Pre- and Post-fertilization Barriers in Interspecific Hybridization between Evergreen Azalea Species and *Rhododendron uwaense* H. Hara & T. Yamanaka

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Reciprocal crosses between nine evergreen azalea species and *Rhododendron uwaense* were performed to clarify the pre- and post-fertilization barriers in this interspecific hybridization for the purpose of obtaining fragrant evergreen azaleas. Unilateral incompatibility appeared in this hybridization. When evergreen azalea species were used as a seed parent, many pollen tubes stopped elongating in a style and no seed could be obtained. The reverse crosses exhibited inhibition of pollen tube penetration into ovules, chlorophyll deficiency in cotyledons, and death of young seedlings in some cross combinations. These pre- and post-fertilization barriers reduced hybridization but did not arrest it completely. As a result, many putative hybrid seedlings could be obtained. RAPD analysis revealed that 10 of the putative hybrid seedlings examined (two vigorous plants selected from respective crosses of *R. uwaense* #1 × five evergreen azalea species) possessed specific bands derived from both parents. The plastid DNA (ptDNA) of these seedlings was inherited maternally except for seedlings of *R. uwaense* #1 × *R. yedoense* var. poukhanense, and the ptDNA of hybrid ones obtained from *R. uwaense* #1 × *R. yedoense* var. poukhanense was inherited paternally.

**Key Words:** histological observation, plastid DNA inheritance, pollen tube growth, unilateral incompatibility.

**Introduction**

Evergreen azaleas are important ornamental plants in Japan and are used in gardens, street plantings, and containers (Kunishige, 2002). A few species offer fragrance: *Rhododendron mucronatum* (Blume) G. Don, *R. yedoense* Maxim. var. poukhanense (H. Lév.) Nakai, and *R. macrosepalum* Maxim. have a delicate scent, the last like red clover (Galle, 1987). In cross breeding for obtaining high fragrance, North American azaleas [e.g., *R. arborescence* (Pursh) Torr., *R. atlanticum* (Ashe) Rehder, and *R. viscosum* (L.) Torr.] are often used as donor parents (Akabane et al., 1971; Kobayashi et al., 2008). Evergreen and North American azaleas differ from each other in subgenus: the former belong to subgenus *Tsutsusi* section *Tsutsusi* and the latter belong to subgenus *Pentanthera* section *Pentanthera*. In intersubgeneric crosses between these two, there are pre- and post-fertilization barriers, so obtaining a hybrid is not easy (Akabane et al., 1971; Eeckhaut et al., 2003). There are some reports on the production of healthy seedlings from this cross combination, but no really worthwhile plant for commercial use has been obtained (Jaynes, 1976; Kehr, 1966; Pryor, 1973).

*Rhododendron uwaense* H. Hara & T. Yamanaka, belonging to subgenus *Azaleastrum* section *Azaleastrum*, has a woody floral scent. The main scent compounds are nerolidol, \(\alpha\)-farnesene, methyl anisate, and methyl cinnamate (Ikeda and Oyama-Okubo, 2008). Section *Azaleastrum* is an evergreen shrub and has the novel feature that a 1–2-flowered axillary inflorescence is formed in the uppermost 1–4 leaves (Davidian, 1992). These are interesting characteristics possessed by a donor parent for evergreen azalea breeding. For these reasons, it is probable that *R. uwaense* is a species worthy of becoming breeding material for fragrant and multiflorous evergreen azaleas. However, the crossability between evergreen azaleas and *R. uwaense* is unclear because *R. uwaense* is a comparatively new species, registered in 1984 (Hara and Yamanaka, 1984), and its intra- and inter-specific crosses have not yet been reported. A noteworthy fact is that *R. uwaense* is distributed in a limited area of the western part of Shikoku, one of the four main islands of Japan, and has decreased in number to a critical level. This species is now classified as “Endangered (IB)” in the Red Data

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Book of Japan. Interspecific hybridization is one useful method to create new cultivars with novel characteristics. Using *R. uwaense* as the breeding material, we need to indicate the source and to treat the plants carefully. In addition, we have to conserve this rare species from the point of view of the genetic resources and biodiversity.

Interspecific pollinations within the *Rhododendron* genus are usually only successful when the parents belong to the same subgenus (Eeckhaus et al., 2003), and cross incompatibility is commonly recognized in wide crosses between subgenera (Kehr, 1977). From cladistic analysis using molecular data, the subgenus *Azaleastrum* was shown to be polyphyletic, and section *Azaleastrum* species showed a sister group relationship to evergreen azalea species (Kurashige et al., 2001). Goetsch et al. (2005) proposed that the genus *Rhododendron* was subdivided into five subgenera, and the subgenus *Tsutsusi* was reduced to a section of the subgenus *Azaleastrum*. So far, we have investigated pre- and post-fertilization barriers and have evaluated the potential for inter-subgeneric crosses of evergreen azalea species × *R. japonicum* (A. gray) J. V. Suringar f. *flavum* Nakai, belonging to the subgenus *Pentanthera* (Okamoto and Suto, 2004; Ureshino et al., 2000), and Kurume azalea (*R. × obtusum* Planch. cultivars) × scaly rhododendrons, belonging to the subgenus *Rhododendron* (Okamoto and Ureshino, 2010). In the present study, reciprocal crosses between evergreen azalea species and *R. uwaense* were undertaken to clarify the pre- and post-fertilization barriers in this hybridization.

**Materials and Methods**

**Parental plants and crosses**

From 2009 to 2012, nine species of wild evergreen azalea and two plants of *R. uwaense* were used (Table 1), and reciprocal crosses were conducted under unheated greenhouse conditions. Concerning nine evergreen azalea species, one individual was used for the respective species. All plants were five to 21 years old, derived from rooted cuttings and grown at the National Agriculture and Food Research Organization (NARO) Kyushu Okinawa Agricultural Research Center, Kurume, Fukuoka Prefecture, Japan. The function of both male and female gametophytes was checked by self-pollination, and all plants produced germinable seeds.

Flower buds were emasculated at the beginning of anthesis. Anthers were collected from the flower buds at the beginning of anthesis. At a stigma covered with exudates, three flowers for respective crosses were pollinated with fresh and one-year-old pollen stored at 5°C.

Capsules were collected in November, and the number of mature seeds per capsule was counted. In late February, approximately 200 seeds were sown on seedling beds consisting of Kanuma volcanic sand covered with a layer of sphagnum moss. The seedling beds were placed in an unheated greenhouse that reduced the photosynthetic photon flux density (PPFD) by 28% and under intermittent mist. Germinating seeds were counted once every two weeks from April to May, and the color of cotyledons was noted. In June, the seedling beds were transferred to another greenhouse where the PPFD was reduced by 17%. The number of viable seedlings was counted nine months after sowing. For the hybridity, putative hybrid seedlings were distinguished from non-hybrid ones by the color of unexpanded leaves in spring. The unexpanded leaves of the evergreen azalea species and *R. uwaense* were yellow green (the Royal Horticultural Society (RHS) colour chart No. 144C) and greyed red (RHS colour chart No. 184B), respectively, and those of the putative hybrids were olive green (RHS colour chart No. 152C). In this study, *R. eriocarpum* (Hayata) Nakai × *R. uwaense*

| Species                        | Source                  | Year |
|-------------------------------|-------------------------|------|
| *R. eriocarpum* (Hayata) Nakai| Isl. Yakushima, Kagoshima  | 1993 |
| *R. indicum* (L.) Sweet       | Kozu River, Wakayama    | 1992 |
| *R. kaempferi* Planch.        | Mt. Kannon-iwayama, Hokkaido | 2005 |
| *R. kiusianum* Makino         | Mt. Takachiho, Kagoshima | 1993 |
| *R. macrosepaldum* Maxim.     | Kyoto, Kyoto            | 1997 |
| *R. ripense* Makino           | Shimanto River, Kochi   | 1993 |
| *R. serpyllifolium* Miq.      | Gobo, Wakayama          | 1992 |
| *R. simsii* Planch.           | Isl. Amami, Kagoshima   | 1992 |
| *R. yedoense* Maxim. var. *poukhanense* (H. Lév.) Nakai | Isl. Tushima, Nagasaki | 1998 |
| *R. uwaense* H. Har & T. Yamanaka #1 | Uwajima, Ehime | 2003 |
| *R. uwaense* H. Har & T. Yamanaka #2 | Uwajima, Ehime | 2007 |

* Cuttings were performed.

**Table 1.** *Rhododendron* species used in this study.

* A rough estimate (purchased at Yamato Noen Co., Ltd., in 2005).

* From Kurume-shi World Azalea Center.
was excluded because contamination by self-pollination could not be prevented.

**Microscopic observation before and after fertilization**

Pollination utilized the same procedure as described above, and six flowers were pollinated for respective crosses between evergreen azalea species and *R. uwaense* #1 in 2011. The style length of the parental plants was measured for 10 pistils. The style length ratio (SLR) was calculated as follows: pollen parent/seed parent (male/female) style lengths. Ten days after pollination, three pistils were collected from respective seed parents, fixed in FAA (1 formalin:1 acetic acid:18 70% ethanol, volume) for 24 h, after which they were transferred to 70% ethanol and stored at room temperature. The fixed pistils were softened by soaking in 1 N NaOH at 38°C for 16 h, washed in distilled water, and stained with 0.1% aniline blue in 0.1 N K$_3$PO$_4$ for a minimum of 3 h. After the styles and ovaries were separated, the latter were cut lengthwise in half and carpels were picked out from each ovary using tweezers. The styles and carpels were placed on separate microscope slides and subjected to gentle pressure on a coverslip. The number of pollen tubes in the lower style and the number of ovules penetrated by pollen tubes were counted, the latter were cut lengthwise in half and carpels were picked out from each ovary using tweezers. The styles and carpels were placed on separate microscope slides and subjected to gentle pressure on a coverslip. The number of ovules penetrated by pollen tubes were counted (Fig. 1).

**Molecular analysis of hybridity and plastid DNA inheritance**

In 2011, 10 putative hybrid seedlings (two vigorous plants selected from respective crosses of *R. uwaense* #1 × five evergreen azalea species, *R. eriocarpum* (Hayata) Nakai, *R. kaempferi* Planch., *R. macrosepalum*, *R. ripense* Makino, and *R. yedoense* var. *poukhanense*) and their parents were used. Total genomic DNA was extracted from approximately 70 mg of frozen leaves by a modified CTAB method (Kobayashi et al., 1998). Random amplified polymorphic DNA (RAPD) analysis was conducted with CMN-A02 (5'-GCCAGCTGTACG-3') and CMN-B27 (5'-CAGGGCCAG-3') common primers (BEX, Co., Ltd., Tokyo, Japan) to confirm the hybridity of the seedlings. Amplifications were performed in 25 μL of reaction solution containing 20 ng of genomic DNA, 0.5 μM primers, 0.1 mM dNTPs, 2 mM MgCl$_2$, 1 × the original reaction buffer, and 0.5 units *Taq* DNA polymerase (La Roche Ltd., Basel, Switzerland). DNA was amplified with a DNA thermal cycler (TP240; Takara Bio Inc., Shiga, Japan) as follows: 1 cycle of 30 sec at 90°C; 45 cycles of 30 sec at 94°C, 2 min at 37°C, and 3 min at 72°C; and 1 cycle of 7 min at 72°C. The amplified products were electrophoresed in 1.5% agarose gels (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) with 1 × TBE. The gel was stained with an ethidium bromide solution and observed under ultraviolet illumination. To clarify the plastid DNA (ptDNA) inheritance of the seedlings, polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis at the chloroplast *trnL–trnF* and *trnG–trnM* intergenic regions was conducted as in a previous report (Itabashi et al., 2008). For the investigation of ptDNA inheritance, molecular marker techniques such as RFLP and PCR-RFLP have been used (Chat et al., 1999; Yao et al., 1994). These methods are very powerful tools to detect polymorphism among species. However, to identify polymorphism with these methods, mutation in a restriction site is required. Therefore, it is difficult to detect polymorphism among closely related species. As a more sensitive polymorphic marker, the PCR-SSCP method has been used to detect polymorphism within species or among some closely related species and to investigate the inheritance pattern of ptDNA (Chen et al., 2002; Shiraishi et al., 2015).
2001). Furthermore, the PCR-SSCP method has been used to study chloroplast DNA inheritance in azaleas (Itabashi et al., 2008; Ureshino et al., 2006, 2010). Therefore, we used this method to clarify ptDNA inheritance.

**Results**

Capsules set only when *R. uwaense* was used as a seed parent (Table 2). In crosses using *R. uwaense* as the seed parent, the number of mature seeds per capsule varied from 73 to 313, and the control cross (*R. uwaense* #2 × *R. uwaense* #1) had 518 (Table 2). The germination rate varied from 40.0% to 83.0%, with 53.0% in the control (Table 2). The cotyledons were green, pale green, and white. The albino rate varied from 0% to 28.3%, and 10 out of 16 crosses had a rate of less than 5% (Table 3). Albino seedlings were not found in the control. The pale green and white seedlings wilted until the formation of the first true leaf. The percentage of viable seedlings/germinating seedlings excluding albinos (survival rate) varied from 22.8% to 67.5%, with 84.9% in the control (Table 3). From these data, we calculated the estimated number of viable seedlings per pollinated flower for respective crosses. This value ranged from 14.3 to 68.3, with 233.1 in the control (Table 3), and showed variation among the pollen parents (Table 4); more viable seedlings were produced when *R. uwaense* was pollinated by

| Seed parent | Pollen parent | Year | No. of capsules set/pollinated flower | No. of mature seeds per capsule | Seed germination (%) |
|-------------|---------------|------|--------------------------------------|---------------------------------|----------------------|
| *R. indicum* | *R. uwaense* #1 | 2011 | 0/3                                  | 0                               | —                    |
| *R. kaempferi* | *R. uwaense* #1 | 2009 | 0/3                                  | 0                               | —                    |
| *R. kiusianum* | *R. uwaense* #1 | 2009 | 0/3                                  | 0                               | —                    |
| *R. macrosepalum* | *R. uwaense* #1 | 2009 | 0/3                                  | 0                               | —                    |
| *R. ripense* | *R. uwaense* #1 | 2009 | 0/3                                  | 0                               | —                    |
| *R. serpyllifolium* | *R. uwaense* #1 | 2011 | 0/3                                  | 0                               | —                    |
| *R. simsii* | *R. uwaense* #1 | 2009 | 0/3                                  | 0                               | —                    |
| *R. yedoense* var. poukhanense | *R. uwaense* #1 | 2009 | 0/3                                  | 0                               | —                    |
| *R. indicum* | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. kaempferi* | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. kiusianum* | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. macrosepalum* | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. ripense* | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. serpyllifolium* | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. simsii* | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. yedoense* var. poukhanense | *R. uwaense* #2 | 2012 | 0/3                                  | 0                               | —                    |
| *R. uwaense* #1 | *R. eriocarpum* | 2012 | 3/3                                  | 313                             | 47.8                 |
| *R. indicum* | *R. eriocarpum* | 2011 | 3/3                                  | 143                             | 66.5                 |
| *R. kaempferi* | *R. eriocarpum* | 2009 | 3/3                                  | 162                             | 67.1                 |
| *R. kiusianum* | *R. eriocarpum* | 2009 | 3/3                                  | 85                              | 72.5                 |
| *R. macrosepalum* | *R. eriocarpum* | 2009 | 3/3                                  | 133                             | 83.0                 |
| *R. ripense* | *R. eriocarpum* | 2009 | 3/3                                  | 106                             | 74.3                 |
| *R. serpyllifolium* | *R. eriocarpum* | 2011 | 3/3                                  | 81                              | 53.0                 |
| *R. simsii* | *R. eriocarpum* | 2009 | 3/3                                  | 148                             | 40.0                 |
| *R. yedoense* var. poukhanense | *R. eriocarpum* | 2009 | 3/3                                  | 73                              | 55.3                 |
| *R. uwaense* #2 | *R. eriocarpum* | 2012 | 3/3                                  | 281                             | 61.2                 |
| *R. indicum* | *R. eriocarpum* | 2012 | 3/3                                  | 137                             | 67.3                 |
| *R. kaempferi* | *R. eriocarpum* | 2012 | 3/3                                  | 252                             | 51.0                 |
| *R. kiusianum* | *R. eriocarpum* | 2012 | 3/3                                  | 93                              | 77.7                 |
| *R. macrosepalum* | *R. eriocarpum* | 2012 | 3/3                                  | 123                             | 82.3                 |
| *R. ripense* | *R. eriocarpum* | 2012 | 3/3                                  | 118                             | 65.2                 |
| *R. serpyllifolium* | *R. eriocarpum* | 2012 | 3/3                                  | 156                             | 63.2                 |
| *R. simsii* | *R. eriocarpum* | 2012 | 3/3                                  | 133                             | 66.1                 |
| *R. yedoense* var. poukhanense | *R. eriocarpum* | 2012 | 3/3                                  | 124                             | 64.7                 |

* About 200 mature seeds were sown.
Table 3. Color of cotyledons and seedling survival rate in crosses between evergreen azalea species and *Rhododendron uwaense*.

| Seed parent         | Pollen parent          | % of seedlings | Seedling survival (%) | Estimated number of viable seedlings per pollinated flower¹ |
|---------------------|------------------------|----------------|-----------------------|------------------------------------------------------------|
| R. indicum          | R. uwaense #1          | —              | —                     | 0                                                          |
| R. kaempferi        | —                      | —              | —                     | 0                                                          |
| R. kiusianum        | —                      | —              | —                     | 0                                                          |
| R. macrosepalum     | —                      | —              | —                     | 0                                                          |
| R. ripense          | —                      | —              | —                     | 0                                                          |
| R. serpyllifolium   | —                      | —              | —                     | 0                                                          |
| R. simsii           | —                      | —              | —                     | 0                                                          |
| R. yedoense var. poukhanense | —   | —              | —                     | 0                                                          |
| R. indicum          | R. uwaense #2          | —              | —                     | 0                                                          |
| R. kaempferi        | —                      | —              | —                     | 0                                                          |
| R. kiusianum        | —                      | —              | —                     | 0                                                          |
| R. macrosepalum     | —                      | —              | —                     | 0                                                          |
| R. ripense          | —                      | —              | —                     | 0                                                          |
| R. serpyllifolium   | —                      | —              | —                     | 0                                                          |
| R. simsii           | —                      | —              | —                     | 0                                                          |
| R. yedoense var. poukhanense | —   | —              | —                     | 0                                                          |

Table 4. Analysis of variance on the estimated number of viable seedlings per pollinated flower in *Rhododendron uwaense* × nine evergreen azalea species.

| Source                          | Degree of freedom | Sum of squares | F ratio  | P value   |
|---------------------------------|-------------------|----------------|----------|-----------|
| R. uwaense                      | 1                 | 74.42          | 1.0372   | 0.3383    |
| Evergreen azalea species        | 8                 | 3107.43        | 5.4134   | 0.0139    |
| Error                           | 8                 | 574.03         |          |           |
| Total                           | 17                | 3755.88        |          |           |

R. eriocarpum than by R. kiusianum Makino, R. serpyllifolium Miq., and R. yedoense var. poukhanense on the basis of Tukey’s HSD test. Most of the green seedlings were putative hybrids that had olive green unexpanded leaves and were healthy two years after sowing. The growth of the putative hybrid seedlings compared favorably with that of control ones (Fig. 2).
The male/female SLR of *R. uwaense* #1 × evergreen azalea species varied from 0.71 to 2.65, and that of reverse crosses varied from 0.38 to 1.41, with 0.88 in the control (Table 5). In the crosses of *R. uwaense* #1 × evergreen azalea species, the number of pollen tubes in the lower style exceeded 200 (Table 5). In the crosses of *R. uwaense* #1 × evergreen azalea species, the number of pollen tubes in the lower style exceeded 200 (Table 5). In the reverse crosses, many pollen tubes stopped elongating at the mid-portion of the style and several abnormalities of arrested pollen tube tips were detected, including mainly tapered and swollen types (Fig. 3A, B). In addition, spiraling tubes without defined callose plugs were found occasionally (Fig. 3C). As a result, there were fewer than 10 pollen tubes in the lower style, and the percentage of ovules penetrated by pollen tubes varied from 0% to 2.4% (Table 5). In the control, there were 400 to 600 pollen tubes in the lower style, and 86.1% of ovules were penetrated by pollen tubes (Table 5). In the crosses of *R. uwaense* #1 × evergreen azalea species, the percentage of fertilized ovules per ovule penetrated by pollen tubes varied from 71.5% to 84.3%, with 82.8% in the control (Table 5). For the reverse crosses, microscopic observation was not performed because *R. uwaense*’s tubes seldom penetrated into the ovules of evergreen azalea species.

RAPD analysis with CMN-A02 and CMN-B27 primers indicated that all 10 seedlings examined (*R. uwaense* #1 × evergreen azalea species) were of hybrid origin, having specific bands derived from both parents, that is, the seedlings of *R. uwaense* #1 × *R. eriocarpum*, *R. kaempferi*, *R. macrosepalum*, and *R. ripense* were confirmed as hybrids by CMN-A02 (Fig. 4). Similarly, those of *R. uwaense* #1 × *R. eriocarpum*, *R. kaempferi*, and *R. yedoense* var. *poukhanense* were confirmed as hybrids by CMN-B27 (Fig. 5). A difference in phenotype was detected between evergreen azalea species and *R. uwaense* #1 by PCR-SSCP analysis in the chloroplast *trnL–trnF* and *trnG–trnM* intergeneric regions (Fig. 6). All seedlings examined had the haplotype of *R. uwaense* #1, except for two seedlings of *R. uwaense* #1 × *R. yedoense* var.

![Fig. 2. Putative hybrid seedlings of *R. uwaense* #1 × *R. macrosepalum* three years after sowing.](image)

### Table 5. Style length ratio, number of pollen tubes in a style, percentage of ovules penetrated by pollen tubes, and fertilized ovule rate in crosses between evergreen azalea species and *Rhododendron uwaense*.

| Seed parent | Pollen parent | Style length ratio* | No. of pollen tubes in lower style | % of ovules penetrated by pollen tubes | % of fertilized ovules* |
|-------------|---------------|---------------------|-----------------------------------|----------------------------------------|------------------------|
| *R. indicum* | *R. uwaense* #1 | 0.43 | <5 | 1.4 | — |
| *R. kaempferi* | *R. uwaense* #1 | 0.46 | <5 | 0.2 | — |
| *R. kiusianum* | *R. uwaense* #1 | 1.41 | <5 | 0.1 | — |
| *R. macrosepalum* | *R. uwaense* #1 | 0.45 | <10 | 2.4 | — |
| *R. ripense* | *R. uwaense* #1 | 0.38 | <5 | 0.4 | — |
| *R. serpyllifolium* | *R. uwaense* #1 | 0.88 | <5 | 0 | — |
| *R. sinii* | *R. uwaense* #1 | 0.39 | <5 | 1.4 | — |
| *R. yedoense* var. *poukhanense* | *R. uwaense* #1 | 0.38 | <5 | 0 | — |
| *R. uwaense* #1 | *R. eriocarpum* | 1.88 | 500–700 | 44.3 | 80.5 |
| *R. indicum* | *R. eriocarpum* | 2.32 | 300–500 | 34.4 | 71.5 |
| *R. kaempferi* | *R. eriocarpum* | 2.17 | 400–600 | 27.7 | 84.3 |
| *R. kiusianum* | *R. eriocarpum* | 0.71 | 300–500 | 35.5 | 79.6 |
| *R. macrosepalum* | *R. eriocarpum* | 2.21 | 400–500 | 34.4 | 81.0 |
| *R. ripense* | *R. eriocarpum* | 2.65 | 500–600 | 26.5 | 75.2 |
| *R. serpyllifolium* | *R. eriocarpum* | 1.14 | 200–400 | 29.0 | 72.9 |
| *R. sinii* | *R. eriocarpum* | 2.58 | 200–300 | 26.3 | 76.5 |
| *R. yedoense* var. *poukhanense* | *R. eriocarpum* | 2.65 | 300–400 | 28.4 | 69.7 |

* Pollen parent/seed parent mean style lengths.

* Ovules having a zygote with free nuclear division of endosperm/ovules penetrated by pollen tubes.
Fluorescence microscopy of incompatible pollen tube behavior, *Rhododendron kaempferi × R. uwaense* #1. A, tapered tube tip (arrow); B, swollen tube tip; C, spiraling tube without defined callose plugs. Scale bar = 100 μm.

The genus *Rhododendron* is divided into eight subgenera (Chamberlain et al., 1996). *Tsutsusi* (evergreen azaleas except trifoliolate azaleas), *Pentanthera* (deciduous azaleas), *Rhododendron* (scaly rhododendrons and Malesian rhododendrons), and *Hymenanthes* (non-scaly rhododendrons) are important subgenera for horticultural use, and breeders have attempted crosses among these four classes (Kehr, 1977). Subgenus *Azaleastrum* species, however, were rarely used in interspecific hybridization.

In this study, unilateral incompatibility appeared in the hybridization between evergreen azalea species and *R. uwaense*. Most pollen tubes of *R. uwaense* did not reach the ovaries of the evergreen azalea species. Kho and Baër (1973) demonstrated that unilateral interspecific incompatibility was observed in *Rhododendron* crosses, which was attributed to the inability of pollen of a short-styled species to traverse the style of a longer-styled species. Williams and Rouse (1988) reported that successful interspecific fertilization was closely related to male/female SLR in interspecific crosses among species belonging to subgenus *Rhododendron* section *Vireya*. They determined that the crosses with a male/female SLR of less than 0.2 or greater than 6.0 were all unsuccessful and that the probability of success increased as the SLR approached 1.0. In our unsuccessful crosses, the range of SLR was 0.38 to 1.41, implying that SLR did not restrict *R. uwaense*’s pollen tube growth into evergreen azalea pistils.
Williams et al. (1982) reported that incompatible pollen tubes exhibited errors of tip growth and callose deposition anomalies after foreign pollination in the genus Rhododendron. In the crosses of evergreen azalea species × R. uwaense, many pollen tubes disappeared rapidly at the mid-portion of the style, and abnormal morphology of tube tips was observed. Hence, we consider that pollen tube growth is arrested by errors of tube tip growth. Therefore, crosses of evergreen azalea species × R. uwaense suffer from a pre-fertilization barrier and yield no seed.

Using R. uwaense as the seed parent, the arrest of pollen tube growth was not found in the pistil and many pollen tubes could reach the ovaries. The percentage of ovules penetrated by pollen tubes was relatively low compared with that of the control, indicating that the inhibition of pollen tubes penetrating into the ovules is one cause of the pre-fertilization barrier. Kaul et al. (1986) determined that incompatible interspecific crosses exhibited seed failure until 15 days after pollination because of failure of zygote and/or endosperm development. In our crosses, many ovules penetrated by pollen tubes developed normally 20 days after pollination, indicating that seed failure did not cause the post-fertilization barrier. The number of mature seeds per capsule was relatively low compared with that of the control, and it seems that this mainly arises from the inhibition of pollen tubes penetrating into ovules. Okamoto and Suto (2004) reported that the lack of germination was one cause of a post-fertilization barrier in crosses of evergreen azalea species × R. japonicum. In this study, the germination rate was, on the whole, at a high or similar level compared with that in the control, indicating that no particular incompatibility appears in this location. The white color of the cotyledons was the result of hybrid chlorophyll deficiency, and caused by interspecific incompatibility between the plastid genome and the nuclear genome, that is, plastome-genome incompatibility (Ureshino et al., 1999). Albino seedlings were detected in many crosses, but the albino rate was generally low. Therefore, the chlorophyll defect is one cause of the post-fertilization barrier, although its effect may be limited. All crosses had a survival rate lower than that of the control, indicating that death of young seedlings (hybrid inviability) is one cause of the post-fertilization barrier. Thus, the crosses of R. uwaense × evergreen azalea species have pre- and post-fertilization barriers; each barrier reduces hybrid-
zation, but does not arrest it completely, and healthy put-
tative hybrid seedlings can be obtained. Amongst these
crosses, the cross of R. uwaense × R. eriocarpum ex-
hibited a greater estimated number of viable seedlings
per pollinated flower than those of R. uwaense × R. kiusianum, R. serpilifolium, and R. yedoense var.
poukhanense. In this study, one individual was used for the
respective evergreen azalea species. We consider
that it is necessary to use more plants for the respective
species in order to be able to discuss the differences in
this trait among species.

It is known that the mode of ptDNA inheritance is
biparental in Rhododendron (Corriveau and Coleman,
1988; Miyamura et al., 1987; Nagata et al., 1999).
Ureshino et al. (1999) reported that ptDNA of green
seedlings obtained from evergreen azaleas × R. japonicum was derived from R. japonicum (paternal
inheritance) and that of albino ones was inherited ma-
ternally. Okamoto and Ureshino (2010) reported that
the ptDNA of vigorous seedlings obtained from ever-
green azaleas × scaly rhododendron, R. keiskei Miq.,
was derived from R. keiskei (paternal inheritance).
These phenomena imply that plastome-genome incom-
patibility occurs between a plastid genome from ever-
green azaleas and a nuclear genome from foreign
subgenera. In this study, the hybrid seedlings obtained
from R. uwaense × evergreen azalea species had ptDNA
derived from R. uwaense (maternal inheritance) except
for R. uwaense #1 × R. yedoense var. poukhanense. This
ptDNA inheritance is similar to the above phenomenon.
The ptDNA inheritance detected in the cross of R. uwaense #1 × R. yedoense var. poukhanense is an
exceptional case because plastome-genome incompati-
bility occurs between a plastid genome from R. yedoense var.
poukhanense and a nuclear genome from R. uwaense is
resolved. Ureshino and Miyajima (2002) reported that
a triploid green seedling with 2x and 1x nuclear genomes
from evergreen azalea and R. japonicum, respectively,
had ptDNA derived from the evergreen azalea. In addi-
tion, plastome-genome incompatibility was broken by a
relative increase of the gene from evergreen azaleas in
the nuclear gene of hybrid seedlings (Ureshino et al.,
2010). The mechanism of ptDNA inheritance detected in
the cross of R. uwaense #1 × R. yedoense var.
poukhanense may become clear when ploidy and iso-
zyme analyses are performed.

We have carried out inter-subgeneric crosses of ever-
green azaleas with deciduous azalea, R. japonicum
(Okamoto and Suto, 2004), and scaly rhododendrons,
R. keiskei and R. mucronatum Turcz. var. ciliatum
Nakai (Okamoto and Ureshino, 2010). These hybridizations
had several causes of pre- and post-fertilization barriers,
and the production of viable seedlings per pol-
linated flower was generally low or absent. In the
crosses of R. uwaense × evergreen azalea species, many
viable seedlings were obtained from one pollinated
flower, despite pre- and post-fertilization barriers being
detected. The difficulty of creating interspecific hybrids
increases along with the phylogenetic distance between
the parents (Sharma, 1995). Section Azaleastrum spe-
cies were shown to be nested within evergreen azalea
species based on molecular phylogeny with mark and
trnK (Kurashige et al., 2001) and RPB2-I (Goetsch
et al., 2005) sequence analysis. Our findings in this
study are consistent with their phylogenetic data. A
similar case was found in interfgeneric hybridization be-
tween Menziesia multiflora var. purpurea (Makino)
Ohwi and evergreen azalea species (Kita et al., 2005).
Menziesia species were placed in the same clade of ever-
green azalea species based on molecular phylogenetic
data (Kron, 1997; Kurashige et al., 2001). These results
demonstrate that molecular information may bring about
a better understanding of the rationale for the de-
gree of reproductive isolation observed.

In this study, molecular screening for true hybrids
was not performed for all seedlings because this re-
search focused on pre- and post-fertilization barriers be-
tween evergreen azalea species and R. uwaense. Further
research will focus on hybrid detection using molecular
techniques and the scents of hybrid plants.

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