Flower color and anthocyanin biosynthesis genes analysis in Hirado azalea (*Rhododendron* × *pulchrum* Sweet)

（ヒラドツツジの花色およびアントシアニン合成遺伝子の解析）

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Chapter 1

Introduction

1.1 Background of the research and motivation

1.1.1 Evergreen azalea and cultivars

There are about 50 *Rhododendron* species endemic to Japan. Some of them belong to the subgenus *Tsutsusi*, section Tsutsusi, and are considered to be important genetic resources for breeding ornamental evergreen azaleas (Kobayashi, 2013; Kobayashi and Kurashige, 2018). Since the Edo era (1603-1868), the horticulturalists have selected cultivars and hybrids of the evergreen azaleas from natural population species such as *Rhododendron kaempferi* Planch., *R. macrosepalum* Maxim., *R. indicum* (L.) Sweet, *R. ripense* Makino, and *R. obtusum* (Kobayashi et

al., 1995, 2000). The floral mutant selection contributed to the development of flower colors and flower shapes of various azalea cultivar groups such as Ōkirishima, Ryūkyū, Edo Kirishima, Kurume, Hirado, and Satsuki (Kobayashi, 2013)

Hirado azalea (*R.* × *pulchrum* Sweet) comprises a group of evergreen azaleas bred in Nagasaki Prefecture, Japan, especially in the Hirado region (Galle, 1987; Kobayashi, 2016). Hirado azalea is known for their large flowers varying in colors including white to pink, red, and purple forms (Tamura, 1963). The red-flowered *R. scabrum* G. Don, purple-flowered *R. ripense*, 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* (Tamura, 1962, 1963). They are thought to have developed by natural hybridization between garden plants without artificial crossing (Nakao and Tamura, 1970). At present, there are about 300 Hirado azalea cultivars in Japan (Galle, 1987; Kobayashi, 2016). However, the origin of this color variation is not well understood.

1.1.2 Flavonoid pigments and their synthesis pathway

Flavonoid pigments contribute to various functions in plants varied from coloring flowers and fruits, UV-protection, and insect protector. This flavonoid pathway is one of the most well-studied done by researchers over past decades. The outline of the scheme has been developed, nevertheless, it is still further away from truly understanding it due to its complexity (Mizuta, et al, 2008). Flavonols and anthocyanins share the same pathway. Anthocyanin and flavonol are main

pigments of the evergreen azalea flowers. Anthocyanins are stable and water-soluble pigments (Asen and Budin, 1966; De Loose, 1969; Mizuta et al., 2009).

Anthocyanins are modified anthocyanidins; major anthocyanidins in azaleas are cyanidin and delphinidin derivatives. Flavonol has two major derivatives which are myricetin and quercetin. Flavonol is colorless pigment found in a cytosol but co-pigmentation between flavonols, and anthocyanins contribute to bluing effect (Mizuno, et al., 2015; Aida, et al., 2000; Asen, et al., 1971, 1972). Cyanidin derivatives are cyanidin (Cy) and peonidin (Pn), whereas delphinidin derivatives are delphinidin (Dp), petunidin (Pt), and malvidin (Mv) (Fig. 1.1). Moreover, anthocyanin colors are determined by the degree of oxygenation of anthocyanidins as the number of hydroxyl groups increase the bluer pigments become (Schwinn and Davies, 2004, Tanaka et al., 2008; Fig. 1.1).

The effect of pH on the stability of anthocyanin pigments depends on the compositions of anthocyanins. For red-colored pigments, it is mainly in the form of flavylium cation which is stable at a lower pH solution (Bakowska, 2005, Tanaka et al., 2008). The formation of anionic quinoidal species is formed via the increasing pH with psedobase and chalcone structures; they are are stabilized at neutral pH (Fossen et al., 1998, Tanaka et al., 2008, Casteneda-Ovando at al., 2009). Hence, the bluer pigments are more stable at higher pH. Cy appears red at lower pH <3, violet at pH 7-8 and blue in at pH > 11 (Torskanggerpoll and Andersen, 2005). Similarly, Pn displays the same characteristics with deeper hue and has higher stability at high pH than other anthocyanidins (Khoo et al., 2017). Nevertheless, anthocyanin pigments are mostly stable at lower pH or acidic condition. In the petal of evergreen azalea, the pH is roughly 3-5 which is acidic (Nakatsuka et al., 2015), hence, the increase in pH to produce blue color is nearly impossible. Hence, co-pigmentation between flavonol and anthocyanins are preferable for bluer hue.

Co-pigmentation is the main mechanism of the stabilization of color in plants (Casteneda-Ovando at al., 2009). The interaction between colorless pigment, flavonol, and anthocyanin affect a bathochromic shift in the adsorption spectra. Moreover, De Loose (1970a, b) and Asen et al. (1971) reported that the co-pigmentation effect also important for developing various flower color. Both scarlet and bluish-red flowers of hybrids of *R. simsii* contain the same amount of anthocyanin and pH value. However, co-pigmentation does not occur. Even though, it contains a trace amount of iron and aluminum. The missing factor is flavonols (azaleatin, and quercetnin) indicating metal ion does not affect flower color in the absence of flavonols De Loose (1970a, b). To conclude, flavonoid pigment compositions play an important role in determining flower color. In evergreen azaleas, Cy-derivative can be found in all azaleas, however, presence of Dp derivatives can be found only in some azaleas. Hence, it is important to study Dp-based pigments. Moreover, co-pigmentation also gives rise to new flower color and the presence of flavonol and metal ions is equally important as Dp-based pigments.

The flavonoid pathway has been studied intensively, in spite of that, it does not fully understand by researchers. Further investigation of each gene responsible in the pathway will lead to a better understanding and scheme for breeding floriculture especially flavonoid 3′5′-hydroxylase (*F3’5’H*) as it determines blue color pigments in evergreen azaleas plays an important role in entering flavonoid synthesis pathway by converting 4-Coumaroyl-CoA and 3 Malonyl-CoA to naringeninchalcones. This naringeninchalcones will further convert to naringenin by chalcone isomerase (*CHI*) in Fig. 1.2. This pathway to flavonoid may be blocked at the very first step of chalcone synthase (*CHS*) which results in no accumulation of flavonols or anthocyanins.

Then, flavone 3-hydroxylase (*F3H*) converts naringenin to dihydrokaempferol (DHK). While flavonoid 3′-hydroxylase (*F3'H*) and *F3'5'H* will convert naringenin to dihydroquercetin

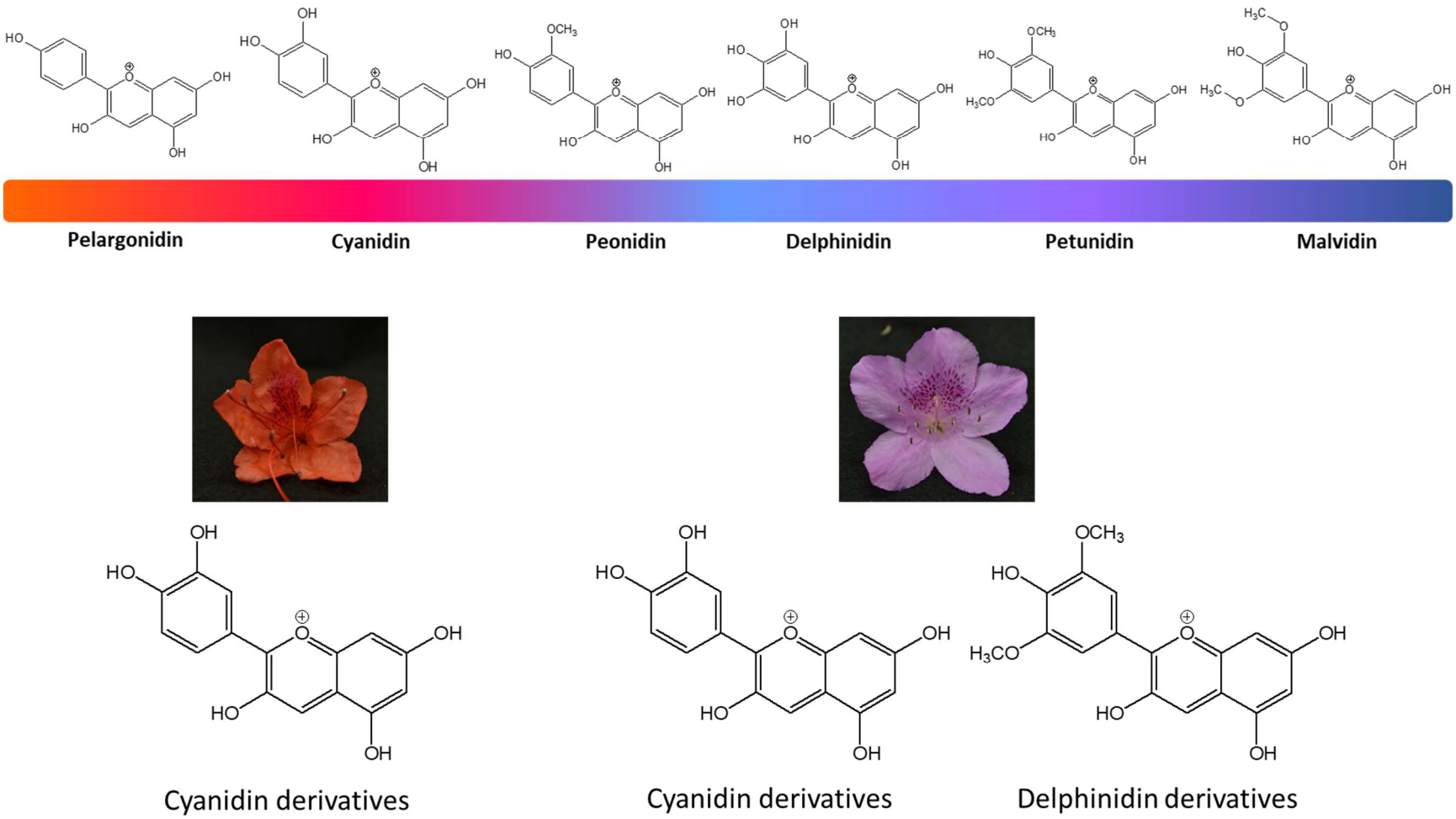

Fig. 1.1 Visible color range of anthocynidins and the pigment compositions found in evergreen azalea. Cyanidin (Cy) derivatives: Cy, Peonidin (Pn). Delphinidin (Dp) derivatives: Dp, Petunidin (Pt), Malvidin (Mv).

(DHQ) and dihydromyricetin (DHM), respectively (Fig. 1.2). Thus, the *F3'5'H* plays an important role for producing substrate which will change to leucodelphinidin entering blue pigment synthesis pathway. Accordingly, many research focus on *F3'5'H* when they want to produce blue-flowered plants. According to Suzuki, et. al. (2000), *Torenia hybrida* 'Summerwave' mainly accumulate malvidin (Dp-based pigments). They reported that co-suppression of *F3'5'H* produced pink-flowered plants mainly containing peonidin. This indicates the importance of *F3'5'H* in producing blue flowers.

Regardless, dihydroflavonol 4-reductase (DFR) also plays an important role for Dp-based pigment synthesis. Due to substrate-specificity of DFR, synthesis of Pelagornidin (Pg), Cy, and Dp also decides at this step. Johnson, et al. (2001) reported that DFR of petunia showed substrate-specificity thus it cannot efficiently reduce DMK which is a precursor to Pg (orange pigment) (Fig. 1.1, 1.2). They transformed petunia with gerbera DFR which resulted in orange flower. Therefore, it is another way to determine Dp-based pigments synthesis.

Anthocyanidin synthase (*ANS*) plays an important role in producing anthocyanidin by converting leucocyanidin to cyanidin, and leucodelphinidin to delphinidin. Anthocyanidin will be further modified by anthocyanidin 3-O-glucosyltransferase (*UFGT*) to produce anthocyanins. However, anthocyanidins may revert to proanthocyanidin which is colorless. Therefore, presence of anthocyanidins may not correlate with flower color in some cases since they contain no anthocyanins (Bogs, et al, 2005). Flavonols are derived from either DMQ or DHM converted by flavonol synthase (*FLS*) (Fig. 1.2). The competition between DFR and *FLS* for limited substrate also plays an important role in determining flavonol concentration, and anthocyanin concentration.

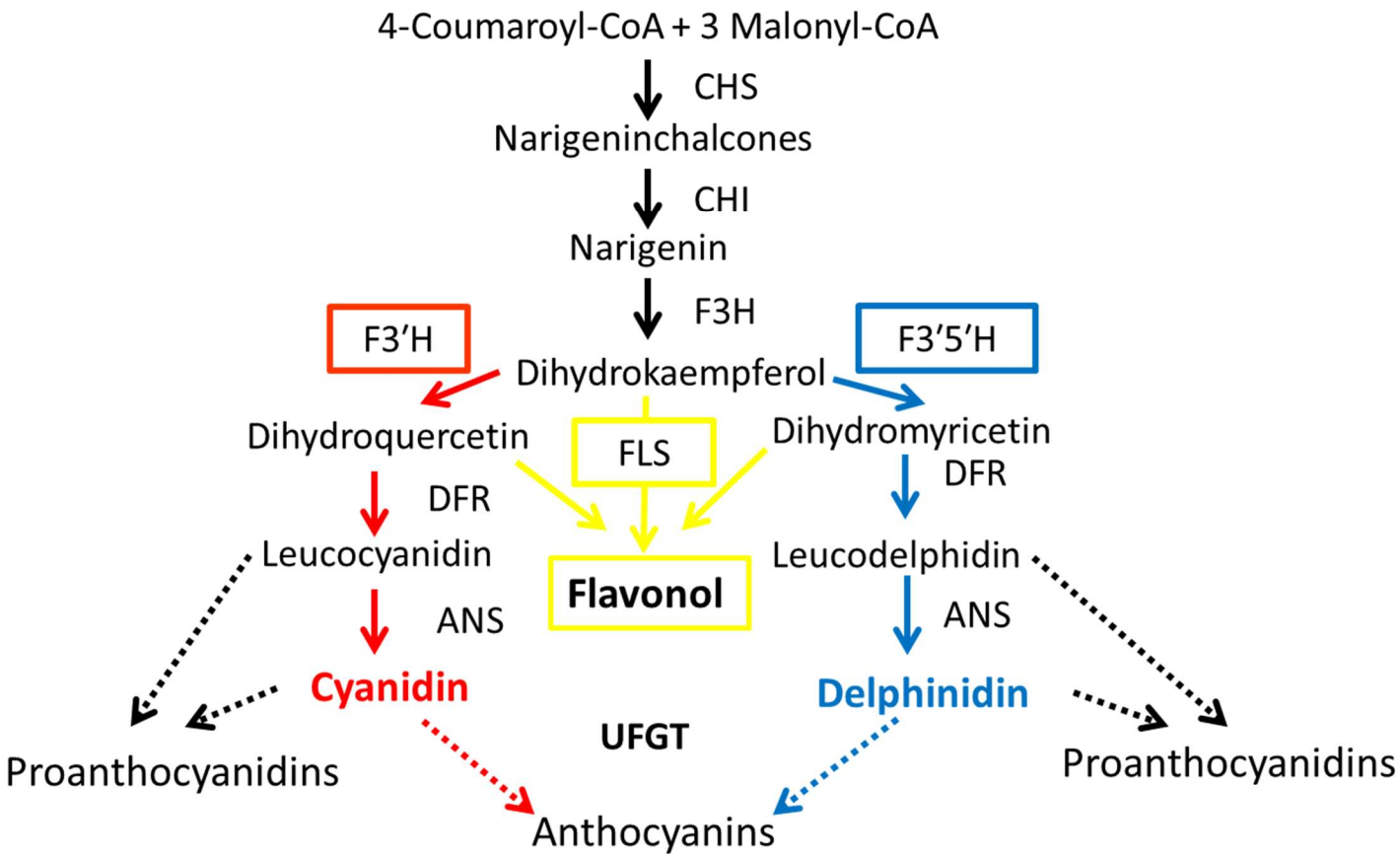

Fig. 1.2 Flavonoid biosynthesis pathway. (CHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavone 3-hydroxylase, F3′H: flavonoid 3′-hydroxylase, F3′5′H: flavonoid 3′5′-hydroxylase, DFR: dihydroflavonol 4-reductase, ANS: anthocyanidin synthase, FLS: flavonol synthase, UFGT: anthocyanidin 3-O-glucosyltransferase)

1.1.3 Flower color and pigments in evergreen azalea

Previous studies have reported that purple-colored flowers of *R. kiusianum* contain both Cy and Dp derivatives, whereas red-colored flowers of *R. kaempferi* contains only Cy derivatives (Sakata et al., 1991, 1993). Natural hybrids, intermediates of the above two Rhododendron species, display a range of flower colors. According to Mizuta, et. al. (2014), they investigated *R. kaempferi*, *R. kiusianum*, and their natural hybrids in Kirishima mountain mass. Their natural hybrids have various flavonoid pigment profile comparing to them. This indicates that various flavonoid pigment led to new flower color caused by the cross of red-flowered and purple-flowered species, especially coexistence of Cy- and Dp-based pigments (Fig. 1.2).

Nakatsuka, et al. (2006) reported that pink-flowered gentian plants which originated from spontaneous mutations of blue-flowered gentians is unknown for its formation mechanism. They found that pink-flowered gentian has a larger size of the *F3'5'H* DNA compared to blue-flowered gentian. This was due to an insertion of transposable element at the first exon of *F3'5'H*. As a result, normal transcript of the *F3'5'H* was interrupted. This suggests the importance of the *F3'5'H*; a key enzyme for synthesizing delphinidin based pigments.

Currently, Hirado azalea cultivars have only been studied for their similar morphology with their putative parents. There has been no reports about their pigment composition, gene expression analysis, and genetic relationship based on the *F3'5'H* gene. Moreover, the genetic relationships among evergreen azaleas inferred from DNA markers, including amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and expressed sequence tag (EST) markers, indicate that Hirado azalea is most closely related to *R. scabrum*, and cluster with *R.* × *mucronatum*, *R. ripense*, and *R. macrosepalum* Maxim (Scariot et al., 2007a, b).

In this study, I investigated the genetic relationships among Hirado azalea and their putative parents, *R. scabrum*, *R. ripense*, and *R.* × *mucronatum* 'Shiro-ryūkyū,' by flower colors, pigment compositions, flavonoid-related biosynthesis gene expression, and identified the correlation amongst them. Furthermore, I assessed the role of the *F3′5′H* gene in the origins of purple and white flowers among the Hirado azalea. I investigated its presence and sequence variation to understand flower color variation and genetic relationships among Hirado azaleas and their putative parents.

1.2 Scope and Objectives

The main purpose of this study was to understand the relationship among putative parents and Hirado azalea cultivars and to identify their flower color variation based on genetic relationship

To achieve these aims, the specific objectives of this were as follow;

1. Analyze the pigment compositions of Hirado azalea cultivars and their putative parents
2. Investigate the gene expression of flavonoid biosynthesis gene such as *F3′H* and *F3′5′H* of Hirado azalea cultivars and their putative parents
3. Analyze genetic relationship by comparing the cDNA *F3′5′H* nucleotide sequences of Hirado azalea cultivars and their putative parents
4. Develop a specific primer to detect F3′5′H2 gene of cultivars with extra 6 amino acids
5.
6. Analyze an 5 bp-insertion in Hirado azalea cultivars using PCR approach

The current study presents the genetic relationship among putative parents and Hirado azalea. An overview of each chapter is described as followed

Chapter 2: This chapter includes the study of flower color, pigment compositions, and flavonoid related biosynthesis gene expression among Hirado azalea and their putative parents.

Chapter 3: This chapter includes the profiling analyses of the *F3'5'H* gene among Hirado azalea and their putative parents by complementary DNA sequence analysis, deduced amino acid sequence, and 5 bp insertion in the *F3'5'H* gene exon 2 analysis.

Chapter 4: This chapter includes the discussion of chapter 2 and 3 and summarize the results

Chapter 2

The Analyses of Pigment Compositions and Anthocyanin Biosynthesis Gene Expressions in Hirado Azalea Cultivars

2.1 Introduction

Currently, Hirado azalea cultivars have only been studied for their similar morphology with their possible parents. There has been no report about their pigment composition and gene expression analysis. In order to develop a new flower color breeding program for large flowered hybrids, it is necessary to understand the pattern of how Hirado azalea cultivars were developed. Hence, I investigated Hirado azalea cultivars and their related parents based on the flower color, pigment composition, and flavonoid-related biosynthesis gene expression and identified the correlation amongst them.

2.2 Experimental procedures

2.2.1 Plant materials

Hirado azalea cultivars were collected as pot plants, which were cutting clones from authorized collections in the Hirado city, Nagasaki prefecture. Fresh petals of fully open flowers of evergreen azaleas (without blotches), including 6 samples of 3 wild species, and 26 cultivars of subgenus Tsutsusi were collected from the experimental field of the Faculty of Life and Environmental Science, Shimane University, Shimane from the middle of April to the end of June in two years; in 2018 and 2019 (Fig. 2.1; Table 2.1).

The fresh petals were boiled in water at 100 °C for 10 s and dried for 20 h at 40 °C. It was then stored in a desiccator at 4 °C until HPLC analysis was performed. For molecular analysis, fresh petals of 6 wild species and 26 cultivars were collected at stage 3 (candle stage, closed flower bud). Petals were frozen in liquid nitrogen and stored at -80 °C until RNA extraction was performed.

2.2.2 Flower color measurement

The flower color of each sample was recorded by photograph (Fig. 2.1). Flower color was measured by the Royal Horticultural Society Color Chart (RHSCC) 6th edition and Color reader (CR-10; Konica Minolta Sensing Inc., Tokyo, Japan) for lightness (L^*) and the two chromatic components a^* and b^* (Mizuta et al., 2009).

Table 2.1 The evergreen azaleas used in this chapter.

	Section or cultivar group	Sample no.	Materials	RHSCC[z]	CIE*L*a*b** coordinates[y]			*h*[x]
					*L**	*a**	*b**	
Wild species	Sec. Tsutsusi	1	*R. scabrum* 1	47D	57.6	49.3	12.4	14.1
		2	*R. scabrum* 2	50B	47.1	52.5	18.5	19.4
		3	*R. ripense* 1	75A	55.1	43.2	-22.3	328.2
		4	*R. ripense* 2	N80D	60.1	31.0	-19.2	332.7
		5	*R. macrosepalum* 1	76B	68.6	24.1	-12.6	332.4
		6	*R. macrosepalum* 2	84C	65.2	28.4	-14.2	333.4
Cultivars	<Ōkirishima group>	7	*R.* × *pulchrum* 'Ōmurasaki'	N74B	45.0	51.5	-18	340.7
	<Hirado azalea group>	8	'Hinomoto'	47C	47.7	55.2	12.7	13.0
		9	'Raijin'	47D	56.3	49.6	13.4	15.1
		10	'Rashōmon'	51B	50.8	51.2	15.3	16.6
		11	'Hiōgi'	52C	50.1	50.8	9.9	11.0
		12	'Kumo-no-ue'	52C	62.0	46.4	11.3	13.7
		13	'Miyo-no-haru'	55B	57.8	48.6	10.2	11.8
		14	'Heiwa-no-hikari'	55C	67.9	42.8	8.0	10.6
		15	'Shinshō'	N57C	57.6	50.0	-2.5	357.1
		16	'Wakakoma'	N57C	51.2	55.4	-3.1	356.8
		17	'Zanshō'	N57D	50.8	56.2	-4.1	355.8
		18	'Hinode'	63B	48.4	55.2	1.3	1.3
		19	'Shō-no-shin'	N66C	52.8	55.0	-3.0	356.9
		20	'Seibo'	N66C	57.7	49.1	-8.7	350.0
		21	'Saotome'	67C	49.0	57.7	-1.6	358.4
		22	'Banzairaku'	68B	54.9	53.2	-5.6	354.0
		23	'Ademurasaki'	72C	47.5	50.5	-16.2	342.2
		24	'Momoyama'	73B	63.9	43.6	-7.0	350.9
		25	'Taihō'	N74D	52.6	46.6	-15.6	341.5
		26	'Hirado-no-homare'	77D	64.4	34.4	-12.1	340.6
		27	'Hakuhō'	NN155C	92.8	1.1	6.5	80.4
		28	'Shiro-kujyaku'	NN155D	92.4	1.4	6.0	76.6
		29	'Tanima-no-yuki'	NN155D	93.6	1.1	4.8	77.6
		30	'Hatsuyuki'	NN155D	93.4	1.4	4.2	71.8
		31	'Hakurakuten'	NN155D	92.6	1.7	5.1	71.1
	<Ryūkyū azalea group>	32	*R.*× *mucronatum* 'Shiro-ryūkyū'	NN155D	92.9	1.5	5.8	75.2

[z] Refer to the Royal Horticultural Society Colour Chart. [y] *L**, lightness; *a** and *b**, chromatic components.[x] *h,* hue angle (degree) = arctan (b*/a*).

2.2.3 HPLC analysis of anthocyanidin and flavonol

The procedures used for the pigment extraction was performed according to Mizuta et al. (2009) with minor modifications. Dried petals (ca. 50 mg) of each sample were extracted for 24 h at 4°C in the absence of light with 4 mL of 50% CH_3COOH in H_2O. The crude extracts were concentrated to small amounts and hydrolyzed with 4 ml of 2N hydrochloric acid at 100°C for 1 h. The hydrolysates were absorbed on a Sep-pak C_{18} cartridge. The cartridge was washed to eliminate the water soluble or hydrophilic contaminants then the anthocyanidins and flavonols were eluted by 50% CH_3COOH in H_2O.

The HPLC system used LC solution (Shimadzu Corp., Kyoto, Japan), an SPD-M20A UV-Vis photodiode array detector, a LC-20AD liquid chromatograph and a CTO-20A column oven with a Poroshell 120 SB-C18 (2.1 mm i.d. x 50 mm, Agilent Technologies, USA). The analytical condition of HPLC for investigating anthocyanidin composition was a ratio of 20% solvent A [MeOH] to 80% solvent B [HCOOH-H_2O (1:99, v/v)]. The samples were run for 40 minutes at 40°C with a flow rate of 0.5 ml/min and monitored at 530 nm. The same HPLC system was used for the existence analysis of flavonols and monitored at 360nm (Mizuta et al., 2014).

2.2.4 RNA extraction and cDNA synthesis

Total RNA was extracted from the petals using Hot-borate method (Wan and Wilkins, 1994). To avoid DNA contamination, DNA digestion was performed according to 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. The first strand of synthesized cDNA was used for gene expression

analysis.

2.2.5 Gene expression analysis by qRT-PCR

The investigation of gene expression involved in anthocyanin biosynthesis was carried out according to Mizuta et al. (2014) with minor modifications. The gene specific primers for *F3'H* (GenBank/EMBL/DDBJ Accession no: AB289597), *F3'5'H* (AB289598), *DFR* (AB289595), and *ANS* (AB289596), and *Histone H3* (AM932886) were used in this study (De Keyser et al., 2009; Nakatsuka et al., 2008).

Table 2.2 Specific primers used in this chapter

Primer	Accession no.	Forward primer	Reverse primer
F3'H	AB289597	AGGATTTGTGCTGGGATGAG	CCGTAGGCTTCATCCATGTT
F3'5'H	AB289598	GTCTTTCGGTCTTGCTTTGC	AGTTTCAGCCGTTGACCTA
DFR	AB289595	TGTTAGTGGTCGGTCCCTTC	CATCATGGGATGAGCAGATG
ANS	AB289596	CCCAAGAAGACCAAAACCAA	CGAGCACAAGTTGTTCAGGA
Histone H3	AM932886	GAAACTCCCATTCAGAGGCT	GCATGGATGGCACAGAGGTT

The cDNA was amplified using TB Green II (Takara Bio Inc.) with a Thermal Cycler Dice Real Time System. Amplification of histone cDNA was used as an internal control and performed under identical conditions to normalize the levels of cDNA. The thermal cycling conditions were 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and finally 30 s at 60°C. Three replications were done for each cDNA sample. Quantitation was performed by using the difference in the cycle threshold values between target genes and the reference genes (*Histone H3*) to calculate the relative amounts of the template presence (Cheon et al., 2017). The mean and standard error were calculated for the value of relative expression.

2.3 Results

2.3.1 Flower color color measurement analysis

The RHSCC numbers of *R. scabrum* 1 and 2 were 47D and 50B, both of which belonged to red group. *R. ripense* 1 and *R. macrosepalum* 1, their RHSCC were 75A and 76B, respectively; belonged to purple group. The RHSCC of *R. ripense* 2 and *R. macrosepalum* 2 were N80D and 84C, which belonged to purple-violet group. The RHSCC of 'Shiro-ryūkyū' and 'Ōmurasaki were NN155D and N74B, which belonged to white and red-purple group, respectively. The Hirado azalea cultivars had RHSCC in the range from 47C (red group) to 84C (purple-violet group), and from NN155C to NN155D (white). This shows that Hirado azalea cultivars had a wide range of flower colors (Fig. 2.1).

In the red group, the a^* and b^* values were in the range of 42.8 to 55.2 and 8.0 to 18.5, respectively. In the red-purple group, the a^* and b^* values were in the range of 43.6 to 57.7 and -18.0 to 1.3, respectively; while these values in the purple and purple-violet groups were in the range of 24.1 to 43.2 and -22.3 to -12.1, respectively. For white group, the a^* and b^* values varied from 1.1 to 1.7 and 4.2 to 6.5, respectively (Table 2.1). The chromatic components a^* and b^* of Hirado azalea cultivars showed that they were divergently distributed into four clusters according to their flower color group. Hirado cultivars' flower colors were more diverse than their related parents (Fig. 2.2). The hue angle (h^*) of red flower group varied from 14.1º to 19.4º, red-purple group varied from 340.7º to 1.3º. Purple and purple-violet flower group varied from 328.2º to 340.6º, and white flower group varied from 71.1º to 80.4º.

The lightness (L^* value) of red flower group was in the range of 47.1 to 67.9, whereas red-purple flower group ranged from 45.0 to 63.9. The L^* values of purple and purple-violet flower group varied from 55.1 to 68.6. In the white flower group, the L^* values varied from 92.4 to 93.6 (Table 2.1).

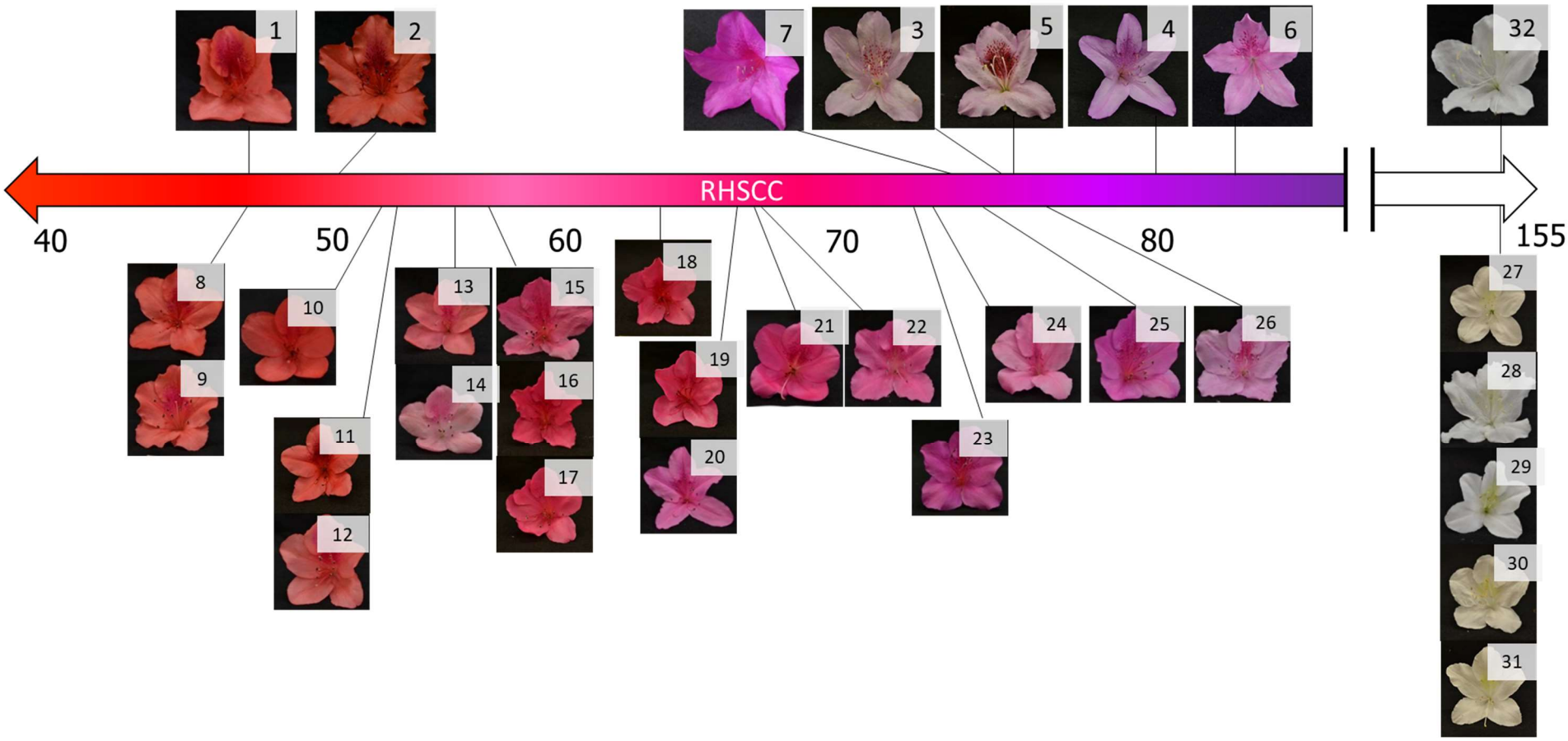

Fig. 2.1 The photograph of wild species and cultivars used in the study. The numbers and Royal Horticultural Color Chart (RHSCC) correspond to those mentioned in Table 2.1.

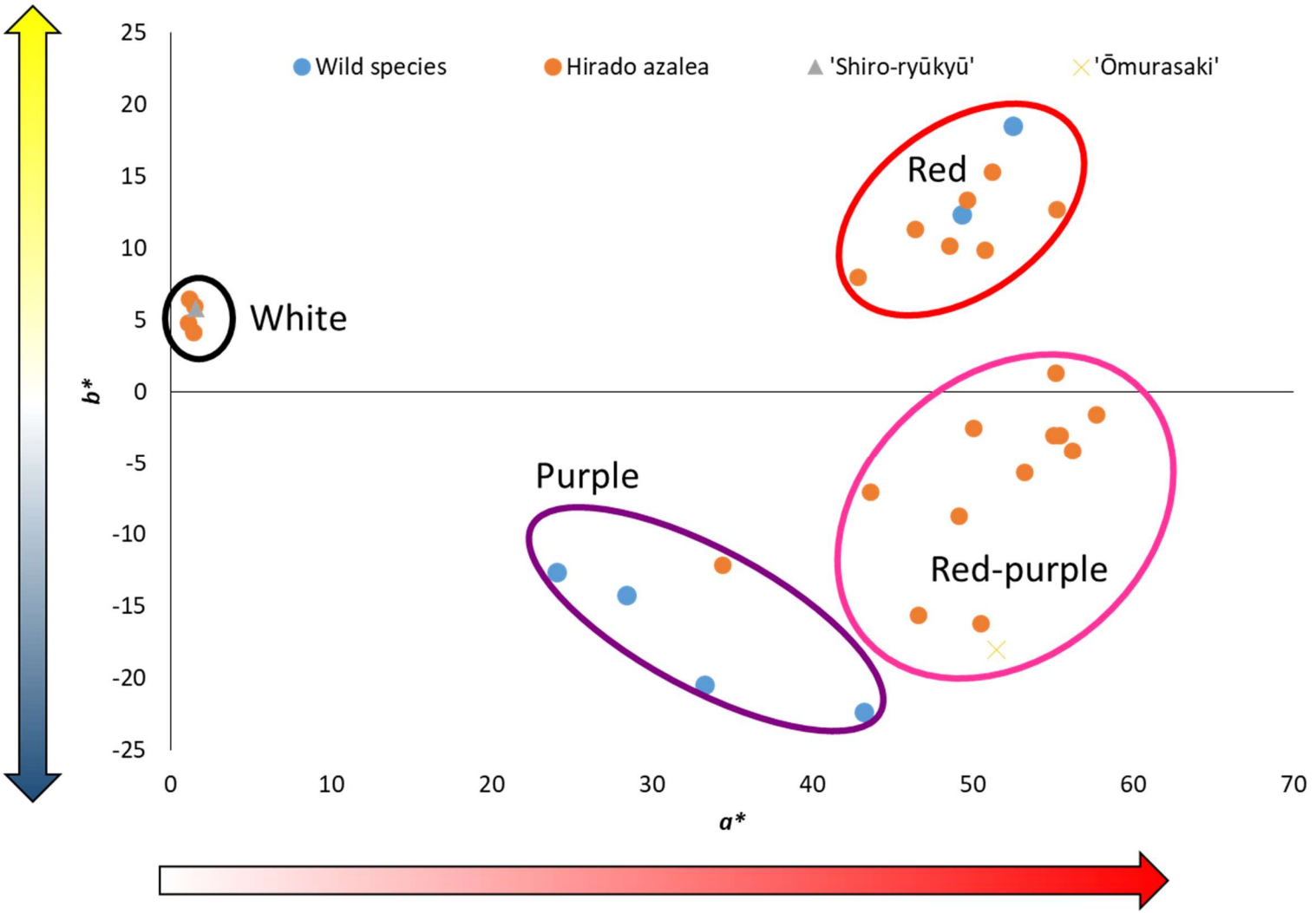

Fig. 2.2 Distribution of wild species and cultivars based on CIE*L*a*b** coordinates

2.3.2 Pigment composition analyses

The HPLC analysis showed that only cyanidin derivatives (Cy and Pn) were found in *R. scabrum* 1 and 2, while Cy-, Dp-derivatives (Dp, Pt, and Mv) and flavonol were found in wild species (*R. ripense* 1 and 2, *R. macrosepalum* 1 and 2) and 'Ōmurasaki' (Table 2.3). 'Shiro-ryūkyū' analyzed by HPLC showed the presence of small anthocyanidin peaks of both Cy and Dp derivatives, however, the anthocyanin content was absent.

Hirado azalea cultivars were divided into four groups according to their pigment compositions. The first group was composed of four red flowers 'Hinomoto', 'Raijin', 'Rashōmon' and 'Hiōgi' pigmented with Cy-derivatives and absence of flavonol. The second group was composed of three red flowered 'Kumo-no-ue', 'Heiwa-no-hikari', and 'Miyo-no-haru', and nine red-purple flowers pigmented with Cy-derivatives and flavonol (Table 2.3). The third group consisting of two red-purple flowers of 'Ademurasaki' and 'Taihō', and one purple flower of 'Hirado-no-homare' contained both Cy- and Dp-derivatives as well as flavonol. The fourth group was consisted of five white flowered Hirado azalea cultivars containing only flavonol.

2.3.3 Anthocyanin synthesis-related gene expression analyses

The four genes, *F3'H*, *F3'5'H*, *DFR*, and *ANS*, extracted from petals were investigated using qRT-PCR. F3′H, the synthesis gene for the Cy-derivative, was expressed in all samples. *F3'5'H*, the synthesis gene for the Dp-derivative, was expressed only in the samples containing Dp-derivatives except 'Wakakoma'. *DFR* and *ANS* were expressed in all the samples, similar to *F3'H* (Fig. 2.3). All white flowers also expressed all the four genes like colored flowers, noting that it has a potential to produce anthocyanidin pigments.

Table 2.3 Percentage of anthocyanidin compositions and presence of flavonol in wild evergreen azalea, and cultivars.

Flower color group	Sample no.	Materials	Anthocyanidin (%)[z]					Flavonol[y]
			Cy	Pn	Dp	Pt	Mv	
Red	1	*R. scabrum* 1	100	0	0	0	0	-
	2	*R. scabrum* 2	100	0	0	0	0	-
	8	'Hinomoto'	100	0	0	0	0	-
	9	'Raijin'	100	0	0	0	0	-
	10	'Rashōmon'	100	0	0	0	0	-
	11	'Hiōgi'	65.4	34.6	0	0	0	-
	12	'Kumo-no-ue'	100	0	0	0	0	+
	14	'Heiwa-no-hikari'	100	0	0	0	0	+
	13	'Miyō-no-haru'	72.7	27.3	0	0	0	+
Red-purple	15	'Shinshō'	100	0	0	0	0	+
	16	'Wakakoma'	100	0	0	0	0	+
	17	'Zanshō'	100	0	0	0	0	+
	18	'Hinode'	100	0	0	0	0	+
	19	'Shō-no-shin'	100	0	0	0	0	+
	22	'Banzairaku'	100	0	0	0	0	+
	24	'Momoyama'	100	0	0	0	0	+
	20	'Seibo'	46.4	53.6	0	0	0	+
	21	'Saotome'	61.6	38.4	0	0	0	+
	7	'Ōmurasaki'	24	21	11	4	40	+
	23	'Ademurasaki'	42.5	3.5	24.7	4.3	25.1	+
	25	'Taihō'	47.4	2.3	27.3	5.9	15.9	+
Purple	3	*R. ripense* 1	35	14	7	0	45	+
	5	*R. macrosepalum* 1	56.6	5.1	21.1	0	17.3	+
	26	'Hirado-no-homare'	39.5	0	60.5	0	0	+
Purple-violet	4	*R. ripense* 2	42.9	6.7	9.0	0	41.4	+
	6	*R. macrosepalum* 2	58.9	7.7	11.4	1.1	20.9	+
White[x]	27	'Hakuhō'	73.8	0	23.2	0	0	+
	28	'Shiro-kujyaku'	72.4	0	27.6	0	0	+
	29	'Tanima-no-yuki'	83.8	0	16.2	0	0	+
	30	'Hatsuyuki'	68.5	0	31.5	0	0	+
	31	'Hakurakuten'	44.7	0	55.3	0	0	+
	32	'Shiro-ryūkyū'	63.0	0	37.0	0	0	+

[z] Cy: Cyanidin, Pn: Peonidin, Dp: Delphinidin, Pt: Petunidin, Mv: Malvidin. [y] (-): absent, (+): present. [x] White flowers contained only little amount of anthocyanidins

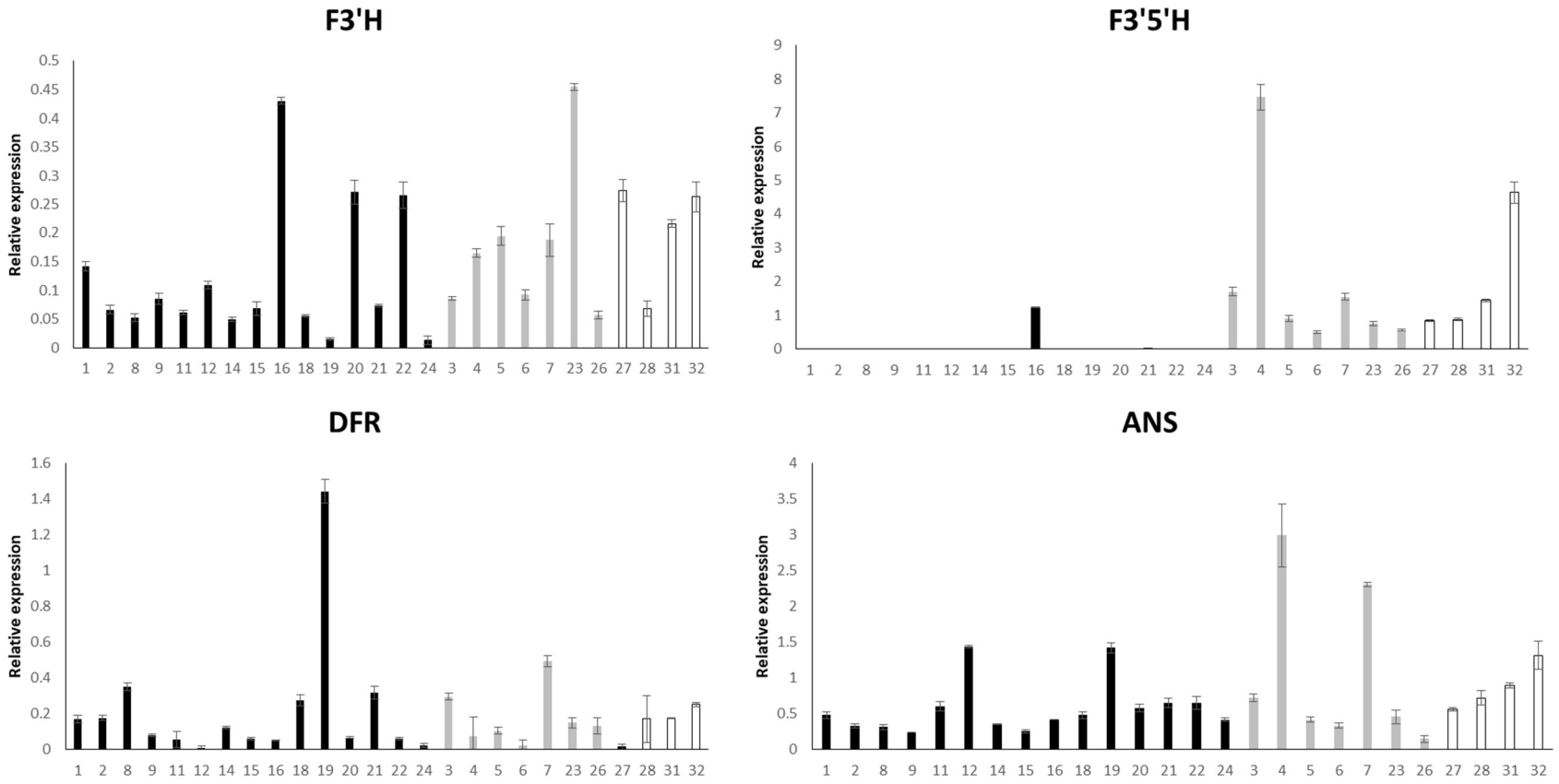

Fig. 2.3 Relative expression of flavonoid biosynthesis genes in the petals of wild species, and cultivars by qRT-PCR analysis. Sample numbers correspond to those in Table 1, 2 and Fig. 1 Quantitative RT-PCR amplification of histone was used to normalize the gene expression under identical conditions. Black and grey columns indicate absence and presence of Dp in flower petals, respectively. White columns indicate white flowers. The vertical bars represent SE of the means of three replication

2.4 Discussion

In order to understand pigment composition and gene expression, I investigated the relationships between Hirado azalea cultivars and their related wild species based on their pigment composition using HPLC and analyzed their gene expression using qRT-PCR. Earlier study had analyzed the pigment composition of *R. scabrum, R. ripense*, *R. macrosepalum*, and 'Shiro-ryūkyū' by HPLC (Mizuta et al., 2009). *R. scabrum* had large red flowers pigmented with Cy-derivatives; *R. ripense* and *R. macrosepalum* had large purple and purple-violet flower, all of them pigmented with Cy- and Dp- derivatives and flavonol (Fig. 2.2; Table 2.3). 'Shiro-ryūkyū' contained only flavonol. Moreover, recent research also confirmed that no anthocyanins detected in white flower of five wild species including *R. mucronatum* (Du et al., 2018).

Hirado cultivar groups were divided into four pigment groups: 1) Cy-derivatives only without flavonol, 2) Cy-derivatives and flavonol, 3) Cy- and Dp-derivatives and flavonol, and 4) flavonol only. The distribution of chromatic components *a** and *b** were diversely distributed. Moreover, the *b** distribution could be divided into two major groups; positive and negative *b** (Fig. 2.2; Table 2.1). The correlation of flower color and pigment were shown. The negative values of *b** indicates that flower shifted toward bluer tone, accordingly, flower with Cy-derivatives and flavonol, and flower with both Cy- and Dp-derivatives, and flavonol shifted toward negative plane.

The correlation of flower colors and pigment compositions between the Hirado azalea cultivars and their parents was studied in this work. The RHSCC of Hirado azalea cultivars varied from 47C to 52C and only Cy-derivatives contributed to their red flower colors (Fig. 2.2; Table 2.1 and 2.3). De Loose (1970b) had investigated the flower color and pigment compositions of natural bud-variants of *R. simsii*. The RHSCC of the cultivar 'Mme Petrick' was 57D with high

levels of both Cy-derivatives and flavonol, while that of its orange sports was 50B, with low levels of flavonol. This corresponds with our result in that Hirado azalea cultivars contained only Cy derivatives and the RHSCC was less than or equal to 52. This suggests that the pigments had the same effects on flower color in Hirado azalea cultivars as observed in small flowered *R. simsii*.

Another group of Hirado azalea cultivars contained Cy-derivatives and flavonol. Their RHSCC varied from 52C to 73B and had wider range of purplish red color (Fig. 2.2; Table 2.1 and 2.3). Moreover, co-pigmentation was observed in the red-purple group. The co-pigmentation between anthocyanin and flavonol is known to contribute to bluing effect, as previously reported in *R. simsii*(Asen et al., 1971, 1972; De Loose, 1970a; Huyen et al., 2016).

Hirado cultivar group pigmented with Cy- and Dp-derivatives and flavonol with RHSCC ranging from 72C to 77D had flowers with reddish purple and purple colors (Fig. 2.2; Table 2.1 and 2.3). They were more bluish than the second group of Hirado azalea cultivars that contained Cy-derivatives and flavonol. This may be due to the presence of Dp-derivatives, which contributed to the bluish coloration in various plants. Normally, *Rosa hybrida* contains Cy derivatives and flavonol; therefore, it lacks blue flower hues, such as purple and blue. In the genetically engineered rose cultivar 'Lavande' carrying *F3'5'H*, the production of Dp derivatives was triggered and resulted in bluer flower hues. The greater the percentage of Dp derivatives, the higher the bathochromic shifts observed towards bluer color (Katsumoto et al., 2007). Similarly, Hirado azalea cultivars that contained Dp derivatives showed a bathochromic shift towards a bluer tone to a greater extent than Hirado azalea cultivars containing only Cy derivatives and flavonol (Fig. 4). The last group was Hirado azalea cultivars containing only flavonol without any anthocyanin. They are white flowered Hirado azalea cultivars with RHSCC range of NN155C to NN155D, which were similar to 'Shiro-ryūkyū'.

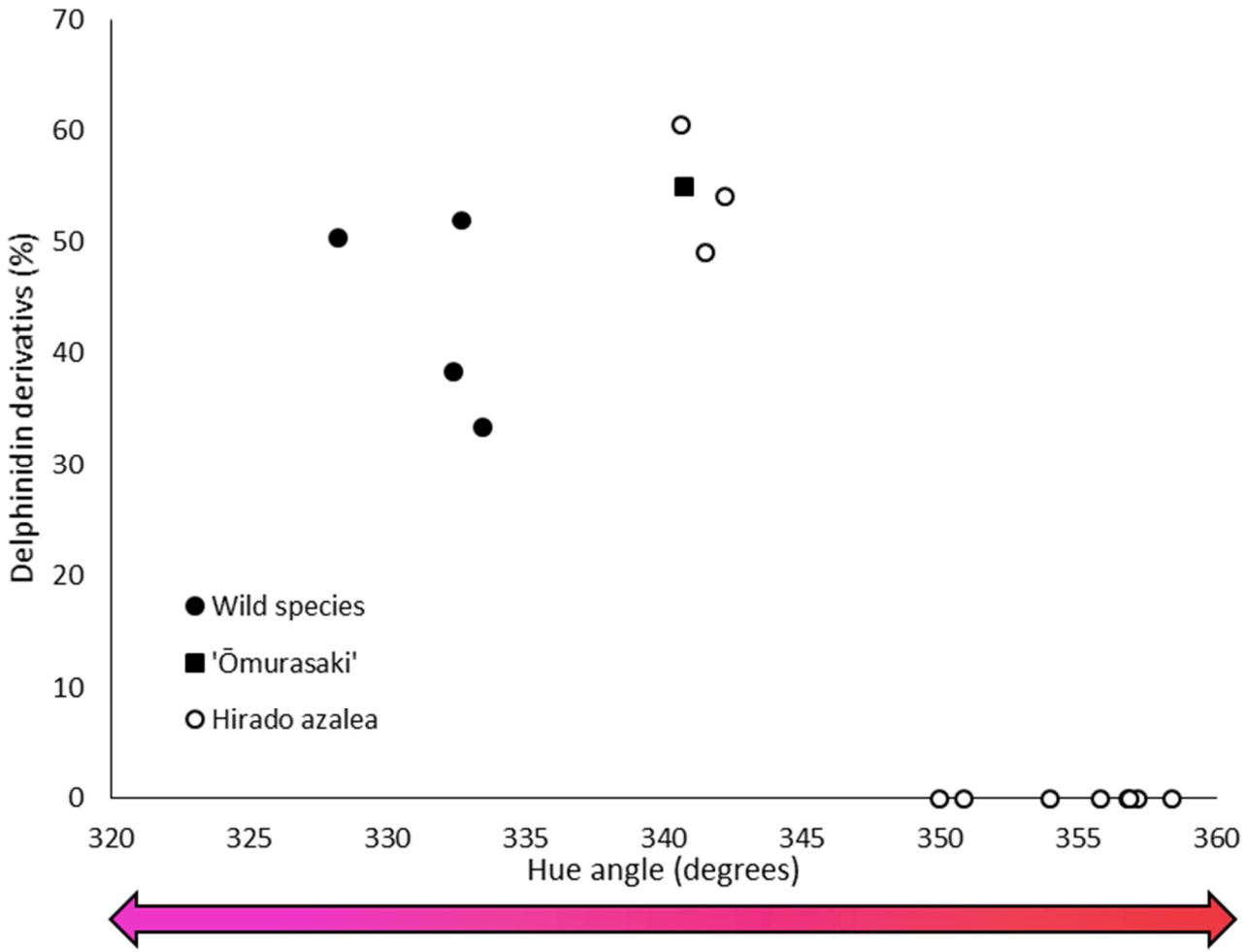

Fig. 2.4 The distribution of wild species and cultivars based on hue angle and percentage of delphinidin derivatives.

The gene expression of four genes was investigated. All samples were shown to express *F3'H, DFR*, and *ANS*, while *F3'5'H* was expressed only in those containing Dp derivatives except 'Wakakoma'. Similar occurrence was found in red sports of 'Ōmurasaki'. The *F3'5'H* was expressed in both red sports of 'Ōmurasaki' and 'Wakakoma' despite the absence of Dp-derivatives. Further investigation of the *F3'5'H* is required to identify the absence of Dp-derivative in 'Wakakoma'. In addition, the level of flavonoid related gene expression do not clarify the difference between the color intensity as shown in Fig. 2.1 and 2.3. Similarly, the related gene expression level in red pears also exhibited the same phenomenal. Red pair cultivars -'Red Zaosu', 'Red Sichou', 'Palacer', and 'Starkinson' had high concentration of anthocyanin especially 'Starkinson'. However, the gene expression level of *F3H*, *DFR*, and *ANS* of 'Starkinson' is lower than the other cultivars despite having the highest anthocyanin concentration (Wu et al., 2019)

Moreover, 'Shiro-ryūkyū' normally expressed *F3'H, F3'5'H, DFR*, and *ANS* like the colored flower despite the absence of anthocyanin (data not shown). White flowered Hirado azalea cultivars also expressed all four genes like 'Shiro-ryūkyū'. Mizuta et al. (2009) also reported that the anthocyanins were not detected but the anthocyanidins were detected in white flower *R. ripense* and 'Shiro-ryūkyū'. Hence, 'Shiro-ryūkyū' and white flowered Hirado azalea cultivar 'Hakuhō', 'Shiro-kujyaku', 'Tanima-no-yuki' and 'Hatsuyuki' are able to synthesize the anthocyanin precursor due to the peaks were detected in the anthocyanidin analysis. The absence of anthocyanin may result from malfunction of downstream step of anthocyanin biosynthesis hence the anthocyanidins revert back to proanthocyanidins, colorless pigments (Bogs, et. al, 2005).

Suzuki et al., (2015) reported the mutations found in regulatory gene and anthocyanin biosynthesis gene in *Lillim speciosum*. They investigated two regulatory genes and eight anthocyanin biosynthesis related genes of two *L. speciosum* phenotypes; white tepals with dark

red anthers and white tepals with yellow anthers. The whites tepal with dark red anthers did not express *F3H, F3'H, DFR*, and *ANS*, and showed lower expression of *MYB12* and *bHLH2.* The cDNA sequence analysis of *MYB12* showed that one amino acid substitution in *R2R3MYB* caused white tepals with dark red anthers phenotype to reduce the anthocyanin production. Whereas white tepals with yellow anthers expressed all genes normally except *DFR*. The *DFR* in white tepals with yellow anthers had a nonsense mutation or stop codon at the middle of its sequence resulting in malfunction, hence, no anthocyanin accumulation.

Similarly, 'Shiro-ryūkyū' has white petals with green blotches and other white flowered Hirado azaleas either have green blotches or yellow blotches. This suggests that the mutations in anthocyanin pathway genes may be related to the white color in these plants as shown in no-anthocyanin pigmented lily mutant (Suzuki et al., 2015). However, the blotches of white flowered Hirado azaleas and 'Shiro-ryūkyū' were not pigmented with anthocyanin. The gene expression analysis of white flowered evergreen azalea expressed anthocyanin biosynthesis related genes normally for *F3'H, F3'5'H, DFR,* and *ANS* (Fig. 2.3). In this study, only four genes were studied for gene expression analysis, therefore, the cause of no anthocyanin accumulated in the petals are unclear. The gene expression analysis of the upstream and downstream structural genes should be investigated to confirm whether the mutation occurs at which part. Also, it has been known that white is homozygote recessive in *R.* × *mucronatum* (Heursel and Horn, 1977). I assumed that old white flower cultivar such as *F3'5'H* expressed 'Shiro-ryūkyū' but *F3'5'H* non-expressed *R. scabrum* was used for development of white Hirado cultivars.

Delphinium flowers have white, red, and blue flower which contain flavonol, pelagornidin (Pg), and Dp, respectively. However, it does not contain any Cy which is synthesized by flavonoid 3' hydroxylase (*F3'H*). *Delphinium zalil* had white flower and contained only flavonol. However,

it had a functional *F3'H* and lacked of ANS which resulted in absence of anthocyanins. *Delphinium cardinal* has red flower pigmented with Pg and lack of *F3'H*, hence, it did not contain any Cy. The hybridization of these two resulted in purple flowered progenies which had hydroxylation ability, therefore, it could synthesize Cy. This suggests that *D. zalil* has a functional *F3'H* which can be passed down to its progenies (Sakaguchi et al., 2019).

Similarly, 'Shiro-ryūkyū' was able to synthesize Dp-derivatives. This suggests that the hydroxylation ability of 'Shiro-ryūkyū' may be passed down to progenies like delphinium. Red flowered Hirado azalea cultivars exhibited similar patterns like *R. scabrum*, as they contained only Cy-derivatives and showed no expression of *F3'5'H*. On the hand, non-red colored flowers of Hirado cultivars also contained either flavonol or both Dp-derivatives and flavonol. This indicates that the cross combination of *R. scabrum* with other wild species or cultivars may have contributed to wider flower color variation in Hirado azalea cultivars.

2.5 Conclusion

The Hirado azalea is a large flowered in which *Rhododendron scabrum*, *R. ripense*, *R.* × *mucronatum*, and other related cultivars are considered to be its parents. This study investigated the correlation of the Hirado azalea cultivars with the wild species and old cultivars by analyzing anthocyanidin composition patterns and the expression of anthocyanin biosynthesis genes. Hirado azalea cultivars were divided into four groups according to their pigment compositions.

Hirado azalea cultivars with only Cy derivatives had red colored flowers similar to those of *R. scabrum*. Hirado azalea cultivars with both Cy and Dp derivatives as well as flavonol exhibited similar flower colors to those of *R. ripense* and *R. macrosepalum*. Hirado azalea

cultivars with only flavonol had white colored flowers similar to those of *R. mucronatum* 'Shiro-ryūkyū'. Hirado azalea cultivars with Cy derivatives and flavonol exhibited wider flower colors from their parents.

All samples expressed *F3'H, DFR*, and *ANS* genes, as determined by qRT-PCR. However, the *F3'5'H* gene was expressed only in samples containing Dp derivatives. Moreover, 'Shiro-ryūkyū' also expressed all four genes, as did cultivars with colored flowers; despite its flowers is white. These results suggested that the hybridization of Hirado azalea using *R. scabrum* as the base may produce wide range of flower colors besides red owing to the presence of the *F3'5'H* gene from *R. ripense, R. macrosepalum*, 'Shiro-ryūkyū'.

Chapter 3

The genetic relationship of Hirado azalea cultivars and their putative parents based on flavonoid 3′, 5′ hydroxylase gene

3.1 Introduction

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

3.2 Experimental procedures

3.2.1 Plant materials

Cutting clones of Hirado azalea cultivars were obtained from genuine collections in Hirado city, Nagasaki Prefecture. Evergreen azaleas, including 27 individuals of 4 wild species, and 19

cultivars from the subgenus *Tsutsusi* were obtained from the experimental field of Shimane University, Japan (Table 3.1; Chapter 2). For genomic DNA extraction, 70 mg of young leaves were sampled from each plant. Closed flower buds were collected for RNA extraction and cDNA sequencing. Each sample was immediately frozen in liquid nitrogen and stored at −80 °C until extraction for genomic DNA and total RNA.

3.2.2 DNA extraction and DNA analysis

DNA amplification was carried out for all samples to assess 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 includes UTR regions in *R.* x *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'-CACATCTAAGGCAAAACCAC-3' and P2: 5'-AGAGCTGCAAGAGGCACA-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'-AGCAATGTATGTTGCTATC-3' and reverse 5'-TGATCGAGTTGTAGGTAGT-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, I attempted to amplify exon region 1 with P3 (5'-CTAAAAATGGGCACCCTTGA-3') and P4 (5'-CCAAAAGGAGTGCTTTAATGTT-3') and exon region 2 with P5 (5'-TGAACGGCTACTACATACCCAAGAAC-3') and P6 (5'-AGTTTCAGCCGTTGAGCCTA-3') (Fig. 3.2A; FASMAC). The PCR mixture (10 μL) contained 1× Extaq 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.

3.2.3 RNA extraction and cDNA analysis

RNA extraction and cDNA synthesis were conducted by the methods of Chapter 2.2.4 for an analysis of the length of coding sequences and untranslated regions, and for deduced amino acid sequence analysis.

Table 3.1 The samples used for sequencing and genotyping in this Chapter.

		Anthocyanin composition [y]		
		Dp derivatives (-)	Dp derivatives (+)	
Wild species	Ser. *Scabra*		*R. ripense* 1, 2 *R. macrosepalum* 1, 2 *R. yedoense* var. *poukhanese* 1	PCR amplification and gene cloning
Cultivars	Ryūkyū azalea group		*R.*× *mucronatum* 'Shiro-ryūkyū'	
	Hirado azalea group		'Ademurasaki' 'Hirado-no-homare' 'Hakuhō' 'Shiro-kujyaku'	
Wild species [z]	Ser. *Scabra*	*R. scabrum* 1–8	*R. ripense* 3–13 *R. macrosepalum* 3, 4, 5	PCR amplification
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 in the Chapter 2.

3.2.4 Isolation of *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 + P2; FASMAC). The PCR mixture (10 μL) contained 1X Ex-taq buffer, 200 μM dNTPs, 0.2 μM of each primer, 0.25 μL 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, Madison, MI, 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., 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 a neighbor-joining (NJ) and bootstrap analysis (1000 replicates).

3.2.5 Detection of F3′5′H genotype in wild species and cultivars

As I found polymorphism in the azalea F3′5′H gene, I attempted used PCR to describe the variants of this gene. To confirm the presence of a 5 bp insertion at Exon 2 of the Rp*F3′5′H2* gene (accession no. AB488484), samples from *R. ripense*, *R. macrosepalum,* and Hirado azalea were

analyzed by PCR using a primer set of *F3′5′H2* specific primer set (P7: 5'-CCAAGTGCTTATGCTATGTAGGC-3' and P8: 5'-GAAACGACGGGAACATTCA-3'; FASMAC). The PCR mixture (10 μL) contained 1X 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% gel.

3.3 Results

3.3.1 Amplification of *F3′5′H* gene in genomic DNA

DNA amplification to assess the presence of the *F3′5′H* gene among wild species and cultivars detected the full-length gene (approximately 4 kb; Fig. 3.1A) in genomic DNA of the taxa with Dp derivatives, i.e. *R. ripense*, *R. macrosepalum*, and *R. yedoense v*ar. *poukhanense* (Table 3.1; Fig. 3.1B). 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’, ‘Hatsuyuki’ and ‘Shiro-kujyaku’) 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. 3.1B). 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’, ‘Kumono-ue’, and ‘Heiwa-no-hikari’), and six red-purple flowered Hirado azalea cultivars (‘Shinshō’, ‘Hinode’, ‘Banzairaku’, ‘Momoyama’, ‘Seibo’, and ‘Saotome’) (Fig.

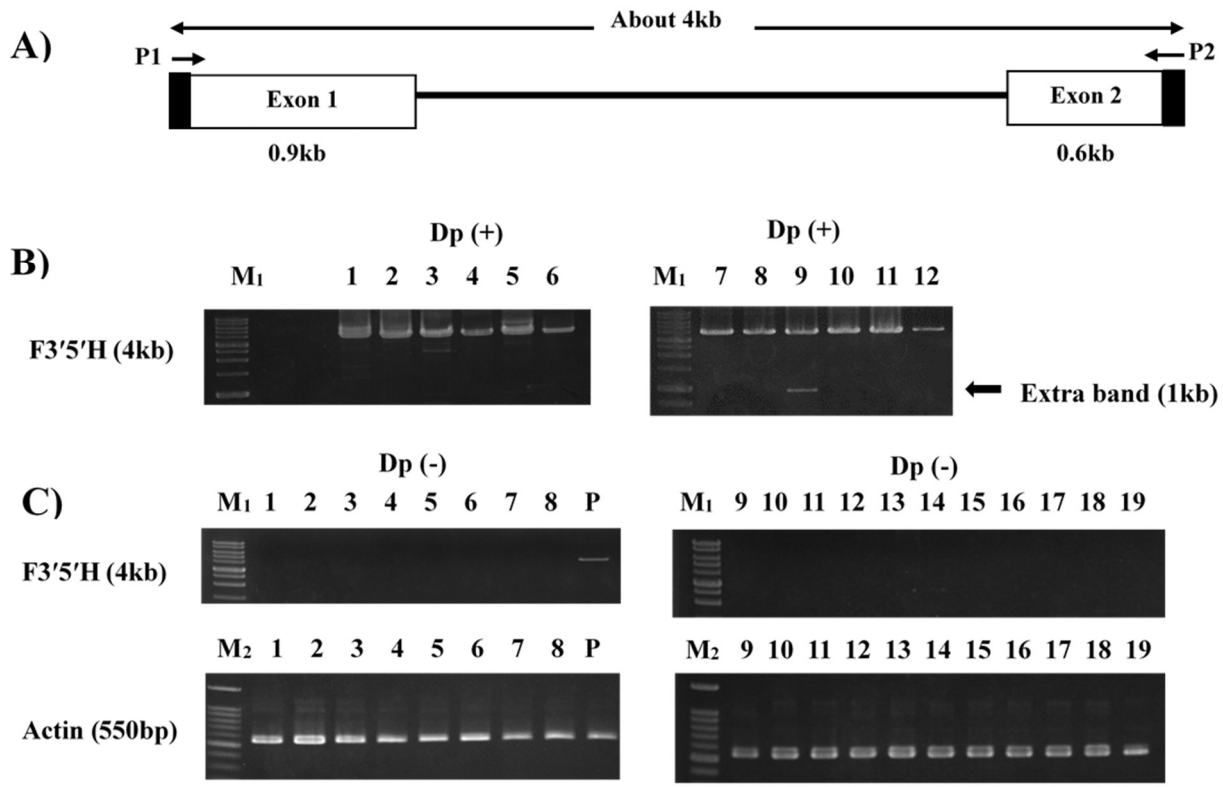

Fig. 3.1 gDNA analyses of the F3′5′H gene. (A) Diagram of the F3′5′H gene, indicating the primers used for each amplification. (B) and (C) Amplification to assess the presence of the F3′5′H gene among wild species and cultivars. Dp: Dp derivative production; M_1: 1kb DNA marker (DM3100; SMO Bio) and M_2: 100bp 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', 'Shinsho', 'Hinode', 'Banzairaku', 'Momoyama', 'Seibo', and 'Saotome'; P: positive control ('Ōmurasaki').

3.1C). These 19 DNA templates of samples without Dp could normally amplify the actin gene (approximately 550 bp) as a positive control gene (Fig. 3.1C). Furthermore, we investigated the CDS using specific primers for each exon in eight *R. scabrum* individuals (Fig. 3.2A). Exon 2 of the *F3′5′H* gene was amplified for all these plants, but exon 1 failed to amplify (Fig. 3.2B, C). Amplification of gDNA from the five red-flowered and six red-purple flowered Hirado azalea cultivars also failed to amplify exon 1 (Fig.3.2B), but did amplify exon 2, for which the band size was approximately 400 bp (Fig.3.2C).

3.3.2 *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 azalea cultivars; '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. After cloning the amplified PCR products from wild species, I 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; Support data S2.1-2.6).

The full-length sequences of the F3′5′H homologue genes contained 27 bp of 5′ untranslated regions (UTRs), 1533 bp of coding sequence (CDS), and 121 or 135 bp of 3′ UTRs in *R. ripense* and *R. macrosepalum*, respectively (Table 3.2). 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

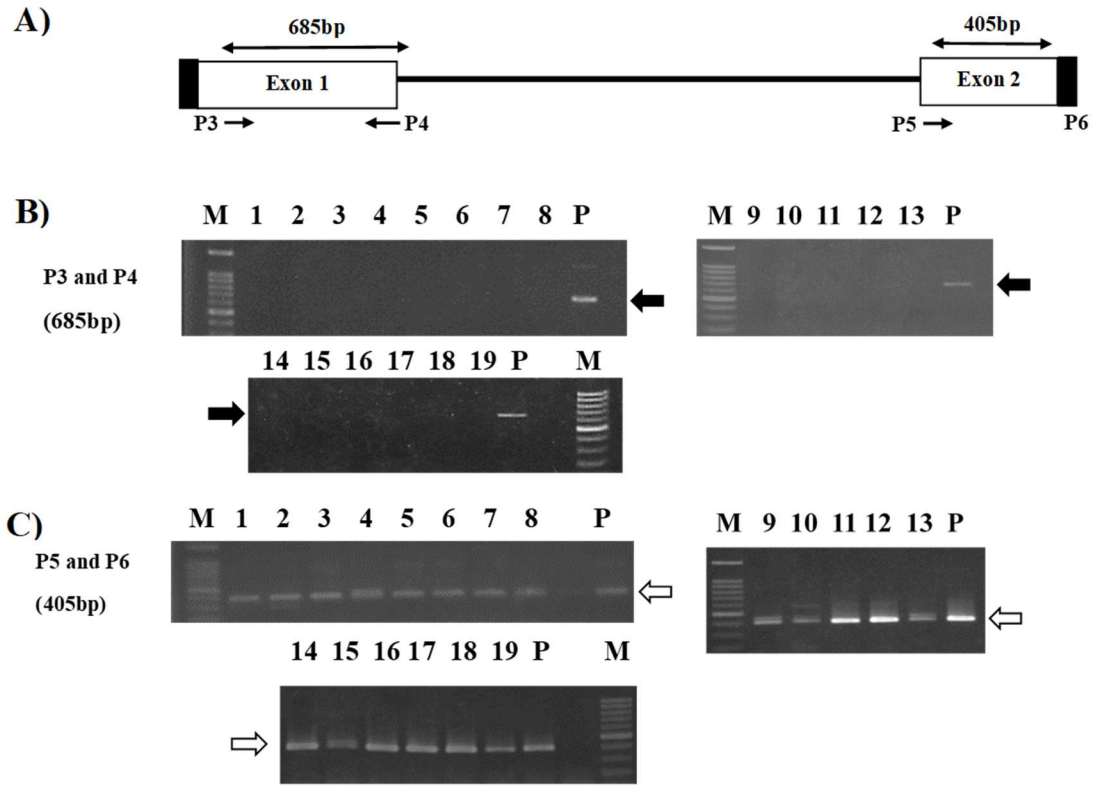

Fig. 3.2 gDNA analyses of F3′5′H gene exon 1 and exon 2. (A) Diagram of F3′5′H gene, indicating the primers used for each amplification. (B) Amplification of F3′5′H exon 1. (C) Amplification of F3′5′H exon 2. For (B) and (C) M: 100bp DNA marker (DM2100; SMO Bio); lanes 1-8: eight individuals of *R. scabrum*; lanes 9-19: Hirado azalea cultivars ‘Hinomoto’, ‘Raijin’, ‘Hiōgi’, ‘Kumo-no-ue’, ‘Heiwa-no-hikari’, ‘Shinsho’, ‘Hinode’, ‘Banzairaku’, ‘Momoyama’, ‘Seibo’, and ‘Saotome’; P: positive control (‘Ōmurasaki’). Black and white arrows indicate 685bp and 405bp, respectively.

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 3.2). The *F3′5′H* gene had a length of 510 amino acid residues in the three wild 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. 3.4C; Table 3.2). The *F3′5′H* gene in Hirado azalea cultivars also displays two different CDS lengths: 1533 bp from 'Hirado-no-homare' and 'Shiro-kujyaku' (DDBJ accession no. LC547913-LC547914) compared to 1551 bp from 'Ademurasaki' and 'Hakuhō', corresponding to 510 and 516 amino acid residues, respectively (Fig. 3.4C; Table 3.2). The *F3′5′H* genes in 'Ademurasaki' and 'Hakuhō' are identical to that of 'Ōmurasaki'. Moreover, Hirado-no-homare' and 'Hakuhō' have different nucleotide sequence, which is shorter as a result of deletion.

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

The *F3′5′H* cDNA of wild species, *R.* × *mucronatum* 'Shiro-ryūkyū', Hirado azalea cultivars were chosen for phylogenetic analysis due to the similarities of 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 azalea,

Table 3.2 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
R. ripense 1		RrF3′5′H1a	27	1533	121	LC547905
R. ripense 2		RrF3′5′H1b	27	1533	135	LC547906
R. macrospalum 1		RmF3′5′H1a	27	1533	121	LC547907
R. macrospalum 2		RmF3′5′H1b1	27	1533	135	LC547908
		RmF3′5′H1b2	27	1533	121	LC547909
R. yedoense var. *poukhanense*		RyF3′5′H1	27	1533	135	LC547910
R.× *mucronatum* '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
	'Hakuhō'	RpF3′5′H2	27	1551	108	In this study
Ōkirishima group	'Ōmurasaki'	RpF3′5′H2				AB488484 (Mizuta et al. , 2010)

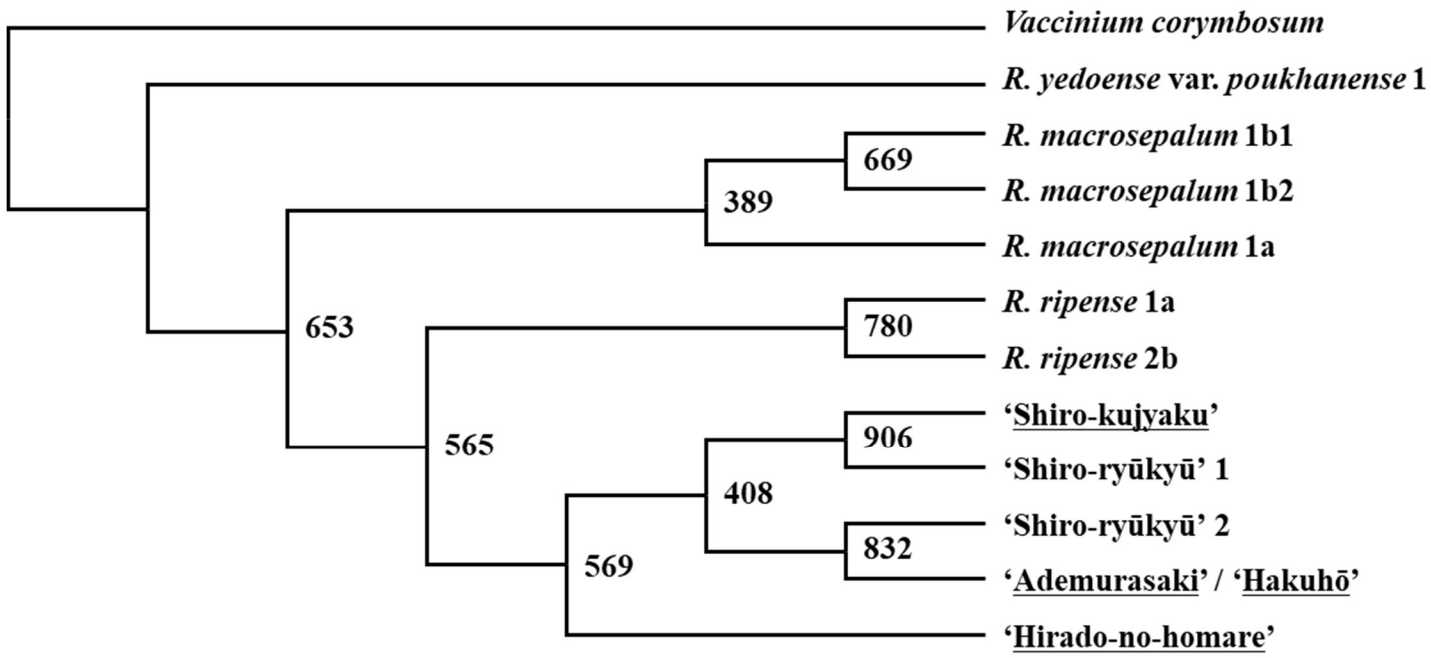

Fig. 3.3 Neighbor-joining tree of F3′5′H nucleotide sequences among wild species, *R.* × *mucronatum* 'Shiro-ryūkyū', and four Hirado azalea cultivars (underlined). The numbers at the nodes are bootstrap values. *Vaccinium corymbosum* was set an outgroup.

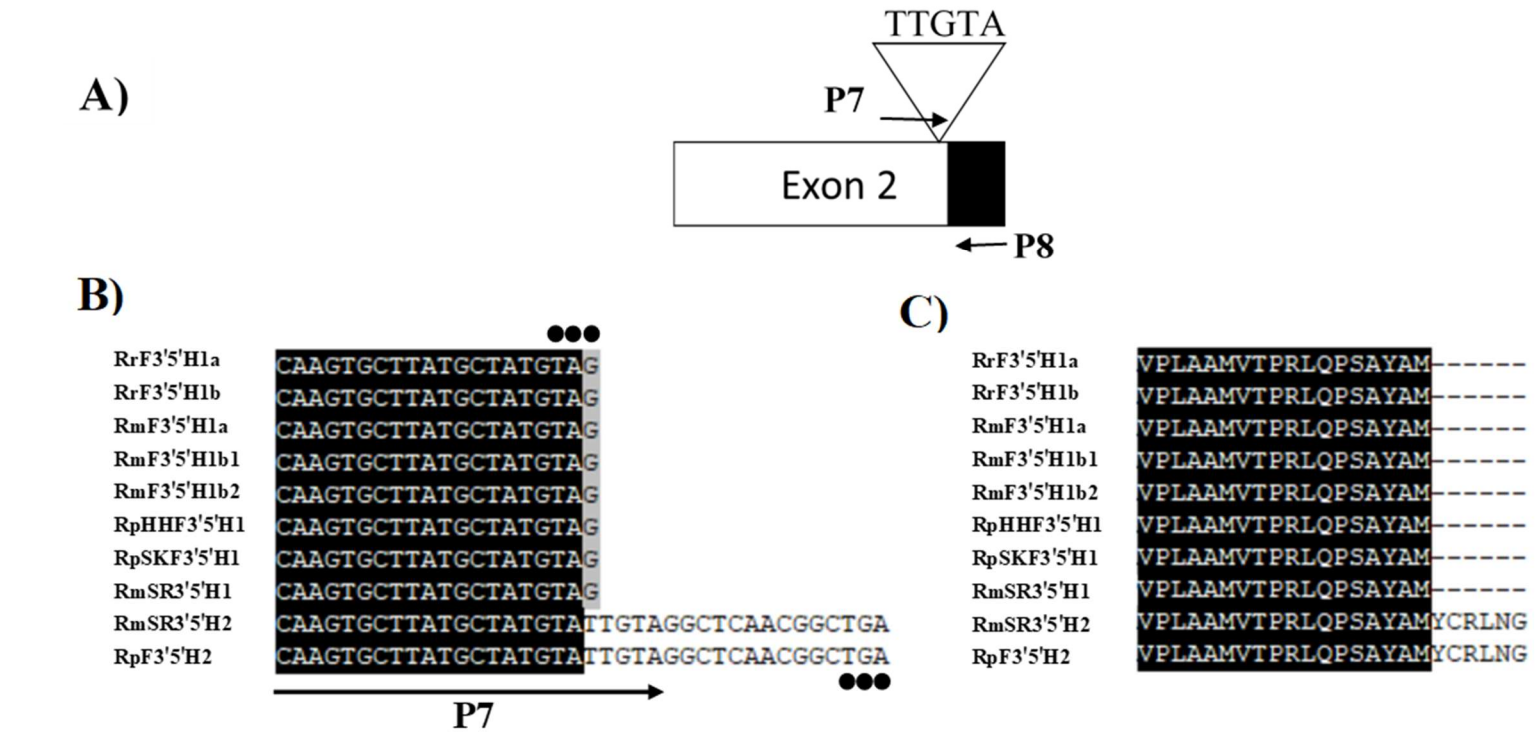

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Fig. 3.4 cDNA sequence analysis. (A) Diagram showing the 5 bp insertion in exon 2 of the *F3′5′H* gene. Nucleotide sequence (B) and translated amino acid (C) alignments of exon 2 region of the F3′5′H near the insertion. Dots indicate the stop codon (TAG and TGA).

3.3.4 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. 3.4). 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 and the cultivars 'Taiho', 'Hirado-no-homare', and 'Shiro-kujyaku', suggesting that these individuals did not have this insertion (Table 3.3). 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', 'Hakuho' and 'Hatsuyuki', suggesting that the 5 bp insertion is present in these cultivars (Table 3.3).

3.4 Discussion

Although recent genetic studies have found that Hirado azalea is closely related to the red-flowered *R. scabrum* (Scariot et al., 2007a, b), this does not explain the wide flower color variation in this cultivar group. The 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; Chapter 2). 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*

Table 3.3 PCR analysis of the *F3'5'H* Exon 2 insertion in *R. scabrum*, *R. ripense, R. macrosepalum* and Hirado azalea cultivars with delphinidin derivatives.

		No insertion	5bp insertion
Wild species[z]		*R. scabrum* (8) *R. ripense* (8) *R. macrosepalum* (5)	*R. ripense* (5)
Cultivars	Ryūkyū azalea group		*R.*× *mucronatum* 'Shiro-ryūkyū'
	Hirado azalea group	'Hirado-no-homare' 'Shiro-kujyaku 'Taihō'	'Ademurasaki' 'Hakuhō' 'Hatsuyuki'
	Ōkirishima group		'Ōmurasaki'

[z] The parentheses show individual numbers of wild species.

nucleotide, similar to *R. ripense* or *R.* × *mucronatum* 'Shiro-ryūkyū' (Fig. 3.1). Eight plants of *R. scabrum* lacked the exon 1 region after PCR using P3 and P4 primers (Fig. 3.2B). Similarly, Exon 1 of in red- and red-purple flowered Hirado azalea lacking Dp-derivatives, was not amplified but exon 2 was (Fig. 3.2B, 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'. 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, the upstream function should be investigated including promotor

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' might 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* and *R. ripense* are closely related to Hirado azalea (Fig. 3.3). 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 '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 'Ōmurasaki'. In combination, our results and those of previous studies suggest that purple-flowered Hirado azaleas '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. 3.3). 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 *MYB12* gene that regulates anthocyanin accumulation in tepals. In lilies, the 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, I found that nucleotide sequence alignments of the *F3'5'H* gene of wild species and cultivars are different, resulting in the *F3'5'H* gene polymorphisms. A 5 bp insertion in the *F3'5'H* gene is found in some cultivars. Therefore, I 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 for all *R. scabrum* and *R. macrosepalum*, whereas it was present for some *R. ripense* plants and *R.* × *mucronatum* 'Shiro-

ryūkyū' (Table 3.3). This suggests that the wide range of flower color in Hirado azalea may be partially due to the introduction of the 5 bp insertion in the *F3'5'H* gene from *R. ripense* and *R.* × *mucronatum* 'Shiro-ryūkyū' to *R. scabrum*.

In this study, I investigated gDNA and cDNA sequences of the *F3'5'H* gene in wild species and cultivars. The results suggest that Hirado azaleas lacking Dp derivatives have an *F3'5'H* gene derived from *R. scabrum*, whereas Hirado azalea with Dp derivatives, which show 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*—I are investigating the *F3'H* gene in additional species and cultivars because the putative parents only make a limited genetic contribution of *R. scabrum* to Hirado azalea with Dp derivatives.

3.5 Conclusion

The putative parents of the Hirado azalea are *R. scabrum*, *R. ripense*, *R.* × *mucronatum*, and other related cultivars. Hirado azalea cultivars show a wide range of flower color variation, but the genetic basis of this color variation is not well understood. In this study, I investigated the *F3'5'H* gene by genomic DNA analysis, cDNA sequence analysis, and deduction of amino acid sequences to assess the genetic relationships between these taxa and to investigate the genetic basis of color variation in this group. In *R. scabrum*, in which Dp derivatives are absent, I found that the *F3'5'H* gene lacks the Exon 1 region. Red and pink flowered Hirado azalea cultivars lacking Dp derivatives showed DNA structure similar to *R. scabrum*, suggesting that *R. scabrum* may be closely related to Hirado azalea cultivars without Dp derivatives.

The lengths of the *F3'5'H* cDNA nucleotide sequences of *R. ripense, R. macrosepalum*, and *R. yedoense* var. *poukhanense were* 1533 bp, whereas in *R.* × *mucronatum* 'Shiro-ryūkyū,' two different sequence lengths were observed—1533 and 1551 bp. *R. ripense, R.* × *mucronatum* 'Shiro-ryūkyū', and four Hirado azalea cultivars were grouped in the same cluster in the F3′5′H gene phylogeny. Among the four Hirado azalea cultivars, 'Ademurasaki' and 'Hakuhō' had a 5 bp insertion in the *F3'5'H* ORF region adjacent to the stop codon whereas 'Hirado-no-homare' and 'Shirokujyaku' lacked this insertion. This 5 bp insertion was found in some *R. ripense* individuals but not in *R. scabrum* and *R. macrosepalum*. These results suggest that the wide range of flower color in Hirado azalea cultivars may be caused by variation in the *F3'5'H* gene derived from hybridization between *R. scabrum* and either *R. ripense or R.* × *mucronatum* 'Shiro-ryūkyū.'

Chapter 4

General Discussion

4.1 Introduction

In this chapter, the main findings in this research are summarized and general conclusion based on the findings of the studies presented are described. Furthermore, the strengths and limitations of this research are considered and suggested for further investigation are presented.

4.2 Links between hypotheses and research

Evergreen azalea has various species, however, mostly cultivars were bred in Japan through natural hybridization and selective breeding (Kobayashi et al., 1995, 2000, 2013). Hirado azalea is one of the well-known cultivars with large flower. Hirado azalea was bred in

Nagasaki Prefecture, Japan. During Edo period, Nagasaki was known as a port city where international trade occurred. Many plants had been traded from abroad or within Japan. Hirado azalea was naturally bred in the garden of Samurai residences, hence, the putative parents were not identified.

Tamura (1962, 1963) suggested that large flowering wild species such as *R. scabrum, R. ripense*, or *R.* × *pulchrum* 'Ōmurasaki' were putative parents of Hirado azalea. This was due to their similarities in morphologies. Nevertheless, the research was mainly observed their morphologies and did not investigate their pigment compositions nor flavonoid biosynthesis related genes. The putative parents of Hirado azalea was hypothesized based on previous research and to determine the relationship among them. Morphological marker may not suffice to clearly distinguish their putative parents. According to Scariot et al. (2007 a,b), they investigated the genetic relationship among evergreen azalea using DNA markers including amplified amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and expressed sequence tag (EST) markers, indicate that Hirado azalea is most closely related to *R. scabrum*, and cluster with *R.* × *mucronatum*, *R. ripense*, and *R*. *macrosepalum* Maxim

However, Nakao and Tamura (1962, 1963, and 1970) does not focus on the flavonoid biosynthesis related gene which is the main feature for developing a new novelty cultivar. In this study, I hypothesized that the *F3'5'H* gene may be used as a molecular marker to identify the origin of the *F3'5'H* gene in Hirado azalea. In 2010, Mizuta et al. reported that the Dp derivative is responsible for color diversity in evergreen azalea. Therefore, this research focused on pigment compositions analyses and flavonoid biosynthesis related gene analyses to identify the relationship among Hirado azalea and their putative parents. To develop a breeding program for large flowering cultivars, this study speculates the evidence of putative parents of Hirado azalea.

4.3 Findings regarding to the research

This study investigated the correlation of the Hirado azalea cultivars with the wild species and old cultivars by analyzing anthocyanidin composition patterns and the expression of anthocyanin biosynthesis genes. Hirado azalea cultivars were divided into four groups according to their pigment compositions. Hirado azalea cultivars with only Cy derivatives had red colored flowers similar to those of *R. scabrum*. Hirado azalea cultivars with both Cy and Dp derivatives as well as flavonol exhibited similar flower colors to those of *R. ripense* and *R. macrosepalum*. Hirado azalea with only flavonol had white colored flowers similar to those of *R.* × *mucronatum* 'Shiro-ryūkyū'. Hirado azalea cultivars with cyanidin derivatives and flavonol exhibited wider flower colors from their parents.

All samples expressed *F3'H, DFR*, and *ANS* genes, as determined by qRT-PCR. However, the *F3'5'H* gene was expressed only in samples containing Dp derivatives. Moreover, *R.* × *mucronatum* 'Shiro-ryūkyū' also expressed all four genes, as did cultivars with colored flowers; despite its flowers is white. These results suggested that the hybridization of Hirado azalea using *R. scabrum* as the base may produce wide range of flower colors besides red owing to the presence of the *F3'5'H* gene from *R. ripense, R. macrosepalum*, *R.* × *mucronatum* 'Shiro-ryūkyū'.

Secondly, this study investigated the genetic relationship among Hirado azalea cultivars and their putative parents via sequence analysis. Hirado azalea cultivars show a wide range of flower color variation, but the genetic basis of this color variation is not well understood. This study investigated the anthocyanin pathway gene; *F3'5'H*, by genomic DNA analysis, cDNA sequence analysis, and deduction of amino acid sequences to assess the genetic relationships between these taxa and to investigate the genetic basis of color variation in this group. In *R.*

scabrum and red and pink flowered Hirado azalea cultivars, in which Dp derivatives are absent, only the Exon 2 region amplified using specific primers in coding region (CDS) of *F3′5′H* gene, except Exon 1, suggesting that DNA structure of exon 1 would be defective in these plants lacked 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 DNA structure of *F3′5′H* gene. 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 purple and white flowered four 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 had a 5 bp insertion in adjacent to the stop codon, in 'Ademurasaki' and 'Hakuhō'. Whereas 'Hirado-no-homare' and 'Shirokujyaku' lacked this insertion and showed 1533 bp CDS. When PCR was performed to distinguish 5 bp insertion, the amplified product was found in some *R*. *ripense* individuals and *R*. × *mucronatum* 'Shiro-ryūkyū' but not in *R*. *scabrum* and *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.'

The genetic relationship among Hirado azalea and putative parents may lead to the understandings of breeding large flowered cultivars. This study found that color variation may stipulate by the hybridization between colored flower and white flower. The hybridization between red, purple, or white flower may develop more complex color.

4.4 General conclusion

Hirado azalea shares similar morphology to *R. scabrum*, however, the color variation was due to the presence of Dp derivatives. Hirado azalea cultivars have shown the relativity between purple flowered wild species like *R. ripense*, *R. macrosepalum*, and etc. due to the presence of Dp derivatives. Nevertheless, this founding do not clearly distinguish these wild species as their putative parents. Hence, the genetic analysis was performed to further differentiate them. Hirado azalea cultivars inherit either the *F3'5'H* gene of *R. ripense* and *R.* × *mucronatum* 'Shiro-ryūkyū' as suggested by my study.

4.5 Recommended further research

In this study, the *F3'5'H* gene of Hirado azalea is either similar to *R. ripens*e or 'Shiro-ryūkyū'. However, the absence of the exon 1 in *R. scabrum* and other Hirado azalea without Dp derivatives are not yet clarified. The specific primers used in this study may not anneal to gDNA as the sequence may differ. Hence, a new approach should be taken to identify the absence or abhorrent in sequences. Currently, there are four Rhododendron species being sequenced which are *R. simsii, R.delavayi, R. willaimsianum*, and *R. lapponicum* for their transcriptome and genome level (Jia et al., 2020 and Yang et al., 2020). The whole genome sequence of Rhododendron is still lacking which results in the hindrance in identifying color formation for breeding program (Yang et al., 2020).

Oxford nanopore sequencing (ONT) and Pacific Biosciences (PacBio) are high performance reads with high accuracy rate for assembly read. ONT ultra-long reads prevent assembly errors due to the repetitive regions. As for PacBio assembly, it is better than ONT with

fewer errors at the level of single nucleotides and small insertion or deletion (Lang et al., 2020). Pacbio would be ideal for identifying a specific gene as it has fewer errors in single nucleotides reading. However, the whole genome assembly of Rhododendron should be done with ONT. Utilizing both techniques would be an ideal approach to decrease the error rates for the assembly. Rhododendron genome research should be further study to identify the transcriptome involving in the color formation. This would result in a better understanding of flower color breeding program in the future.

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Supplemental figure

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTTGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E L G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

GGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATTCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

G M D L Q G I E G G M K R I H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGCGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCGCTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTGCTGAATCCACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N P Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCGTCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGTATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggctgaaactggtcgggagaaacttgaacggatccgcccctcaaccaaattgc

R L Q P S A Y A M *

ttatgctatgctctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 1 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron ripense* 1 (RrF3′5′H1a, accession no. LC547905)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAAACGGGGCACATGGTTCGGGCCATGTGCGAGTCGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E S G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATTCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAAGAGCACGCC

W M D L Q G I E G G M K R I H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGCGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTGCTGAATCCACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N P Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCGTCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggctgaaactggtcgggggaaacttgaacggatccgcccctcaaccaaagtcc

R L Q P S A Y A M *

ttgcttatgctatgcccaagacaactctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 2 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron ripense* 2 (RrF3′5′H1b, accession no. LC547906)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCTTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTCGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E S G R R G E

GCAGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

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S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAATTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D N L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTCATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTACTGAATCTACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N L Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACATGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCATCTGAAGCA

N M P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGTTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggttgaaaatggtcgggagaaacttgaacggatccgcccctcaaccaaattgc

R L Q P S A Y A M *

Ttatgctatgctctatttaatttaatattgttggtattttagctgtgcctcttgcagctctaatcactagtgaatt

S. 3 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron macrosepalum* 1 (RmF3′5′H1a, accession no. LC547907)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCACCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A P A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCTGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTCGGGCAGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E S G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACATTTGCCATGGCGAACATCATCGGCCAGGTGATACTAGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

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W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

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I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

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L L N P R I L K R A H Q E M D Q V I G R S R R L E E S D I K

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N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACAGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

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A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggctgaaactggtcgggggaaacttgaacggatccgcccctcaaccaaagtgc

R L Q P S A Y A M *

ttgcttatgctatgcccaagacaactctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 4 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron macrosepalum* 2 (RmF3′5′H1b1, accession no. LC547908)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCACCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A P A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCTGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTCGGGCAGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E S G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACATTTGCCATGGCGAACATCATCGGCCAGGTGATACTAGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTCATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTACTGAATCTACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N L Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACATGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCATCTGAAGCA

N M P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGTTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggctgaaaatggtcgggagaaacttgaacggatccgcccctcaaccaaattgc

R L Q P S A Y A M *

ttatgctatgctctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 5 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron macrosepalum* 2 (RmF3′5′H1b2, accession no. LC547909)

cacatctaaggcaaaaccactcacaaaATGGCCGTAGACACTCTGTTGTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A V D T L L F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCGCCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P A R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCAAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P K S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTCGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E S G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCAGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTCATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTAGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAACAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTGCTGAATCCACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N P Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCATCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGTGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGccaggttcaacggctgaaactggttgggagaaacttgaacggatccgcccctcaaccaaa

R L Q P S A Y A M *

gtgcttatgctatgcccaagagaactctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 6 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron yedoense* var. *poukhanense* (RyF3′5′H1, accession no. LC547910)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTTTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L F F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGTACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L Y M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTTGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E L G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTACTGAATCTACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N L Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCGTCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGCCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K P V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggctgaaactggtcgggggaaacttgaacggacccgcccctcgaccaaagtgc

R L Q P S A Y A M *

ttgcttatgctatgcccaagacaactctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 7 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron* × *mucronatum* 'Shiro-ryūkyū' (RmSRF3′5′H1, accession no. LC547911)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTTGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E L G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTGTTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTACTGAATCTACAAATCCTAAAACGGGCACACCAGGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N L Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTGCAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCGTCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGTATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTATTGTAGGCTCAACGGCTGAaactggtagggagaaacttgaacggatccgcccctcaaccaa

R L Q P S A Y A M Y C R L N G *

attgcttgcttatgctatgcccaagacaactctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 8 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron* × *mucronatum* 'Shiro-ryūkyū' (RmSRF3′5′H2, accession no. LC547912)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGAGCACATGGTTCGGGCCATGTGCGAGTCGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T E H M V R A M C E S G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTACTGAATCTACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N L Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCGTCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTTTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G F A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggctgaaactggtcgggggaaacttgaacggacccgcccctcaaccaaagtgc

R L Q P S A Y A M * A Q R

ttgcttatgctatgcccaagacaactctatttaatttaatattgttgatattttagctgtgcctcttgcagctct

S. 9 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron* × *pulchrum* 'Hirado-no-homare' (RpHHF3′5′H1, accession no. LC547913)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTTTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L F F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCACCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTTGGGCCGGAGGGGCGAG

G K A L D D S V G T R H T E T G H M V R A M C E L G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTACTGAATCTACAAATCCTAAAACGGGCACACCAAGAGATGGGTCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N L Q I L K R A H Q E M G Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTACAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCGTCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGACCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGGATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTAGgctcaacggctgaaactggtcgggggaaacttgaacggacccgcccctcaaccaaaatgc

R L Q P S A Y A M *

ttgcttatgctatgcccaagacaactctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 10 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron* × *pulchrum* 'Shiro-kujyaku' (RpSKF3′5′H1, accession no. LC547914)

cacatctaaggcaaaaccactcacaaaATGGCCGCAGACACTCTGTTGTTTAGAGAGATTGCAGCAGCAACTGTAATCTTTTTCCTCACT

M A A D T L L F R E I A A A T V I F F L T

AGGCTGTTCCTCCGTTCCCTCCTCCTCAAACCCACCCGTAAACTCCCGCCCGGTCCGAAAGGGTGGCCGATCATCGGCGCCCTCCCCCTT

R L F L R S L L L K P T R K L P P G P K G W P I I G A L P L

CTTGGAACCATGCCCCATGTTGCCCTAGCCCAAATGGCCAAGAAATATGGACCCATCATCTACCTAAAAATGGGCACCCTTGACATGGTG

L G T M P H V A L A Q M A K K Y G P I I Y L K M G T L D M V

GTGGCCGCGACGCCCGAGTCGGCCCGAGCCTTCCTGAAAACCCTGGACATGAACTTCTCAAACCGGCCACCTAATGCCGGCGCGACCCAC

V A A T P E S A R A F L K T L D M N F S N R P P N A G A T H

TTGGCATACAACAGCCAAGACATGGTTTTCGCCGACTACGGCCCGAAGTGGAAGTCGTTGCGCAAGTTGAGCAACCTGCACATGCTGGGC

L A Y N S Q D M V F A D Y G P K W K S L R K L S N L H M L G

GGGAAGGCGCTCGACGACTCGGTTGGCATCCGGCATACGGAGACGGGGCACATGGTTCGGGCCATGTGCGAGTCGGGCCGGAGGGGCGAG

G K A L D D S V G I R H T E T G H M V R A M C E S G R R G E

GCGGTGGTGGTGGCGGAGATGCTGACGTTCGCTATGGCGAACATCATCGGCCAGGTGATACTCGGGCGGAGGGTATTCGTCCAGGCGGGT

A V V V A E M L T F A M A N I I G Q V I L G R R V F V Q A G

TCGGAGTCCAACGAGTTCAAGGACATGGTGGTGGAGCTGATGACCTCGGCCGGTCTGTTCAACGTGGGCGACTTTATACCGGCGCTGGCG

S E S N E F K D M V V E L M T S A G L F N V G D F I P A L A

TGGATGGACTTGCAAGGGATTGAGGGCGGGATGAAACGGATGCATCGCAAGTGGGACAGTTTGATAACGAGGATGGTGAAGGAGCACGCC

W M D L Q G I E G G M K R M H R K W D S L I T R M V K E H A

GAGACGGCTCATGAGCGTCGAGGGAACCCTGATTTTCTTGATGTGCTTATGGCTAATAGAGAGACTTCTCAAGGCGGCCCCGGGCTTAGC

E T A H E R R G N P D F L D V L M A N R E T S Q G G P G L S

ATCACCAACATTAAAGCACTCCTTTTGAATCTATTTACTGCCGGTACCGATACCTCTTCCAGCGTAATCGAATGGGCACTGGCTGAAATG

I T N I K A L L L N L F T A G T D T S S S V I E W A L A E M

CTACTGAATCTACAAATCCTAAAACGGGCACACCAAGAGATGGATCAAGTCATTGGAAGAAGCAGGAGATTACAGGAGTCTGACATTAAA

L L N L Q I L K R A H Q E M D Q V I G R S R R L Q E S D I K

AACCTGCCTTACCTGCAAGCCATATGCAAAGAAAGCTTCCGAAAGCACCCTTCCACCCCCCTCAACCTCCCCCGAATCTCGTCCGAAGCA

N L P Y L Q A I C K E S F R K H P S T P L N L P R I S S E A

TGCGAGGTGAACGGCTACTACATACCCAAGAACGCGCGACTGAGCGTGAACATCTGGGGCATCGGGAGGGACCCCGACGTCTGGGAGAAC

C E V N G Y Y I P K N A R L S V N I W G I G R D P D V W E N

CCTCTGGAGTTCAACCCGGAGAGGTTTCTGACCGAGAAAAACGCCAAGATCGATCCGCGGGGGAACGATTTCGAGCTGATCCCGTTTGGC

P L E F N P E R F L T E K N A K I D P R G N D F E L I P F G

GCGGGGCGGAGGATATGCGCGGGGGCTAGGATGGGAGTTGTGATGGTTGAGTACTTCTTGGGCACGTTGGTTCACTCATTTGACTGGAAA

A G R R I C A G A R M G V V M V E Y F L G T L V H S F D W K

TTGCCTGATGGTATGGGTGAGCTAAACATGGATGAGTCTTTCGGTCTTGCTTTGCAAAAGGCTGTGCCTCTCGCGGCTATGGTTACCCCG

L P D G M G E L N M D E S F G L A L Q K A V P L A A M V T P

CGGCTGCAACCAAGTGCTTATGCTATGTATTGTAGGCTCAACGGCTGAaactggtagggagaaacttgaacggatccgcccctcaaccaa

R L Q P S A Y A M Y C R L N G *

attgcttatgctatgctctatttaatttaatattgttggtattttagctgtgcctcttgcagctct

S. 11 The alignment of nucleotide sequence and amino acid sequence of *Rhododendron* × *pulchrum* ‘Ademurasaki’ and ‘Hakuhō’ (RpF3′5′H2, accession no. AB488484)

List of publication

Meanchapiboon, S., Kobayashi, N., & Nakatasuka, A. 2020. Analyses of Pigment Compositions and Anthocyanin Biosynthesis Gene Expression in Hirado Azalea Cultivars. Hort. J. Vol. 89(3) 284-291.

Meanchapiboon, S., Kobayashi, N., & Nakatasuka, A. Genetic relationships among Hirado azalea cultivars a nd their putative parents inferred from flavonoid 3′,5′ hydroxylase gene sequences. Hort. J. (Accepted).

Attended Conference

Meanchapiboon, S., Kobayashi, N., & Nakatasuka, A. 22 July 2017. Comparison of flower color phenotyp e and gene expression between wild species and progenies in evergreen Azalea. The Chugoku horticultural journal annual conference 2017. Matsuyama city, Ehime. Oral presentation.

Meanchapiboon, S., Hitomi, M., Kobayashi, N., & Nakatasuka, A. 25 November 2017. Effect of pigment composition and flavonoid hydroxylation related genes on flower color in evergreen azalea hybrids. The plant breeding annual conference. Higashi-Hiroshima, Hiroshima. Poster presentation.

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Meanchapiboon, S., Kobayashi, N., & Nakatasuka, A. 15 September 2019. Pigment composition patterns and expression analyses of anthocyanin biosynthesisgenes in collora of Hirado azaleacultivar group. The horticultural journal autumn conference 2019. Matsue city, Shimane. Oral presentation.

Abstract

Hirado azaleas (*R.* × *pulchrum*) are known for their large flowers varying in colors including white to pink, red, and purple forms. However, the origin of this color variation is not well understood. Their putative parents are *Rhododendron scabrum* G. Don, *R. ripense* Makino, and white-flowered *R.* × *mucronatum* (Blume) G. Don 'Shiro-ryūkyū'. This study investigated the correlation of Hirado azalea cultivars with their putative parents via genetic analyses and pigment compositions. The aim is to identify the origin of Hirado azalea cultivars.

Hirado azalea cultivars were divided into four groups according to their pigment compositions. Three out of four groups were similar to either one of the putative parents as suggested by their morphological evidence except the group with cyanidin derivatives and flavonol exhibited wider flower colors from their parents. All samples expressed F3′H, DFR, and ANS genes, as determined by real-time quantitative RT-PCR. However, the F3′5′H gene was expressed only in samples containing delphinidin derivatives. 'Shiro-ryūkyū' and white flowered cultivars also expressed all four genes. This may suggest the hybridization between *R. scabrum* and other putative parents may not limit to colored flowers.

The F3′5′H gene of *R. scabrum*, *R. ripense*, *R. macrosepalum*, *R. yedoense* var. *poukhanense,* and 'Shiro-ryūkyū' was analyzed by gDNA and cDNA sequence analysis, and deduction of amino acid sequences to assess the genetic relationships between these taxa and to investigate the genetic basis of color variation in this group. In *R. scabrum* and Hirado azalea cultivars without Dp derivatives only exon 2 were amplified using specific primers. The exon 1 was absent in all of them suggesting defective DNA. On the other hand, *R. ripense*, *R. macrosepalum*, *R. yedoense* var. *poukhanense* and *R.* × *mucronatum* 'Shiro-ryūkyū,' and Hirado

azalea cultivars with Dp derivatives have normal DNA structure of F3′5′H gene. 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 purple and white flowered four 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 had a 5 bp insertion in adjacent to the stop codon, in 'Ademurasaki' and 'Hakuhō'. Whereas 'Hirado-no-homare' and 'Shirokujyaku' lacked this insertion and showed 1533 bp CDS. When PCR was performed to distinguish 5 bp insertion, the amplified product was found in some *R*. *ripense* individuals and *R*. × *mucronatum* 'Shiro-ryūkyū' but not in *R*. *scabrum* and *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.'

Primary Reader and Advisor: Associate Professor Akira Nakatsuka

Secondary reader: Professor Nobuo Kobayashi and Associate Professor Yoshihiro Takemura

Summary in Japanese

「ヒラドツツジ」(Rhododendron× pulchrum)は，赤色，紫色，ピンク色から白色まで幅広い花色を有する大輪系の代表的なツツジ園芸品種群である．これまでに行われた花色や花形，雌蕊や雄蕊の数などの広範な形態調査の結果に基づいて，赤花のケラマツツジ（ R. scabrum G. Don），紫花のキシツツジ（ R. ripense Makino），白花のリュウキュウツツジ‘白琉球’（ R. × mucronatum (Blume) G. Don）などが「ヒラドツツジ」品種の成立に関与した植物種と推定されている．しかしながら，花色発現に関与する遺伝子の配列等はほとんど知られておらず，分子生物学的手法を用いた系統発生学的研究の進展が期待される．本論文では，アントシアニジン色素構成，アントシアニン合成関連遺伝子の発現とフラボノイド 3 ′5 ′水酸化酵素遺伝子（F3’5’H）の DNA 構造解析を行ない，「ヒラドツツジ」品種における花色の多様化の仕組みと推定親との遺伝的関係を考察した．

1.「ヒラドツツジ」品種における色素構成とアントシアニン合成遺伝子の発現解析.

「ヒラドツツジ」は長崎県平戸市を中心に発達した大輪系の園芸品種群で，その成立にはケラマツツジ，キシツツジ，リュウキュウツツジ‘白琉球’などの野生種や園芸品種が関与したと考えられている．本論文では，「ヒラドツツジ」品種と野生種について，花色とアントシアニジン組成およびアントシアニン合成関連遺伝子発現を比較した．「ヒラドツツジ」品種は色素構成により大きく 4 つのグループに分類された．赤花の園芸品種とケラマツツジはシアニジン系色素のみを有していた．紫花の園芸品種とキシツツジやモチツツジは，シアニジン系色素とデルフィニジン系色素およびフラボノール色素を有していた．白花の園芸品種とリュウキュウ

ツツジ‘白琉球’はフラボノール色素のみを有していた．野生種と異なる花色のピンク色から赤紫色を示す「ヒラドツツジ」品種の色素は，シアニジン系色素およびフラボノールであった．すべての調査個体はリアルタイム定量 RT- PCR 法によりフラボノイド 3 ′水酸化酵素，ジヒドロフラバノール還元酵素およびアントシアニン合成酵素の遺伝子発現が検出されたが，デルフィニジン系色素を有する野生種および品種のみが F3’5’H を発現した．加えて白花個体も全ての遺伝子を発現していた．これらの結果は赤花のケラマツツジに紫花のキシツツジ，モチツツジや白花のリュウキュウツツジ‘白琉球’などから F3’5’H が導入され，「ヒラドツツジ」の紫・赤紫およびピンクなどの多様な花色が作られたことを示唆した．

2.「ヒラドツツジ」品種とその成立に関連するツツジにおける F3’5’H に基づく遺伝的関係.

「ヒラドツツジ」品種群は大輪赤花のケラマツツジをもとに，キシツツジやリュウキュウツツジ‘白琉球’などの野生種や園芸品種が関与することによって花色が多様化したと推定されるが，その遺伝的関係は不明な点が多い．本論文では，「ヒラドツツジ」品種とその成立に関連する野生種・園芸品種について，花色の多様化に重要な F3’5’H のゲノム構造および塩基配列を調査し，遺伝的関係を評価した．F3’5’H のコード領域（CDS）については，デルフィニジン系色素を有しないケラマツツジと「ヒラドツツジ」品種では，第 2 エキソン領域のみが PCR 増幅されたことから，ケラマツツジとこれらの「ヒラドツツジ」品種において第 1 エキソン領域の構造変異の可能性が示唆された．一方，デルフィニジン系色素を有するキシツツジやモチツツジ，チョウセンヤマツツジの F3’5’H は正常な DNA 構造であり，単離した遺伝子の CDS 配列は 1533bp（510 アミノ酸），リュウキュウツツジ‘白琉球’では 1533bp（510 アミノ酸）と 1551bp（516 アミノ酸）であることが確認できた．F3’5’H 配列に基づいて系統樹を作成したところ，今回調査した紫花品種‘艶紫’と‘平和の光’，白花品種の‘白峰’と‘白孔

雀’は，キシツツジやリュウキュウツツジ‘白琉球’と同じグループを形成した．また F3’5’H の CDS 配列は‘艶紫’と‘白峰’では終始コドンの近くに 5bp の挿入がある 1551bp であったが，‘平和の光’と‘白孔雀’では挿入がない 1533bp であった．5bp の挿入を識別するために PCR 増幅したところ，ケラマツツジやモチツツジになく，一部のキシツツジとリュウキュウツツジ‘白琉球’に確認された．これらの結果は，「ヒラドツツジ」の花色多様性は，ケラマツツジに対してキシツツジやリュウキュウツツジ‘白琉球’が交雑することによる F3’5’H 構成の多様化が原因であることを示唆した．

本論文では，特にデルフィニジン系色素の有無に着目して，その合成に重要な F3’5’H の発現や DNA 構造解析を行い，「ヒラドツツジ」品種群における花色の多様化の仕組みとともに推定親について遺伝的背景を示すことができた．これらの知見は，遺伝子情報に基づいたツツジ花色の創出に貢献することが期待される．

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