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INTROGRESSIVE HYBRIDIZATION BETWEEN *RHODODENDRON KIUSIANUM* AND *R. KAEMPFERI* (*ERICACEAE*) IN KYUSHU, JAPAN BASED ON CHLOROPLAST DNA MARKERS

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Wild evergreen azalea populations of *Rhododendron kiusianum* and *R. kaempferi* (*Ericaceae*) were analysed using a chloroplast DNA (cpDNA) PCR-RFLP marker that was used to detect introgressive hybridization in our previous study of the Kirishima Mts populations.

The populations of the intermediate region in the Unzen Mts, which show phenotypic variation, were demonstrated to result from interspecific hybridization between *Rhododendron kiusianum* and *R. kaempferi*, possessing cpDNA from either *R. kiusianum* (1030/420 bp) or *R. kaempferi* (950/420/80 bp).

Most individuals of *Rhododendron kiusianum* in the Kujyu Mts, the Aso Mts and the surrounding mountains exhibited the PCR-RFLP pattern of *R. kaempferi*. These results from the Kujyu Mts and the Aso Mts indicate that natural hybridization and cytoplasmic introgression from *Rhododendron kaempferi* to *R. kiusianum* have occurred in the relatively distant past. In the case of Mt Yufudake and Mt Haneyama, the *Rhododendron kiusianum* population retains the effects of natural hybridization with *R. kaempferi* in the cpDNA as well as in the variation in flower characteristics.

All individuals of *Rhododendron kiusianum* on Mt Onogaradake in the Takakuma Mts exhibit *R. kiusianum* cpDNA (1030/420 bp), in spite of variation in flower colour.

Keywords. Chloroplast DNA variation, introgressive hybridization, *Rhododendron kaempferi*, *Rhododendron kiusianum*.

INTRODUCTION

In the genus *Rhododendron* (*Ericaceae*), the species in the subgenus *Tsutsusi*, section *Tsutsusi*, are an important genetic resource for evergreen azaleas used as ornamental shrubs or pot azaleas. Since the 18th century, rhododendrons and azaleas have been hybridized extensively to produce new varieties (Kron *et al.*, 1993). Especially in Kyushu, the south main island of Japan, wild evergreen azaleas in *Rhododendron*

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section *Tsutsusi* are widely and abundantly distributed. *Rhododendron kiusianum* Makino is endemic to the upper areas of volcanic mountains on Kyushu, and *R. kaempferi* Planch. is the most commonly occurring species at the bottom of these mountains (Yamazaki, 1996). These azaleas have been used as a gene source for azalea breeding and hundreds of azalea cultivars have been produced since the Edo era (1603~1867) (Kunishige & Kobayashi, 1980).

There have been studies to investigate the relationships among these azalea populations in terms of morphology, pigment characteristics, pollinators and chloroplast DNA (cpDNA). These two species have been clearly distinguished by morphological and floral pigment features. *Rhododendron kiusianum* has small pink-purple flowers including anthocyanin of the cyanidin and delphinidin series, and small elliptic leaves, whereas *R. kaempferi* has larger red-orange flowers including only anthocyanin of the cyanidin series with dark blotches, and oblong leaves. Interspecific hybrids distributed in the intermediate regions of these two species in the Kirishima and Unzen mountain ranges show a wide variety of phenotypes within the ranges of the two species, especially with regard to flower colour and leaf shape (Kunishige & Tamura, 1961; Sakata *et al.*, 1991, 1993; Miyajima *et al.*, 1995, 2001; Kobayashi *et al.*, 2000). Also, these wild azalea populations have common pollinators as an effective route for gene introgression (Yokokawa & Hotta, 1995). In a previous study examining introgressive hybridization between *Rhododendron kiusianum* and *R. kaempferi* in the Kirishima Mts, PCR-RFLP analysis of cpDNA revealed specific bands for two species in the 16S rDNA region when digested with the restriction enzyme *Hha*I (Kobayashi *et al.*, 2000). Populations of interspecific hybrids were composed of individuals that displayed the banding pattern of either *Rhododendron kiusianum* or *R. kaempferi*. These results for morphological characters and cpDNA patterns indicate that *Rhododendron kiusianum* and *R. kaempferi* are clearly distinct species and that natural hybrid populations are the result of crosses between the two species.

Hybridization and introgression have been suggested by previous workers as important factors in the evolution of the southeastern USA azaleas (*Rhododendron* sect. *Pentanthera*). After analysis of cpDNA, Kron *et al.* (1993) found evidence of extensive localized cytoplasmic introgression into *Rhododendron flammeum* from *R. canescens*.

In this study we analysed some of the wild evergreen azalea populations of *Rhododendron kiusianum* and *R. kaempferi* using the same cpDNA marker as that used to detect introgressive hybridization in the case study on the Kirishima Mts populations. Introgression and relationships between wild azalea populations on Kyushu are discussed in relation to previously obtained morphological data for each population.

Materials and Methods

Plant materials

Leaf samples of wild *Rhododendron kiusianum*, *R. kaempferi* and natural hybrid populations of these two species were collected from their natural habitat and

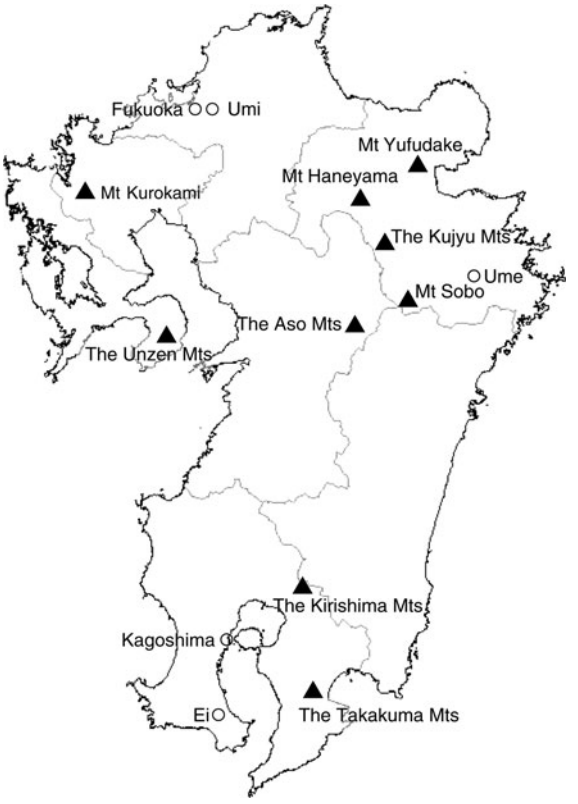

FIG. 1. Sampling sites of wild evergreen azalea populations in Kyushu, Japan.

from some experimental fields in March and June of 1993, 1994 and 1995 (Fig. 1, Table 1).

DNA extraction

Newly expanded leaves were collected from each individual and stored at –80°C until use. Total genomic DNA was extracted from 150 mg of frozen leaf sample using a modified CTAB method (Kobayashi *et al.*, 1998).

PCR-RFLP analysis

In this study we used a PCR-RFLP marker that could detect polymorphism between *Rhododendron kiusianum* and *R. kaempferi* from Mt Kirishima in the previous study (Kobayashi *et al.*, 2000). Out of 41 sets of primers designed using the tobacco cpDNA sequence (Shinozaki *et al.*, 1986), and out of 20 restriction endonucleases

TABLE 1. Wild evergreen azalea populations used in the DNA analysis in this study

Origin	Source	Species	No. of individuals
The Unzen Mts			
Mt Fugendake	Kurume branch, NIVOT	*R. kiusianum*	20
Mt Myokendake	Kurume branch, NIVOT	*R. kiusianum*	11
Nita pass	Kurume branch, NIVOT	Natural hybrid	21
Ikenohara	Kurume branch, NIVOT	Natural hybrid	22
Zigoku hot spring	Kurume branch, NIVOT	*R. kiusianum*	2
Mt Mayuyama	Kurume branch, NIVOT	Natural hybrid	22
The Kujyu Mts			
Mt Daisen	Kurume branch, NIVOT	*R. kiusianum*	3
Mt Yufudake	Kurume branch, NIVOT	*R. kiusianum*	18
Mt Haneyama	Kurume branch, NIVOT	*R. kiusianum*	9
The Aso Mts			
Mt Nekodake	Kurume branch, NIVOT	*R. kiusianum*	6
Mt Ebosidake	Sampling in field	*R. kiusianum*	15
Sensuikyou	Kyushu University	*R. kiusianum*	15
Mt Sobo	Akagi Nature Park	*R. kiusianum*	3
The Kirishima Mts			
Mt Karakuni, Ebino plateau	Sampling in field	*R. kiusianum*	63
Mt Takachihonomine	Sampling in field	*R. kiusianum*	76
Takachihogawara, Hybrid area	Sampling in field	Natural hybrid	96
Shinyu hot spring	Sampling in field	Natural hybrid	22
Kirishima-cho	Sampling in field	*R. kaempferi*	70
The Takakuma Mts			
Mt Onogaradake	Kyushu University	*R. kiusianum*	45
Mt Onogaradake	Kyushu University	*R. kaempferi*	26
Fukuoka-city (Fukuoka pref.)	Kyushu University	*R. kaempferi*	10
Umi-town (Fukuoka pref.)	Sampling in field	*R. kaempferi*	18
Takeo-city (Saga pref.)	Kyushu University	*R. kaempferi*	9
Mt Kurokami (Saga pref.)	Kyushu University	*R. kaempferi*	10
Ume-town (Ohita pref.)	Kyushu University	*R. kaempferi*	10
Kagoshima-city (Kagoshima pref.)	Sampling in field	*R. kaempferi*	13
Ei-town (Kagoshima pref.)	Sampling in field	*R. kaempferi*	7
Total			425

tested, only digestion of the 16S rDNA region with *Hha*I could be used to successfully detect polymorphism between *Rhododendron kiusianum* and *R. kaempferi*. *Rhododendron kiusianum* displayed a banding pattern of 1030 and 420 bp in 16S rDNA digestion with *Hha*I, while *R. kaempferi* displayed a banding pattern of 950, 420 and 80 bp. A pattern of 1030, 230 and 190 bp was also detected in a few

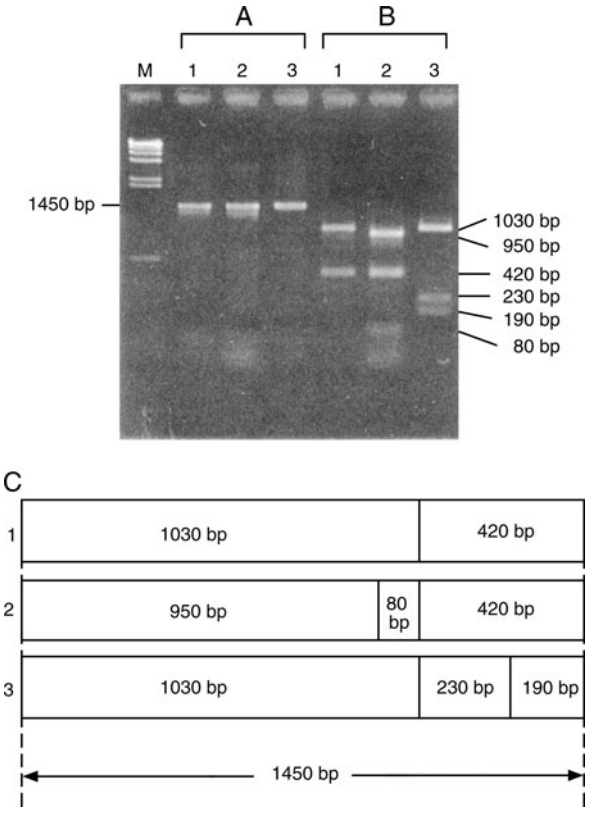

FIG. 2. Amplified products of the 16S rDNA region of chloroplast DNA (A) and polymorphic PCR-RFLP patterns after digestion of (A) with *Hha*I (B). *Hha*I restriction site map in the 16S rDNA region corresponding to the upper figure (C). 1, *R. kiusianum* pattern (1030/420 bp); 2, *R. kaempferi* pattern (950/420/80 bp); 3, minor pattern (1030/230/190 bp); M, molecular marker (*Hind* digested λDNA). This figure is quoted from the previous report of Kobayashi *et al.* (2000).

populations (Fig. 2). The reaction mixtures were prepared in a volume of 25 μl containing 20 ng genomic DNA, 0.5 μm primers, 0.1 mM dNTPs, 2 mM $MgCl_2$, 10% of the original reaction buffer (Boehringer Mannheim Co.) and 0.5 units of Taq polymerase (Boehringer Mannheim Co.). DNA was amplified using a DNA thermal cycler PJ-2000 (Perkin Elmer Cetus-Takara) with the following regime: initial denaturation for 4 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 40 seconds at 57°C and 3 minutes at 72°C. Amplified products were checked by electrophoresis in 2% agarose gels under UV light after staining with ethidium bromide. Amplified products were digested to detect polymorphism using the restriction endonuclease *Hha*I at 37°C for 4 hours. Restriction fragments were separated by electrophoresis in 2% agarose gels and photographed under UV light after staining with ethidium bromide.

Results and Discussion

In a PCR-RFLP analysis of each azalea population we could detect the same banding pattern as in the previous study (Kobayashi *et al.*, 2000) when the 16S rDNA region was digested with *Hha*I (Fig. 2).

A banding pattern of 1030 and 420 bp (Haplotype A) was detected in 81.8–100% of *Rhododendron kiusianum* individuals from the Unzen Mts, the Kirishima Mts and Mt Takakuma; a pattern of 950, 420 and 80 bp (Haplotype B) was detected in all populations of *Rhododendron kaempferi*, without exception, from the population in the Kirishima Mts; and a pattern of 1030, 230 and 190 bp (Haplotype C) was detected in populations from the Unzen Mts and the Kirishima Mts. However, 83.3–100% of *Rhododendron kiusianum* individuals from the Kujyu Mts, the Aso Mts and other surrounding mountains have Haplotype B as in *R. kaempferi*. All Haplotypes (A, B, C) were detected in natural hybrid populations from the Unzen Mts and the Kirishima Mts (Tables 2–4).

Distribution of PCR-RFLP patterns in wild azalea populations in the Unzen Mts

Most individuals of *Rhododendron kiusianum* on Mt Fugendake, Mt Myokendake and at the Zigoku hot spring exhibited a specific PCR-RFLP pattern, 1030/420 bp (Haplotype A), although Haplotype B was detected in 10–18.2% of individuals from two populations. All *Rhododendron kaempferi* individuals in the Fukuoka and Saga areas exhibited a specific PCR-RFLP pattern, 950/420/80 bp (Haplotype B). Presumed interspecific hybrid populations at Nita pass, Ikenohara and Mt Mayuyama were composed of individuals having a banding pattern of either

Table 2. Distribution of PCR-RFLP patterns of wild azalea populations on the Unzen Mts and neighbouring area

Sampling sites	Species	No. of individuals	PCR-RFLP patterns (bp)		
			1030/420	950/420/80	1030/230/190
The Unzen Mts					
Mt Fugendake	*R. kiusianum*	20	18	2	0
Mt Myokendake	*R. kiusianum*	11	9	2	0
Nita pass	Natural hybrid	21	13	8	0
Ikenohara	Natural hybrid	22	12	9	1
Zigoku hot spring	*R. kiusianum*	2	2	0	0
Mt Mayuyama	Natural hybrid	22	10	12	0
Fukuoka-city (Fukuoka pref.)	*R. kaempferi*	10	0	10	0
Umi-town (Fukuoka pref.)	*R. kaempferi*	18	0	18	0
Takeo-city (Saga pref.)	*R. kaempferi*	9	0	9	0
Mt Kurokami (Saga pref.)	*R. kaempferi*	10	0	10	0

Rhododendron kiusianum (1030/420 bp: Haplotype A) or *R. kaempferi* (950/420/80 bp: Haplotype B), with an approximately equal percentage in each group (Table 2).

Aburaya *et al.* (1979) and Miyajima *et al.* (1995) indicated that the phenotypic variation of intermediate region populations in the Unzen Mts was caused by interspecific hybridization between *Rhododendron kiusianum* and *R. kaempferi*, based on the study of morphological and pigment variation in the flowers.

These results, based on the distribution of cpDNA patterns, provide evidence that the populations at the Nita pass, Ikenohara and Mt Mayuyama are apparently natural hybrids of *Rhododendron kiusianum* and *R. kaempferi*. Additionally, these interspecific hybrid populations were clarified as being composed of individuals that had cpDNA of either *Rhododendron kiusianum* (Haplotype A) or *R. kaempferi* (Haplotype B). The inclusion of Haplotype B in *Rhododendron kiusianum* indicates the effect of introgressive hybridization.

Even though the altitude of the sampling site is lower than for other sites, each of the two individuals of the Zigoku hot spring exhibited a *Rhododendron kiusianum* PCR-RFLP pattern (1030/420 bp: Haplotype A). This is likely to be related to the volcanic environmental tolerance of *Rhododendron kiusianum* for sulphur dioxide gas or high soil pH. Volcanic hot springs and gas erupt in the Zigoku hot spring area and a population of *Rhododendron kiusianum* remained in the vegetation after volcanic activity in the Unzen Mts. Further study is needed to establish the environmental tolerance of *Rhododendron kiusianum* and other species.

Distribution of PCR-RFLP patterns in wild azalea populations in the Kujyu Mts, the Aso Mts and other mountains

All *Rhododendron kiusianum* individuals in the Kujyu Mts and the Aso Mts, and in the area around these mountains, exhibited the PCR-RFLP pattern of *R. kaempferi* (950/420/80 bp: Haplotype B) except for three individuals on Mt Yufudake and one individual on Mt Nekodake in the Aso Range (Table 3).

Miyajima *et al.* (2001) reported that the populations in the Kujyu Mts, Mt Yufudake and the Aso Mts are pure *Rhododendron kiusianum* populations, without any traits from *R. kaempferi*, since there was little observed variation in morphological and flower colour characters. In contrast, the population on Mt Haneyama, which displayed wide variation in flower characteristics, was strongly affected by *Rhododendron kaempferi* and has produced some horticultural varieties.

Studies of introgression based on cpDNA variation have been published for a number of plant species, such as Louisiana irises (Arnold *et al.*, 1991), deciduous azaleas (Kron *et al.*, 1993), and others. The previous study examining two deciduous azalea species, *Rhododendron flammeum* and *R. canescens*, on Stone Mountain, Georgia, USA (Kron *et al.*, 1993) is informative and recorded results similar to those of the present study. Kron *et al.* reported that many individuals in the sample population that were morphologically indistinguishable from *Rhododendron flammeum* possessed the chloroplast

TABLE 3. Distribution of PCR-RFLP patterns of wild azalea populations on the Kujyu Mts, the Aso Mts and surrounding mountains

Sampling sites	Species	No. of individuals	PCR-RFLP patterns (bp)		
			1030/420	950/420/80	1030/230/190
The Kujyu Mts					
Mt Daisen	*R. kiusianum*	3	0	3	0
Mt Yufudake	*R. kiusianum*	18	3	15	0
Mt Haneyama	*R. kiusianum*	9	0	9	0
The Aso Mts					
Mt Nekodake	*R. kiusianum*	6	1	5	0
Mt Ebosidake	*R. kiusianum*	15	0	15	0
Sensuikyou	*R. kiusianum*	15	0	15	0
Mt Sobo	*R. kiusianum*	3	0	3	0
Ume-town (Ohita pref.)	*R. kaempferi*	10	0	10	0

genome of *R. canescens*. This suggests that some of the observed variation in populations of *Rhododendron flammeum* may be due to past introgression from *R. canescens*.

In the results from the mountain areas in the present study, most individuals identified morphologically as *Rhododendron kiusianum* displayed the *R. kaempferi* PCR-RFLP pattern (950/420/80 bp: Haplotype B). The results from the Kujyu Mts and the Aso Mts indicate that natural hybridization and cytoplasmic introgression from *Rhododendron kaempferi* to *R. kiusianum* have occurred in the relatively distant past among the wild populations of these areas. On the other hand, in the case of Mt Yufudake and Mt Haneyama, the *Rhododendron kiusianum* population retains the effects of natural hybridization with *R. kaempferi* in the cpDNA as well as in the variation in flower characters (Miyajima *et al.*, 2001).

Distribution of PCR-RFLP patterns in wild azalea populations in the Kirishima Mts and the Takakuma Mts

Almost all individuals of *Rhododendron kiusianum* and *R. kaempferi* from the Kirishima Mts, the Takakuma Mts and the neighbouring area exhibited a specific PCR-RFLP pattern, 1030/420 bp (Haplotype A) and 950/420/80 bp (Haplotype B), respectively (Table 4). However, a 5.3% inclusion of Haplotypes B and C, indicating the effect of introgression, was detected in one population of *Rhododendron kiusianum* in the Kirishima Mts. Two natural hybrid populations were composed of individuals that displayed the banding pattern of either *Rhododendron kiusianum* or *R. kaempferi*. These results and phenotypic characters indicate that *Rhododendron kiusianum* and *R. kaempferi* are apparently distinct species and that the populations of the intermediate region are natural hybrids of the two species. The diverse range

TABLE 4. Distribution of PCR-RFLP patterns of wild azalea populations on the Kirishima Mts, the Takakuma Mts and neighbouring area

Sampling sites	Species	No. of individuals	PCR-RFLP patterns (bp)		
			1030/420	950/420/80	1030/230/190
The Kirishima Mts					
Mt Karakuni, Ebino plateau	*R. kiusianum*	63	63	0	0
Mt Takachihonomine	*R. kiusianum*	76	72	1	3
Takachihogawara, Hybrid area	Natural hybrid	96	57	36	3
Shinyu hot spring	Natural hybrid	22	9	11	2
Kirishima-cho	*R. kaempferi*	70	0	66	4
The Takakuma Mts					
Mt Onogaradake	*R. kiusianum*	45	45	0	0
Mt Onogaradake	*R. kaempferi*	26	0	26	0
Kagoshima-city (Kagoshima pref.)	*R. kaempferi*	13	0	13	0
Ei-town (Kagoshima pref.)	*R. kaempferi*	7	0	7	0

of phenotypic characters of the hybrid populations is the result of repetitive natural hybridization between the two species (Kron *et al.*, 1993). Additionally, the morphological cline along an altitudinal gradient is likely to be caused by introgression between the two species. Details of the wild azalea populations in the Kirishima Mts were given in the previous study (Kobayashi *et al.*, 2000).

All individuals of *Rhododendron kiusianum* on Mt Onogaradake in the Takakuma Mts possess cpDNA of *R. kiusianum* (1030/420 bp: Haplotype A), although an influence from *R. kaempferi* is indicated in the flower colour and its wide variability (Miyajima *et al.*, 1997).

CONCLUSION

In this study, we used just one cpDNA marker which can distinguish *Rhododendron kiusianum* and *R. kaempferi*, as used in the previous study in the Kirishima Mts (Kobayashi *et al.*, 2000). For the Unzen Mts the results of the distribution of the cpDNA pattern indicate that the populations of the intermediate region are natural hybrids of *Rhododendron kiusianum* and *R. kaempferi*. On the other hand, in the case of the Aso Mts, the Kujyu Mts and the surrounding mountains, past cytoplasmic introgression from *Rhododendron kaempferi* to *R. kiusianum* is suggested due to the fact that morphologically identified *Rhododendron kiusianum* possessed cpDNA of *R. kaempferi.* Further analysis, using another cpDNA marker as well as a nuclear DNA marker (RAPDs, AFLPs and SSR marker), is needed to confirm the hybrid origin of these populations and to resolve the relationships among these wild azaleas.

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