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Lack of Prezygotic Reproductive Isolation between *Rhododendron eriocarpum* and *R. indicum*; Overlapping Flowering Phenology and Cross Compatibility

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Flowering phenology in *Rhododendron eriocarpum* and *R.indicum* in Yakushima Island and its surrounding islands and cross compatibility between the species were investigated. We found that their flowering periods partially overlapped from May to early June. The flowering peaks of the riverside populations were variable and longer than those of the seaside populations where the flowering patterns synchronized. In artificial intra– and inter–specific outcross experiment, >60% crosses were successful. Weak self–incompatibility was observed in the both species. Their cross compatibility has been maintained in their presumptive hybrids. These results indicate that there is little prezygotic reproductive isolation between the two species and the lack of such reproductive isolation is likely to have contributed the diversification of 'Satsuki' cultivars.

INTRODUCTION

The genus *Rhododendron* L. (Ericaceae) including over 1000 species is widely distributed in the Northern Hemisphere and subdivided into eight subgenera and 12 sections (Champberlain *et al.*, 1996), among which fifty species are native to Japan (Yamazaki, 1996). The species in the section Tsutsusi of subgenus Tsutsusi are the most important evergreen azaleas and used as resources of ornamentals. Satsuki azalea (*Rhododendron indicum* (L.) Sweet) is one of them and used for such many horticultural purpose as gardening, hedge, greening and Bonsai. It is said that there are more than 2,000 cultivars and that the cultivars contain natural hybrids with *R. eriocarpum* (Hayata) Nakai, because the distribution of the two species overlaps in Yakushima Island and there are abundant morphological variations (Kunishige and Kobayashi, 1980).

Rhododendron eriocarpum and *R. indicum* are diploid (2n=26) evergreen azaleas (section Tsutsusi), 0.5–1.5 m tall shrubs and endemic to Japan (Yamazaki, 1996). *Rhododendron eriocarpum* is distributed in Tokara archipelago, Mishima archipelago, Tanegashima Island, Yakushima Island and the southernmost part of Satsuma Peninsula of Kyushu Island, the southwestern mainland of Japan, while *R. indicum* is in Kinki and Chubu districts in the central part of Honshu Island, the largest mainland of Japan and Yakushima Island. They are morphologically distinguishable by corolla color (light purple in *R. eriocarpum* v.s. deep red in *R. indicum*), number of stamina (ten in *R. eriocarpum* v.s. five in *R. indicum*) and leaf shape (obovate, broadly obovate in *R. eriocarpum* v.s. lanceolate in *R. indicum*) (Yamazaki, 1996). *Rhododendron eriocarpum* grows on a sunny rocky stretch and forest margin in seaside, while *R. indicum* grows along mountain streams and sometimes on an open summit in Yakushima Island.

The degree of reproductive isolation among related species is an important factor influencing genetic integrity of a species and the probability of the formation of hybrids (Levin, 1978; Grant, 1981; Harrison, 1993; Avise, 1994; Arnold, 1997; Alarcón and Campbell, 2000; Mráz *et al.*, 2005). There is no knowledge on reproductive isolation mechanisms for *R. eriocarpum* and *R. indicum* in Yakushima Island. In this study, we investigated flowering phenology and cross–compatibility of the two species to determine whether they work as prezygotic barriers or not.

MATERIALS AND METHODS

Study populations

Six (Ys1–2, 4–7), 11 (Yr1–8) and two (Ym1–2) populations of Yakushima Island were selected for the study. They are located in seaside forest margin, along rapid mountain streams and in open summits, respectively (Fig. 1B, Table 1). Two additional populations of *R. eriocarpum* in Nakanoshima Island (NKN) and Kuchinoerabujima Island (KCN) were supplied for artificial cross experiment (Fig. 1A, Table 1).

Individuals in NKN and KCN where only *R. eriocarpum* is distributed were morphologically identified as *R. eriocarpum*. All seaside populations in Yakushima Island seemed to contain hybrids and can be roughly divided into two groups; Ys1–2 and 4–5 are consisted of the individuals morphologically rather similar to *R. eriocarpum* (*Eriocarpum*–like type), whereas Ys6–7 are consisted of individuals rather similar *to R. indicum* (*Indicum*–like type). All riverside and summit popula-

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tions were judged to be *R. indicum*. The riverside population of Yr7 was, however, divided into four subpopulations Yr7(1)–(4) by 200 m distance interval from Torohki fall at the river mouth along Tainoko–river, because Yr7 seemed to have contributed to the hybridization with a seaside population Ys6.

Flowering pattern and fruit set under natural condition

Flowering patterns and natural fruit sets of seven to 21 individuals of each population were observed in 2006 (Table 1, Fig. 1). Number of flowers was counted every two weeks from 18 April to 15 July. Flowering that occurred out of the observation period was excluded in phenology analysis. Number of mature fruits per total flowers was used for evaluation of fruiting success.

Pollinator visitation was investigated in four seaside populations (Ys1–2, Ys4 and Ys6) and three riverside populations (Yr3, Yr5 and Yr7) for about five to 10 plants in each population to examine the relationship to fruit set. Periods of observation varied from one to eight hours during daytime (from 9:00 to 16:00) in 2001 and 2003, because populations with lower pollinator frequency needed longer observation time.

Artificial cross

Plants raised from the cuttings of *R. eriocarpum* of Nakanoshima Island (NKN), Kuchinoerabujima Island (KCN), *R. indicum* (Yr6–7), *Eriocarpum*–like individuals (Ys4) and *Indicum*–like individuals (Ys6) were used. Self– and cross–pollinations were conducted in a plastic house shaded with black cloth in our experimental field in 2005, 2006 and 2007. Self pollination was conducted by hand with fresh pollen of the same plant. Flowers for cross pollination were emasculated before

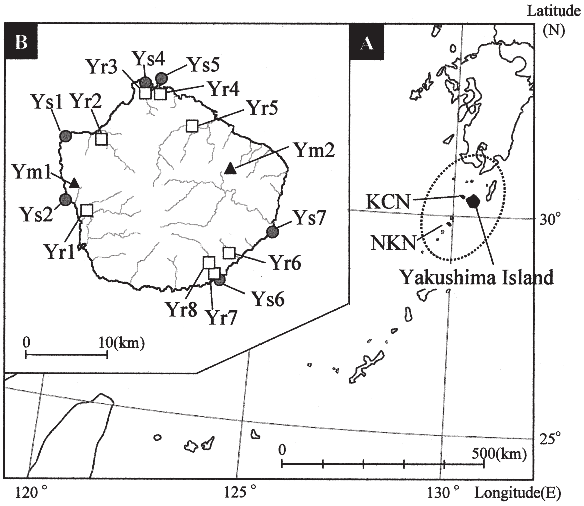

Fig. 1. Geographic distribution of *Rhododendron* populations investigated in this study. A, the location of Yakushima Island and two populations neighboring to Yakushima Island. A circle by dotted lines indicates the distribution of *R. eriocarpum*. *R. indicum* is distributed only in Yakushima Island. B, the location of populations in Yakushima Island. ●; seaside populations, □; riverside populations, ▲; summit populations. See Table 1 for abbreviation of population names.

Table 1. Geographic location of *Rhododendron* populations for the observations of fruit set and flowering patterns in this study

Population ID	Location	Latitude (N)	Longtitude (E)	Altitude (m)	Habitat	No. of accessions for floweirng pattern and fruit set observations	
						individuals	flowers/individuals
NKN	Nakanoshima Isl.	29°51′	129°51′	Unknown	volcanic slope	–	–
KCN	Kuchinoerabujima Isl.	30°26′	130°13′	Unknown	volcanic slope	–	–
Ys1	Nagata cape, Yakushima Isl.	30°23′	130°22′	30	seaside	15	36.1
Ys2	Tategami, Yakushima Isl.	30°19′	130°23′	20	seaside	20	33.6
Ys4	Ooura, Yakushima Isl.	30°27′	130°28′	10	seaside	18	55.8
Ys5	Yahazu cape, Yakushima Isl.	30°28′	130°29′	10	seaside	19	44.4
Ys6	Torohki fall, Yakushima Isl.	30°15′	130°35′	3	seaside	16	9.6
Ys7	Harutahama, Yakushima Isl.	30°17′	130°38′	5	seaside	12	32.1
Ym1	Hidsukushi, Yakushima Isl.	30°19′	130°23′	410	summit	11	9.5
Ym2	Mt. Aikodake, Yakushima Isl.	30°21′	130°36′	1235	summit	18	46.3
Yr1	Ookawa River, Yakushima Isl.	30°18′	130°25′	10	riverside	20	19.5
Yr2	Nagata River, Yakushima Isl.	30°23′	130°26′	30	riverside	21	21.8
Yr3	Isso River, Yakushima Isl.	30°26′	130°28′	40	riverside	17	17.8
Yr4	Nunobiki fall, Yakushima Isl.	30°26′	130°29′	100	riverside	19	21.5
Yr5	Miyanoura River, Yakushima Isl.	30°24′	130°32′	20	riverside	20	26.4
Yr6	Otakumi River, Yakushima Isl.	30°16′	130°36′	300	riverside	15	30.2
Yr7 (1)	Tainoko River, low, Yakushima Isl.	30°15′	130°35′	56	riverside	7	7.6
Yr7 (2)	Tainoko River, low, Yakushima Isl.	30°15′	130°35′	41	riverside	19	21.6
Yr7 (3)	Tainoko River, low, Yakushima Isl.	30°15′	130°35′	24	riverside	14	27.4
Yr7 (4)	Tainoko River, low, Yakushima Isl.	30°15′	130°35′	18	riverside	11	29.8
Yr8	Tainoko River middle, Yakushima Isl.	30°15′	130°34′	340	riverside	18	30.6
Average						33	27.5
Total						620	521.6

anthesis. All the flowers for the crossings were covered by paper bags before anthesis and re–bagged after pollination treatments. Fruit set was determined four months in 2005 and 2006, and 40 days in 2007 after pollination.

RESULTS AND DISCUSSION

Flowering pattern and fruit set under natural conditions

Flowering patterns synchronized in the seaside populations (Ys1–2 and Ys4–7) that bloomed from late April to mid June with a flowering peak in May (Fig. 2). Flowering peak of the riverside populations (Yr1–8) varied ranging from mid May to mid July and the period was longer than that in the seaside populations. Plants of the summit populations flowered later than those of the other populations. Ym2 individuals, which grow at 1200 m altitude, started flowering in early May and the flowering lasted until late July. It is noticeable that the flowering periods among three different habitats overlap during more than two months in spite that their patterns were variable.

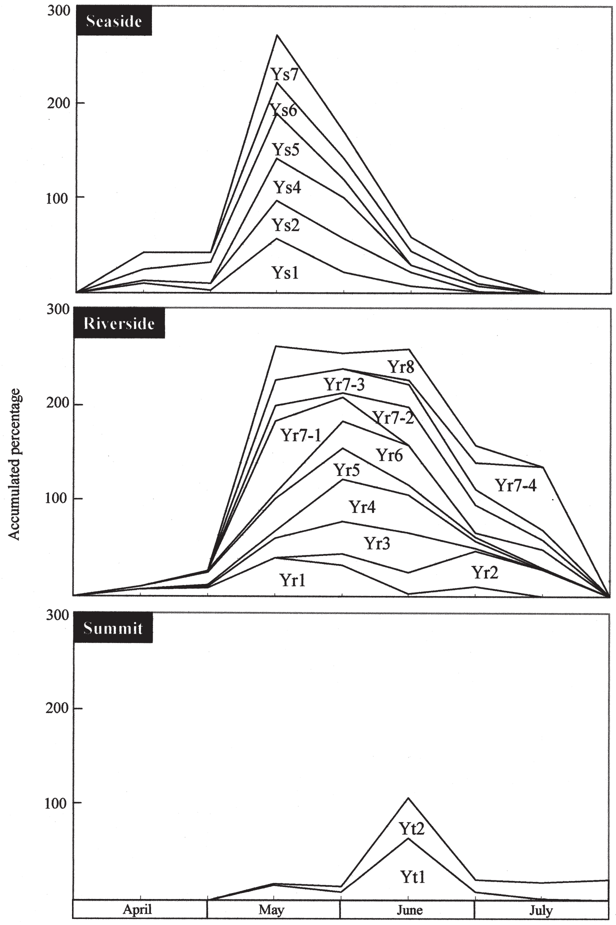

Fig. 2. Flowering patterns in seaside, riverside and summit *Rhododendron* populations. See Table 1 for abbreviation of population names. One diagram expresses the changes in flowering percentage in one population. (number of flowers at the time of observation conducted every two weeks)/(total number of flowers throughout the full observation periods) × 100.

Variations in flowering patters may be due to the differences in microhabitats which strongly influence plant phenology (Tarasjev, 1997). Individuals in the seaside populations grow in a rather uniform environment on sunny rocky stretch near the sea, whereas those in the riverside populations grow in more various environments; they grow not only on sunny moisture rocks nearby the stream but also under a shaded forest edge along the stream, and the direction of valley also influences habitat's daylight and temperature. Four subpopulations, Yr7 (1)–(4) had different flowering patterns, despite that they grew closely with only 200 m distance. The longer flowering period of riverside *R. indicum* seems to be the reproductive strategy as a rheophyte to avoid blooming flowers from being washed away by frequent floods as suggested by Van Steenis (1981) and Kato (2003).

Average fruit set in *Eriocarpum*–like seaside populations (Ys1–2 and 4–5) was about 59%, while those of *Indicum*–like seaside populations (Ys6–7) and of riverside populations (Yr1–8) were around 22%. Summit populations (Ym1–2) showed the lowest fruit set of 3.6% (Fig. 3). Number of pollinators per hour was 9.9 in *Eriocarpum*–like seaside populations (Ys1–2 and Ys4–5), 2.7 in *Indicum*–like seaside populations (Ys6–7) and 1.4 in riverside populations (Fig. 3). Generally, pollinator visitation highly enhanced fruit set in entomophilous flowers (Kudo, 1993; Ng and Corlett, 2000). Considering that both species and presumptive hybrids showed high fertility in artificial cross experiment (see below, Table2), low pollinator frequency seems to cause lower fruit set of *Indicum*–like seaside population (Ys6–7) and riverside populations.

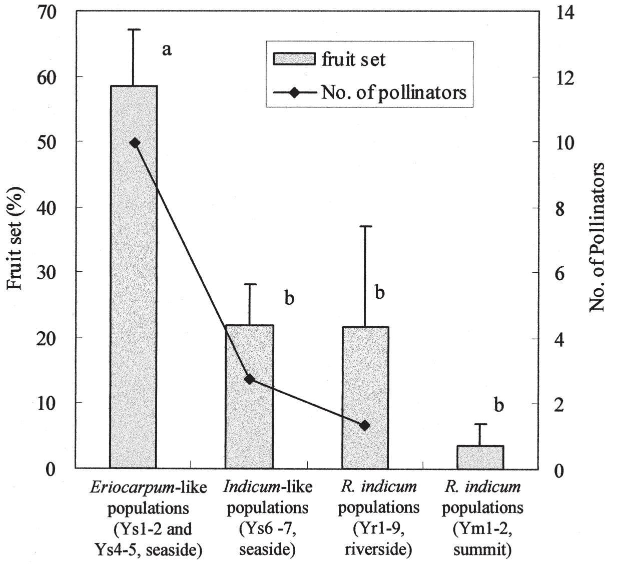

Fig. 3. Average fruit set (%) and number of observed pollinators. Pollinator frequencies represent the results from observation in Ys1, Ys2 and Ys4 for *Eriocrapum*–like hybrids, Ys6 for *Indicum*–like hybrids, and Yr5 for riverside *R. indicum*. Pollinator observation was not conducted in summit populations. Different letters mean significant difference ($P<0.05$) based on Tukey's HSD (honestly significant difference) test.

Table 2. Percentage of fruit set in self– and cross–pollination treatments. Number of fruits formed and number of flowers tested are shown in parentheses

Cross	Seed parent	Pollen parent			
		R. eriocarpum NKN, KCN	Hybrid poplations		*R. indicum* Yr6, Yr7
			Ys4	Ys6	
self		40 (2/5)	20 (2/5)	20 (2/10)	37.5 (3/8)
outcross	*R. eriocarpum* (NKN, KCN)	100 (7/7)	100 (2/2)	100 (5/5)	91.7 (11/12)
	Eriocarpum–like hybrids (Ys4)	–	75 (3/4)	80 (4/5)	60 (3/5)
	Indicum–like hybrids (Ys6)	100 (2/2)	83.3 (5/6)	100 (1/1)	75 (3/4)
	R. indicum (Yr6, Yr7)	80 (4/5)	100 (5/5)	83.3 (5/6)	92.3 (12/13)

Artificial cross

Intra– and interspecific crosses of the two parent species showed >80% fruit set (Table 2). Fruit sets of the presumptive hybrid populations, Ys4 and Ys6, were comparable to that of parent species in crosses with parent species and within the populations. These results indicate that hybrids maintain their fertility and there is no effective isolation mechanism in fertilization between the two species even after interspecific hybridization. Once F_1 hybrids are established, they can be either a maternal or paternal donor and their backcross with sympatric parent species would form offspring with morphologically variable traits.

Lower fruit set by selfing than that by outcross suggest weak self–incompatibility of the both species. Self–incompatibility is one of the mechanisms that have evolved to encourage outbreeding in flowering plants (De Nettancourt, 1977) and known for some closely related *Rhododendron* species of section Tsutsusi (Yamaguchi, 1980; Ng and Corlett, 2000).

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