyama, T. Handa and K. Takayanagi. 1998. A simple and efficient DNA extraction method from the plants, especially from woody plants. *Plant Tiss. Cult. Biotech.* 4: 76–80.
- Meanchaipiboon, S., N. Kobayashi and A. Nakatsuka. 2020. Analyses of pigment compositions and anthocyanin biosynthesis gene expression in Hirado azalea cultivars. *Hort. J.* 89: 284–291.
- Miyagawa, N., Y. Nishizaki, T. Miyahara, M. Okamoto, Y. Hirose, Y. Ozeki and N. Sasaki. 2014. Sequence variations in the *flavonoid 3',5'-hydroxylase* gene associated with reddish flower phenotypes in three delphinium varieties. *Plant Biotech.* 31: 83–87.
- Mizuta, D., T. Ban, I. Miyajima, A. Nakatsuka and N. Kobayashi. 2009. Comparison of flower color with anthocyanin composition patterns in evergreen azalea. *Sci. Hortic.* 122: 594–602.
- Mizuta, D., A. Nakatsuka, T. Ban, I. Miyajima and N. Kobayashi. 2014. Pigment composition patterns and expression of anthocyanin biosynthesis genes in *Rhododendron kiusianum*, *R. kaempferi*, and their natural hybrids on Kirishima mountain mass, Japan. *J. Japan. Soc. Hort. Sci.* 83: 156–162.
- Mizuta, D., A. Nakatsuka, I. Miyajima, T. Ban and N. Kobayashi. 2010. Pigment composition patterns and expression analysis of flavonoid biosynthesis genes in the petals of evergreen azalea ‘Oomurasaki’ and its red flower sport. *Plant Breed.* 129: 558–562.
- Moreau, C., M. J. Ambrose, L. Turner, L. Hill, T. H. N. Ellis and J. M. I. Hofer. 2012. The *b* gene of pea encodes a defective flavonoid 3',5'-hydroxylase, and confers pink flower color. *Plant Physiol.* 159: 759–768.
- Nakao, S. and T. Tamura. 1970. Tutuji-ru. p. 3005–3018. In: R. Ishii and Y. Inoue (eds.). *Encyclopedia of horticulture*, Vol. 6. Seibundoushinkosha, Tokyo (In Japanese).
- Scariot, V., T. Handa and J. De Riek. 2007a. A contribution to the classification of evergreen azalea cultivars located in the Lake Maggiore area (Italy) by means of AFLP markers. *Euphytica* 158: 47–66.
- Scariot, V., E. De Keyser, T. Handa and J. De Riek. 2007b. Comparative study of the discriminating capacity and effectiveness of AFLP, STMS and EST markers in assessing genetic relationships among evergreen azaleas. *Plant Breed.* 126: 207–212.
- Tamura, T. 1962. Kyusyu no tsutsuji. *Shinkaki* 35: 38–42 (In Japanese).
- Tamura, T. 1963. Studies on the Hirado-azaleas, with special reference to their formation. *Bulletin of the Horticultural Research Station D*: 155–185 (In Japanese with English abstract).
- Wan, C. Y. and T. A. Wilkins. 1994. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* 223: 7–12.
- Yamagishi, M. and T. Nakatsuka. 2017. *LhMYB12*, regulating tepal anthocyanin pigmentation in Asiatic hybrid lilies, is derived from *Lilium dauricum* and *L. bulbiferum*. *Hort. J.* 86: 528–533.
- Yamagishi, M., H. Ihara, K. Arakawa, S. Toda and K. Suzuki. 2014. The origin of the *LhMYB12* gene, which regulates anthocyanin pigmentation of tepals, in Oriental and Asiatic hybrid lilies (*Lilium* spp.). *Sci. Hortic.* 174: 119–125.
- Yamamoto, S., T. Nakamura, K. Koiwai, M. Miyano, E. Iizuka, A. Nakayama, Y. Kurashige, N. Kobayashi and T. Handa. 2019. Origin of the *Rhododendron kaempferi* related species and cultivars estimated by SSR analysis. *Acta Hortic.* 1263: 295–297.