EVIDENCE FOR INTROGRESSIVE HYBRIDIZATION BASED ON CHLOROPLAST DNA POLYMORPHISMS AND MORPHOLOGICAL VARIATION IN WILD EVERGREEN AZALEA POPULATIONS OF THE KIRISHIMA MOUNTAINS, JAPAN

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For the purpose of determining the origin of horticultural evergreen azalea cultivars, this study was focused on the natural populations of azalea in Kyushu (south main island of Japan). The Kirishima mountains, the volcanic mountain mass in Kyushu, are an important centre of diversity for the Japanese evergreen azaleas. *Rhododendron kiusianum* Makino grows above 1000m alt., whereas *R. kaempferi* Planch. is distributed below 600m alt. Putative natural hybrid populations of these two species are found in the intermediate region (1000–600m alt.). These two species have been clearly distinguished by their respective morphological features. *Rhododendron kiusianum* has small pink-purple flowers and small elliptical leaves, whereas *R. kaempferi* has larger red-orange flowers with dark blotches and large oblong leaves. Interspecific hybrids show phenotypes within the range of the two species, especially with regard to flower colour and leaf shape. A morphological cline of these characteristics corresponding to altitude has been observed between these two species. PCR-RFLP analysis of chloroplast DNA detected specific bands for the two species in the 16S rDNA region when digested with *Hha*I restriction enzyme. Populations of interspecific hybrids were composed of individuals that had a banding pattern of either *R. kiusianum* or *R. kaempferi*. This indicates that *R. kiusianum* and *R. kaempferi* are clearly distinct species. Furthermore, natural hybrid populations consist of individuals that have one of two cpDNA. Some individuals in the populations of *R. kiusianum* (T-1430 and T-1030) possess the cpDNA pattern of *R. kaempferi*, which suggests that cytoplasmic introgression has occurred in the populations of *R. kiusianum* from *R. kaempferi*.

Keywords. *Rhododendron kaempferi*, *Rhododendron kiusianum*, PCR-RFLP, phenotype.

INTRODUCTION

The genus *Rhododendron* L. (*Ericaceae*), which comprises over 1000 species that are widespread in the Northern hemisphere, is subdivided into 8 subgenera and 12 sections (Chamberlain *et al.*, 1996). Among the fifty *Rhododendron* species native to Japan (Yamazaki, 1995), the species in the subgenus *Tsutsusi* section *Tsutsusi* are the most important evergreen azaleas used as ornamental shrubs. In Japan, hundreds

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of azalea cultivars have been selected by horticulturists; these cultivars stem from the endogenous evergreen azalea species and their natural hybrids from the Edo era (1603–1867) (Kunishige & Kobayashi, 1980). Natural hybridization in native species often generates morphological variations that have been used as an important gene source for azalea breeding. On Kyushu, the south main island, wild evergreen azaleas are distributed widely and abundantly; consequently, Kyushu is the breeding centre of evergreen azalea cultivars.

The Kirishima mountains are a volcanic mountain massif in southern Kyushu. Three wild azalea populations were found and reported as the estimated origin of evergreen azalea cultivars on this mountain mass (Makino, 1917; Miyazawa, 1918; Nakai, 1922). Firstly, *R. kiusianum* Makino is a densely branched dwarf shrub with pink-purple flowers, and is endemic to Kyushu island, where it inhabits open and windy peak areas above 1000m. Secondly, *R. kaempferi* Planch. is a loosely branched shrub with red-orange flowers and endemic to Japan islands, distributed in the edges of secondary forest below 600m. Thirdly, populations with abundant phenotypic variation, such as in flower colour, grow in the open forest of the intermediate region between 1000m and 600m. This region represents the space between the habitats of the two species. Such wild populations have been thought to be the origin of the Japanese evergreen azalea cultivar groups, including the Edo-Kirishima and Kurume azaleas. In the ancient Japanese horticultural literature, the cultivar group named 'Kirishima' indicated the place of origin as the Kirishima mountains (Miyazawa, 1918).

The relationships among these azalea populations in the Kirishima mountains have been investigated in terms of morphology, pigmentation characters, and pollinators (Kunishige & Tamura, 1961; Sakata *et al*., 1993; Yokokawa & Hotta, 1995). Such studies suggested that populations found in the intermediate region consist of individuals showing phenotypic variation, which results from interspecific hybridization between *R. kiusianum* and *R. kaempferi*.

In this study, a DNA marker was used to investigate introgression and the relationships among wild evergreen azalea populations in the Kirishima mountains; extensive sampling was undertaken for these purposes (Arnold *et al*., 1991, Kron *et al*., 1993). A combined method of polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) was used for the analysis of chloroplast DNA (cpDNA) as a marker. Morphological analysis was also performed to support the results of DNA analysis.

MATERIALS AND METHODS

Plant material

Nine populations of *R. kiusianum* from the most highly elevated area of the Kirishima mountains (research sites: K-1650, K-1420, K-1180, T-1560, T-1430, T-1300, T-1210, T-1120, T-1030; numbers indicate altitude), three populations of *R. kaempferi* at the

base (T-600, Ki-520, Ki-440) and four putative hybrid populations in the intermediate region between the two species (S-940, T-820, T-800, T-790) were used for this study. At 16 research sites, 327 individuals were investigated in May and June of 1993, 1994 and 1995 (Fig. 1). Leaf material was collected for further analysis.

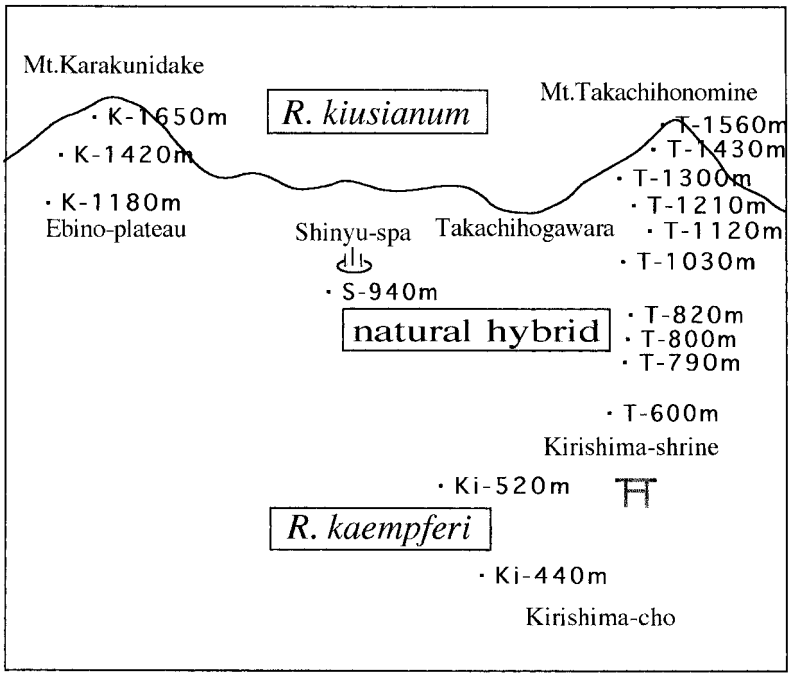


FIG. 1. Research sites sampling wild azalea populations on the Kirishima mountains. Numbers indicate the altitude (m) of each research site. K, Mt Karakuni; T, Mt Takachihonomine; S, Shinyu-spa; Ki, Kirishima-cho.

Investigation of phenotypic characteristics

Leaf size and shape (length, width and width/length), flower size (diameter) and colour (according to the RHSCC, Royal Horticultural Society Colour Chart), expression of petal blotch (percentage of the individuals which have petal blotch in each sample population), length of previous year's shoot and plant height were recorded for each individual plant.

DNA extraction

Newly expanded leaves were collected from each plant and stored at $-80^{\circ}C$ until used. Total genomic DNA was extracted from 150mg of frozen leaf sample by a modified CTAB method (Kobayashi *et al.*, 1995, 1998).

PCR-RFLP analysis

DNA amplification by polymerase chain reaction (PCR). Forty-one sets of primers designed from a tobacco cpDNA sequence (Shinozaki *et al.*, 1986) were used for amplification of 41 regions within the cpDNA of *R. kiusianum* and *R. kaempferi.* Regions of amplified single band products with the same molecular size in both *R. kiusianum* and *R. kaempferi* were investigated by restriction endonuclease analysis (Table 1). The reaction mixtures were prepared in a volume of 25μl containing 20ng genomic DNA, 0.5μM primers, 0.1mM dNTPs, 2mM $MgCl_2$, 1 × the original reaction buffer (Boehringer Mannheim Co.), and 0.5 unit Taq polymerase (Boehringer Mannheim Co.). DNA was amplified with DNA thermal cycler PJ-2000 (Perkin Elmer Cetus-Takara) by the following program: initial denaturation lasted 4min at 94°C followed by 35 cycles of 30s at 94°C, 40s at 57°C and 3min at 72°C. Amplified products were checked by electrophoresis in 2% agarose gels under UV light after being stained with ethidium bromide.

Restriction endonuclease analysis. Amplified products were digested to detect polymorphism between *R. kiusianum* and *R. kaempferi* using 20 kinds of restriction endonucleases (*Alu*I, *Bam*HI, *Bgl*II, *Dde*I, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinc*II, *Hin*fI, *Hin*dIII, *Mbo*I, *Msp*I, *Rsa*I, *Sac*I, *Scr*FI, *Sty*I, *Taq*I, *Tsp*509 and *Xba*I). Digestion was carried out at 37°C or 60°C (*Taq*I) for 4h. Restriction fragments were separated by electrophoresis in 2% agarose gels and photographed under UV light after they were stained with ethidium bromide.

RESULTS

Morphological characteristics

The results of morphological analysis are indicated in Table 2 and Fig. 2. Populations of spreading dwarf shrubs, *R. kiusianum* near the top area of Kirishima mountains had smaller elliptical leaves and smaller pink-purple flowers (19.2–26.3mm diam., and in the 70 to 80 range according to the RHSCC flower colour number); the corolla bore few petal blotches. This phenotype was more common at Mt

TABLE 1. The cpDNA regions with the same molecular size in both *R. kiusianum* and *R. kaempferi*, as determined by PCR

*trn*H–*psb*A	*psb*J–*trn*P
*rps*16	*pet*B–*pet*D
*trn*S–*trn*R	*trn*L–rps122
*trn*G–*atp*A	*rpl*2
*rpo*C1–*rpo*C2	16S
*psb*D–*psb*C	*trn*I–23S
*trn*G–*psa*B	*ndh*D
*trn*T–*psb*G	*ndh*H–*ndh*I

TABLE 2. Morphological characteristics of wild azalea populations on the Kirishima mountains

Research site	Species	No. of individuals	Leaf			Flower		Shoot length (mm)	Tree height (cm)
			Length (mm)	Width (mm)	Width/length	Diameter (mm)	Expression of blotch		
K-1650*	*R. kiusianum*	26	7.9±0.18**	4.8±0.12	0.61±0.01	19.2±0.40	8.7%	20.6±0.95	32.8±3.68
K-1420	*R. kiusianum*	10	9.3±0.37	5.6±0.24	0.6±0.02	20.7±0.63	10.0%	20.2±0.58	87.5±3.35
K-1180	*R. kiusianum*	27	8.5±0.21	5.0±1.12	0.6±0.01	20.7±0.38	8.7%	16.2±1.64	55.6±2.58
T-1560	*R. kiusianum*	23	10.6±0.31	6.2±0.16	0.59±0.01	21.5±0.64	33.3%	12.3±0.55	66.7±3.54
T-1430	*R. kiusianum*	10	10.3±0.57	6.1±0.29	0.6±0.02	21.7±0.39	60.0%	21.2±3.12	48.0±8.14
T-1300	*R. kiusianum*	23	9.1±0.25	5.3±0.13	0.59±0.01	21.2±0.62	44.4%	16.0±0.98	8.3±0.91
T-1210	*R. kiusianum*	10	9.5±0.48	5.7±0.35	0.6±0.02	22.1±0.53	30.0%	15.9±0.82	28.7±5.28
T-1120	*R. kiusianum*	10	14.0±1.18	7.3±0.48	0.54±0.02	24.2±0.92	20.0%	26.2±0.96	126.5±12.02
T-1030	*R. kiusianum*	35	16.0±0.88	8.7±0.35	0.56±0.01	26.3±0.59	77.8%	38.9±2.12	168.1±7.51
S-940	Natural hybrid	22	24.3±0.96	11.7±0.45	0.49±0.01	29.2±0.79	95.5%	39.4±2.21	219.3±8.61
T-820	Natural hybrid	9	20.8±0.82	11.0±0.53	0.53±0.02	31.2±1.22	90.0%	30.1±1.55	224.5±10.94
T-800	Natural hybrid	52	20.5±0.58	10.6±0.29	0.52±0.01	30.6±0.66	90.2%	40.2±2.69	153.1±5.80
T-790	Natural hybrid	10	23.4±1.22	11.5±0.54	0.5±0.02	36.3±1.95	80.0%	45.4±4.60	236.0±18.25
T-600	*R. keampferi*	29	31.4±1.31	15.3±0.76	0.48±0.01	39.0±0.85	96.8%	52.1±2.69	253.5±15.13
Ki-520	*R. keampferi*	26	39.0±2.35	18.3±0.28	0.47±0.01	39.6±0.99	100.0%	54.4±4.48	184.0±9.06
Ki-440	*R. keampferi*	5	27.6±0.62	13.3±1.18	0.48±0.03	39.8±1.60	100.0%	58.9±8.12	212.5±16.55

* Number indicates the altitude (m) of each research site, ** Mean±standard error.

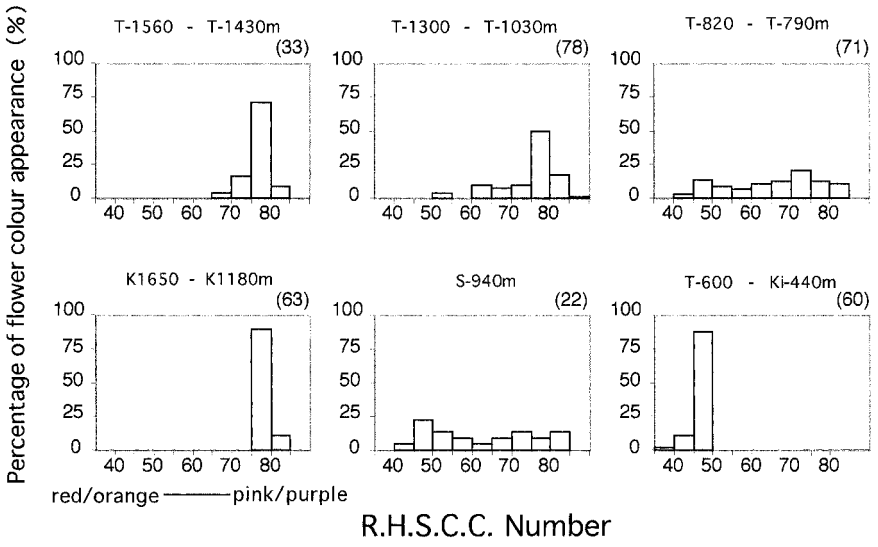


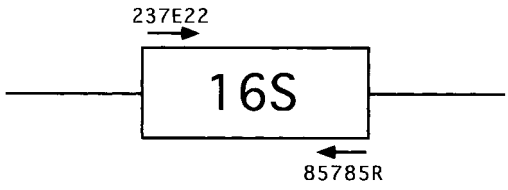
FIG. 2. Frequency of flower colour based on RHS colour chart in wild azalea populations on the Kirishima mountains. Numbers in parenthesis show the total number of individuals investigated in terms of flower colour.

Karakunidake and on the Ebino plateau (K-1650, K-1420, and K-1180). Populations of *R. kaempferi* at the foot of the mountain range were loosely branched shrubs with larger oblong leaves and larger red-orange flowers (39.0–39.8mm diam., and in the range of the 40s as regards RHSCC number); darkly coloured blotches were commonly observed in this population. The putative hybrid populations (S-940, T-820, T-800, T-790) showed wide phenotypic variation within the range of the two species, especially as regards flower colour and leaf shape. The lengths of the previous year's shoot and plant height were shorter in *R. kiusianum*, especially in T-1300: 8.3cm plant height; lengths were longer in *R. kaempferi*. In the putative hybrids, length was intermediate.

The morphological characteristics of wild azalea populations varied according to altitude.

Screening of PCR-RFLP marker

Out of 41 sets of PCR primers for cpDNA, 16 amplified single band products which had the same molecular size in both *R. kiusianum* and *R. kaempferi* which were identified by morphological features (Table 1). After cutting with 20 restriction endonucleases for these 16 regions, only the 16S rDNA region digested with *Hha*I could detect polymorphism between *R. kiusianum* and *R. kaempferi* (Fig. 3). *Rhododendron kiusianum* possessed a banding pattern of 1030 and 420bp in 16S rDNA digested with *Hha*I. *Rhododendron kaempferi* had a banding pattern of 950,



primer

237E22 : 5' – A C G G G T G A G T A A C G C G T A A G – 3'

85785R : 5' – C C A G T A C G G C T A C C T T G T T A C – 3'

FIG. 3. 16S rDNA region of chloroplast DNA and sequences of primers used for PCR amplification.

420 and 80bp. A pattern of 1030, 230 and 190bp was also detected in a few of the populations (Fig. 4).

Distribution of PCR-RFLP patterns in wild populations

Almost all *R. kiusianum* and *R. kaempferi* individuals exhibited a specific PCR-RFLP pattern, namely 1030/420bp and 950/420/80bp respectively. Populations of putative interspecific hybrids of the intermediate region of the Kirishima mountains were composed of individuals which had a banding pattern similar to either *R. kiusianum* or *R. kaempferi* (Table 3, Fig. 5).

In addition, 8 *R. kiusianum* individuals had a PCR-RFLP pattern of 950/420/80bp. However, none of the *R. kaempferi* samples showed a pattern of 1030/420bp.

DISCUSSION

Previous studies of azalea populations in the Kirishima mountains have indicated that the phenotypic variation observed in the intermediate region might be a result of interspecific hybridization between *R. kiusianum* and *R. kaempferi* (Kunishige & Tamura, 1961; Sakata *et al.*, 1993; Yokokawa & Hotta, 1995). The results of the present study support this hypothesis in terms of chloroplast genome analysis and morphological analysis.

Our results indicate that the populations of the intermediate region are natural hybrids of *R. kiusianum* and *R. kaempferi*. Furthermore, these hybrid populations were shown to consist of individuals that had a cpDNA of either *R. kiusianum* or *R. kaempferi*.

It will need further investigation using other DNA markers to explain the minor PCR-RFLP pattern of 1030/230/190bp that was detected in the populations of *R. kiusianum*, *R. kaempferi* and natural hybrids.

Although 16 regions of chloroplast DNA with combinations of 20 restriction

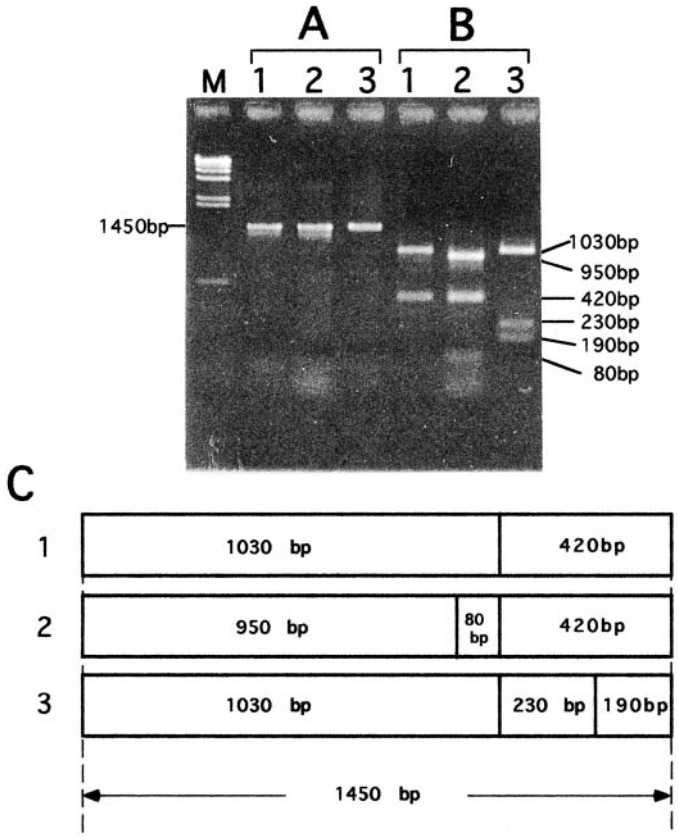


FIG. 4. Amplified products of the 16S rDNA region of chloroplast DNA (A) and polymorphic PCR-RFLP patterns after digestion of (A) with *Hha*I (B). *Hha*1 restriction site map in the 16S rDNA region corresponding to the upper figure (C). 1, *R. kiusianum* pattern (1030/420bp); 2, *R. kaempferi* pattern (950/420/80bp); 3, minor pattern (1030/230/190bp); M, molecular maker (*Hin*d III digested λDNA).

endonucleases were analysed, in only one region of cpDNA was it possible to detect polymorphism between *R. kiusianum* and *R. kaempferi*. Species-specific RAPD markers could not be detected in these populations, which corroborates a study of Louisiana irises (Arnold *et al.*, 1991; Kobayashi, 1996). Results of the present study support the phylogenetic close relationship of the two species in the section *Tsutsusi*.

In a case study of introgression of deciduous azalea in Georgia, USA (Kron *et al.*, 1993), it was found that numerous individuals in the sample population that were morphologically indistinguishable from *R. flammeum* possessed the chloroplast genome of *R. canescens.* That study indicated further that this phenomenon suggests that observed variations in some populations of *R. flammeum* may a result of past introgression from *R. canescens.* In the case of evergreen azaleas in the Kirishima mountains, some individuals in the populations of *R. kiusianum* (T-1430 and T-1030)

TABLE 3. Distribution of PCR-RFLP patterns of wild azalea populations on the Kirishima mountains

Research site	Species	No. of individuals	PCR-RFLP patterns (bp)		
			1030/420	950/420/80	1030/230/190
K-1650*	*R. kiusianum*	26	26	0	0
K-1420	*R. kiusianum*	10	10	0	0
K-1180	*R. kiusianum*	27	27	0	0
T-1560	*R. kiusianum*	23	23	0	0
T-1430	*R. kiusianum*	10	7	1	2
T-1300	*R. kiusianum*	23	23	0	0
T-1210	*R. kiusianum*	10	10	0	0
T-1120	*R. kiusianum*	10	9	0	1
T-1030	*R. kiusianum*	35	28	7	0
S-940	Natural hybrid	22	9	11	2
T-820	Natural hybrid	9	6	3	0
T-800	Natural hybrid	52	23	26	3
T-790	Natural hybrid	10	0	10	0
T-600	*R. keampferi*	29	0	29	0
Ki-520	*R. keampferi*	26	0	23	3
Ki-440	*R. keampferi*	5	0	4	1

* Number indicates the altitude (m) of each research site.

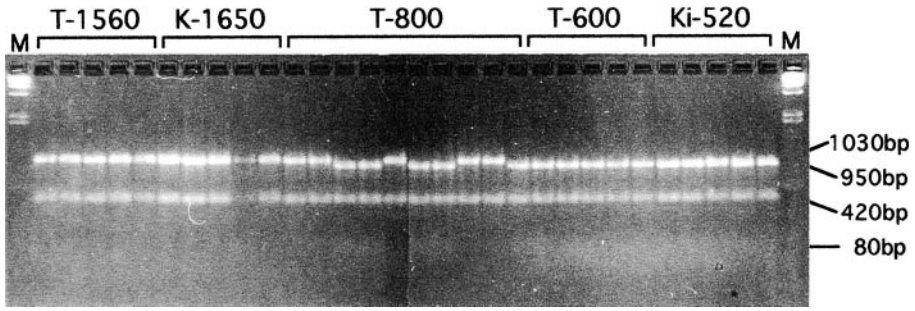


FIG. 5. Polymorphic PCR-RFLP patterns of selected azalea populations on the Kirishima mountains, as detected by *Hha*I digestion of the 16S rDNA region.

that were morphologically indistinguishable from *R. kiusianum* possess the cpDNA pattern of *R. kaempferi*. This suggests that cytoplasmic introgression occurred in populations of *R. kiusianum* from *R. kaempferi.*

A diverse range of phenotypic characteristics of hybrid populations is the result of repetitive natural hybridization between two species (Kron *et al.*, 1993). Moreover, a morphological cline corresponding to altitude would be caused by introgressive hybridization between two species (Anderson, 1949: 1–11).

Environmental factors of habitats, the volcanic activity, road construction and so on, are important for the recognition of the natural hybridization of two species. *Rhododendron kiusianum* is a pioneer plant in volcanic mountains with an ability to adapt environmentally to acid soil and hydrogen sulfide gas, whereas *R. kaempferi* prefers open forest and tolerates slight shade (Sakata *et al*., 1993; Yokokawa & Hotta, 1995).

The composition of cpDNA markers in *R. kiusianum* and its related species in other Kyushu mountains, as well as the relationship of these species to older azalea cultivars, was also investigated. Future studies using nuclear DNA and isoenzyme analysis will be necessary to confirm the nuclear introgression of these populations.

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