

Investigation of Clonal Structure and Self-incompatibility in Japanese Endemic Snow Camellia (*Camellia rusticana*)

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Abstract

We investigated the clonal structure, self-incompatibility, and number of seeds per fruit in *Camellia rusticana*, a Japanese endemic species, in comparison to the closely related *C. japonica*. Clonal propagation was more vigorous in *C. rusticana* than in *C. japonica* and the clonal structure of *C. rusticana* varied among populations. *C. rusticana* can maintain genets for extended periods, even in harsh environments such as high-altitude areas with considerable snow accumulation, because even a single surviving genet can propagate clonally. However, sexual reproduction (*i.e.*, reproduction by seed) is advantageous for dispersal to distant suitable habitats. An artificial crossing test revealed high self-incompatibility in *C. rusticana*, as observed in *C. japonica*. In addition, the number of seeds per fruit was lower in *C. rusticana* than in *C. japonica*. Self-incompatibility in *C. rusticana* may play a role in production of high-quality seed, despite low seed production.

Keywords: *Camellia japonica*, *Camellia rusticana*, self-incompatibility, microsatellite

Introduction

Camellia rusticana, an endemic species of Japan, is an evergreen shrub tree distributed in heavy snow areas up to

approximately 1,000 m elevation from Tohoku to the Hokuriku region along the shore of the Sea of Japan (Iokawa 2017). In Japan, four *Camellia* species (*C. rusticana*, *Camellia japonica*, *Camellia sasanqua*, and *Camellia lutchuensis*) are distributed. Of these species, only *C. rusticana* and *C. japonica* are distributed in northern Japan (Nagamasu 2006). *C. japonica* is a subcanopy tree distributed in warm temperate forests of the Honshu, Shikoku, Kyushu, and Okinawa regions, as well as in Taiwan and China (Shandong and Zhejiang) (Min and Bartholomew 2007). There are differences in the petal color, filament color and shape of the flowers between *C. rusticana* and *C. japonica* (Orikawa et al., 1998, Abe et al., 2021). The rate, which is calculated by dividing the length of the uniting basal part of the stamen by the maximum length of the stamen, is significantly longer in *C. japonica* than in *C. rusticana* (Orikawa et al., 1998). *C. japonica* has darker and purer red petals than does *C. rusticana*, and *C. japonica* filaments are close to white in color, whereas *C. rusticana* filaments are closer to yellow (Abe et al., 2021). The pollinators of *C. japonica* are mainly birds, such as the Japanese white eye (*Zosterops japonica*) (e.g., Abe et al. 2011, Kunitake et al. 2004, Yumoto 1988); Tanaka (1988) reported that flower-visiting species of *C. rusticana* in Niigata Prefecture were mainly insects, including bees and small beetles. Therefore, *C. rusticana* and *C. japonica* have different pollinators.

C. rusticana grows in regions where snow accumulation exceeds 150 cm (Ishizawa, 1978) and rapidly produces adventitious roots from the stem. Clonal propagation in this species has been confirmed from observations of root connected to an

adjacent stem (Ishizawa 1988). Clonal propagation is an important morphological characteristic that distinguishes *C. rusticana* and *C. japonica*. Other species that occur in snowy regions also exhibit a capacity for clonal propagation, such as *Cryptomeria japonica*, *Prunus padus*, *Fagus crenata*, *Ilex leucoclada*, and *Quercus crispula* var. *horikawae* (Aizawa et al. 2017, Ogasawara et al., 1988; Torimaru and Tomaru 2005; Taira et al., 1997; Worth et al., 2021). In *Cryptomeria japonica*, investigations from old-growth stands have shown that the snow depth (i.e., snow pressure) strongly affects clonal propagation. However, efforts to investigate genet size (the area occupied by a single clone) in multiple populations and linkages to environmental factors have been limited.

Sexual reproduction is important for the maintenance of genetic diversity. Ishizawa (2005, 2009) reported that the number of fruits and seeds per fruit was lower in *C. rusticana* than in *C. japonica*. Species of the genus *Camellia* generally have high self-incompatibility (Tanaka 1985). Although self-incompatibility is high in *Camellia sinensis* and *C. japonica* (Chen et al., 2012; Shibata and Ieyumi 1991), it is unknown whether *C. rusticana* has self-incompatibility.

In this study, we clarified the reproductive ecology of Japanese-endemic *C. rusticana* in the comparison with *C. japonica*. First, we analyzed the clonal structures of multiple populations and evaluated the relationships of clonal structure with snow pressure and individual size. Second, we investigated self-incompatibility using artificial crossing tests. Third, we surveyed the amount of fruits and the number of seeds per fruit of both species.

Materials and Methods

Study site

Based on the distributions of *C. japonica* and *C. rusticana* in Niigata Prefecture (Ishizawa 1990), two populations of *C. japonica* (Murakami and Maze) and three populations of *C. rusticana* (Ninouji, Irihirose, and Itoigawa) (Table 1) were selected. The *C. japonica* population was located at an altitude of ≤ 40 m along the Sea of Japan; the *C. rusticana* population was located in a mountainous area with an altitude of ≥ 500 m.

The canopy of the two study sites for *C. japonica* was mainly occupied by *C. japonica*. There were few seedlings in the Maze population, where a dense canopy of *C. japonica* heavily shaded the understory. By contrast, seedlings were found in a gap in the understory of the Murakami population, where the canopy comprised large *C. japonica* and also *Magnolia obovata* and *Castanea crenata* (Fig. 1). The canopy of the three study sites for *C. rusticana* was *Fagus crenata* with some evergreen shrubs (e.g., *C. rusticana*, *Lindera umbellata*, and *Ilex leucoclada*) on the forest floor. To estimate the snow pressure, which affects clonal structure, the subsidence pressure and slope snow pressure were calculated; these are applicable at flat and sloped sites, respectively. Snow depth data for each plot were estimated using a 1-km mesh representing average snowfall data for the 30-year period of 1981–2010, obtained using climate data

from the National Land Numerical Information published by the Japanese Ministry of Land, Infrastructure, Transport and Tourism (Table 1). The average slope angle was calculated based on six slope angles measured from top to bottom at 2-m intervals (5-m intervals in Murakami) using TruPulse (Tissue Technologies). The F_{\max} value, indicating sedimentation pressure, was calculated using the equation of Nakamata and Sudo (New Handbook for Snow Protection Engineering 1988): $F_{\max} = 1.4 \times HW_{\max}$ (1)

F_{\max} , maximum sedimentation pressure (t/m); factor of proportionality, 1.4; HW, maximum payload (t/m²)

The S value, indicating the slope snow pressure, was calculated using the Haefeli equation (Haefeli 1965).

$$S = \gamma H^2 / 2 \times (0.36 \cos^3 \phi + \sin 2\phi \times K \times N) \quad (2)$$

S , snow pressure (t/m); γ , snow density (g/cm³), 0.4 g/cm³

H , snow depth (m); ϕ , slope angle (°), K , creep coefficient, 0.83

N , glide coefficient, 2.3

This formula calculates the snow pressure in a direction parallel to the slope and ignores static snow pressure on steep slopes; it determines the magnitude of force applied to a structure 1 m wide, with a height identical to that of the snow depth. The maximum snow depth data were obtained from the 2010 Mesh Annual Average of the Japan Meteorological Agency; the snow density and creep coefficient data were obtained from the New Snow Protection Engineering Handbook (1988).

Analysis of clonal structure

A 10 × 10 m plot was established in each of the four populations. Because the ramet density of the Murakami population was low, the plot size was set at 25 × 25 m. For the *C. japonica* population, all ramets with a height of ≥ 50 cm in the plot were used (54 ramets in Maze, 123 ramets in Murakami). Because the *C. rusticana* population had a very high ramet density, the 10 × 10 m plot was divided into 100 quadrats of 1 × 1 m; ramets with the longest stem length in the quadrat were used. The numbers of ramets were 79, 90, and 76 in Ninouji, Irihirose, and Itoigawa, respectively and the basal diameter of each ramet was measured. The positions of ramets were also measured with TruPulse (Tissue Technologies) and used to create a tree position map.

Total DNA was extracted from leaves by the modified CTAB method (Murray and Thompson 1980). Based on polymorphisms, shapes of allele peaks, extent of PCR amplification, and presence of null alleles, the 14 EST-SSR markers developed by Toju et al. (2011) were selected (CV066969, TMSE-11H10S, TMSE-5F09T, TMSE-25E07T, TMSE-2E04T, TMSE-30A04S, TMSE-12B09T, TMSE-28B04T, TMSE-11D02T, TMSE-17E06S, TMSE-25H09T, TMSE-27B03T, TMSE-29B08S, and TMSE-CV14520). For two markers (TMSE-30A04S and TMSE-29B08S), primers were re-designed to adjust the length of the amplified fragment (TMSE-29B08S: forward GCGTTTATTCTCTCTCGCTCTC, reverse GTTCTTGTGATCGCGAGCATTGATTT; TMSE-30A04S: forward CACTCATCTCAATCACTAACC, reverse GTTCTTGGCTCCTAATCTCACATCTTCTTCT). Two primer mixes that encompassed eight loci (TMSE-12B09T [final concentration, 0.2 μ M], TMSE-28B04T [0.2 μ M], TMSE-11D02T [0.2 μ M], TMSE-27B03T [0.2 μ M], TMSE-29B08S [0.2 μ M], CV14520 [0.2 μ M],

Table 1
Populations of *C. japonica* and *C. rusticana* used in this study.

	Population	Latitude	Longitude	Elevation (m)	Average slope (°)	Plot size (m×m)	Number of ramets ^{※1}	Average basal Diameter (mm)	Snow depth (m) ^{※2}	Slope snow pressure (t/m)	Sedimentation Pressure (m)
<i>C. japonica</i>	Maze	N37°49'35.44"	E138°51'25.39"	39	30.71±2.82	10×10	54	39.62±16.38	0.20	0.02	0.11
	Murakami	N38°14'35.91"	E139°26'51.14"	30	48.84±1.16	25×25	123	53.80±59.45	0.68	0.18	0.38
<i>C. rusticana</i>	Ninouji	N37°54'16.72"	E139°27'59.16"	720	43.82±4.84	10×10	337(79)	25.68±10.16	1.40	0.80	0.78
	Irihirore	N37°20'55.28"	E139°09'25.76"	530	19.28±1.70	10×10	305(90)	16.51±6.14	2.73	2.23	1.52
	Itoigawa	N36°51'59.35"	E137°49'55.37"	870	24.74±1.76	10×10	310(76)	28.84±11.06	1.19	0.49	0.67

※1: Values in parentheses are numbers of ramets investigated.

※2: Values from climate data obtained from the National Land Numerical Information published by the Japanese Ministry of Land, Infrastructure, Transport and Tourism.

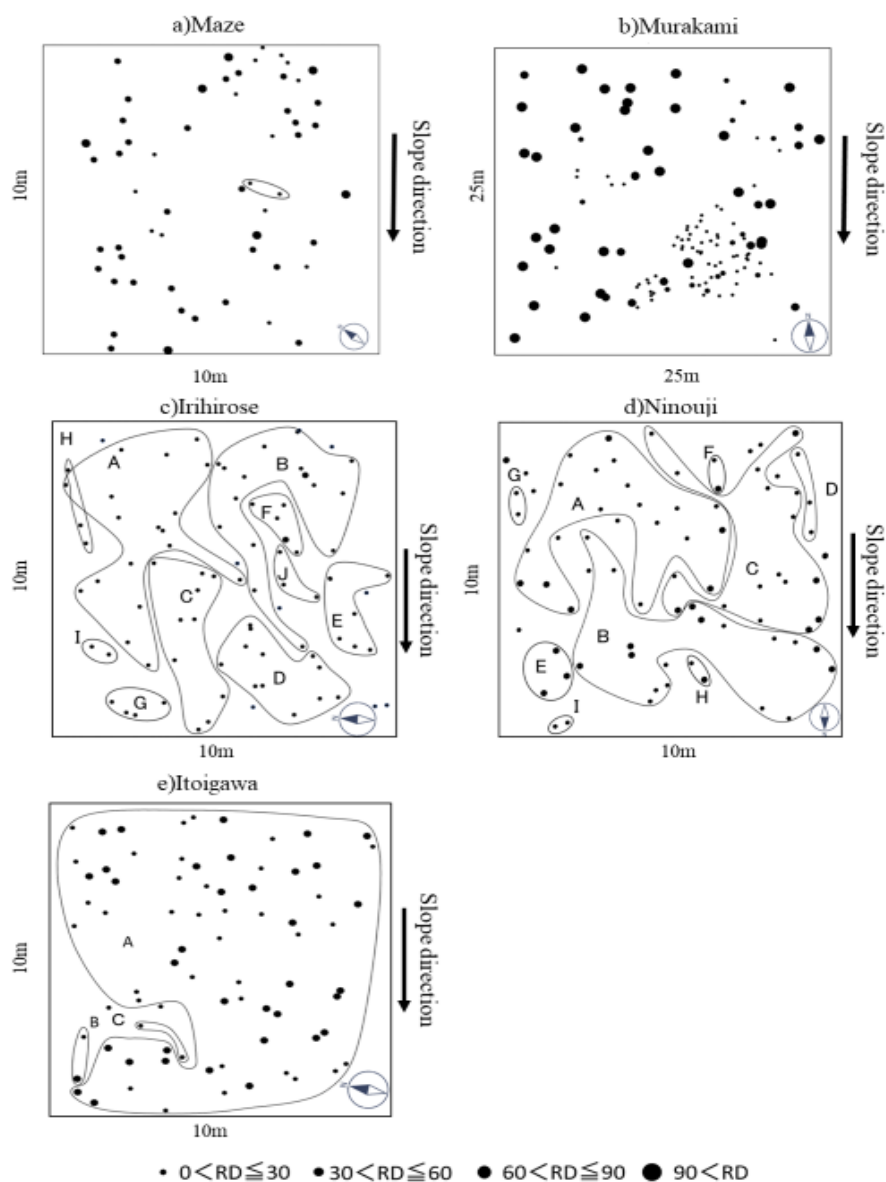


Figure 1
Clonal structures detected at study sites (a: Maze, b: Murakami, c: Irihirore, d: Ninouji, e: Itoigawa). Sets of ramets enclosed by solid lines show genets of the same genotype. Size of the circle indicates basal diameter.

TMSE-17E06S [0.2 μ M], and TMSE-25H09T [0.2 μ M]) and six loci (CV066969 [0.2 μ M], TMSE-11H10S [0.2 μ M], TMSE-5F09T [0.4 μ M], TMSE-25E07T [0.2 μ M], TMSE-2E04T [0.42 μ M], and TMSE-30A04S [0.2 μ M]) were prepared. Multiplex PCR amplification was performed using each primer mix.

Multiplex PCR reactions were carried out in a total volume of 6.0 μ L containing 1 μ L of DNA (5 ng/ μ L), 3 μ L of 1 \times Multiplex PCR Master Mix (Qiagen), and 0.8 μ L of primer mix. PCR amplification was performed using a Thermal Cycler Dice (TaKaRa) with the following program: 30 s at 95°C; 35 cycles of 30 s at 95°C, 90 s at 57°C, and 30 s at 72°C; and 30 min at 60°C. Polymorphisms in PCR products were assessed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems); alleles were identified using Gene Marker software ver. 2.4.0 (Soft Genetics). For clonal analysis, GenAEx software ver. 6.503 (Peakall and Smouse 2010) was used. The probability of identity (PI) was estimated using genotype data from all individuals, to evaluate the accuracy of the analysis. In this study, pairs that differed by only one allele with a difference of ≤ 2 bp were considered to be the same clone, considering the possibility of mutation.

Artificial crossing test

To investigate the self-incompatibility of *C. rusticana*, an artificial crossing test was performed on *C. rusticana* trees in the Irihiro population. Although high self-incompatibility has been reported for *C. japonica* (Shibata and Ieyumi 1991), an artificial crossing test was performed on *C. japonica* in the Maze population as a control for *C. rusticana*. In the Maze population, a total of 104 flowers from 10 ramets was used (self-pollination, 30 flowers from 10 ramets [3 flowers per ramet]; cross-pollination, 30 flowers from 10 ramets [3 flowers per ramet]; natural pollination, 44 flowers from 10 ramets [4.9 ± 1.6 flowers per ramet]). Maternal ramets were randomly selected from around the study plot. For self-pollination, on February 25, 2019, flowers were covered with crossing bags (25 \times 20 cm, Morifuji Co., Ltd., Japan) before flowering to prevent visits by pollinators. On March 5th, pollen from stamen in the crossing bag was attached to the stigma and the bag was replaced. For cross-pollination, on February 25, 2019, the petals and anthers of unflowered flowers of maternal ramets were removed and the anthers of flowers of paternal ramets were touched to the remaining stigma; the crossing bag was then replaced. Cross-pollination was repeated on March 5, 2019, using the same paternal and maternal ramets. The paternal ramet was at least 20 m from the maternal ramet to avoid inbreeding. For *C. rusticana*, an artificial crossing test was performed on the Irihiro population on May 23, 2019. Because the number of flowers per ramet in *C. rusticana* was small, a total of 95 flowers from 25 ramets was used (self-pollination, 30 flowers from 17 ramets [1.8 ± 0.9 flowers per ramet]; cross-pollination, 30 flowers from 18 ramets [1.7 ± 0.6 flowers per ramet]; natural pollination, 35 flowers from 23 ramets [1.7 ± 1.3 flowers per ramet]). Pollination was performed as for *C. japonica*. Because of the small amount of pollen per flower in *C. rusticana*, paternal ramets differed between the first and second cross-pollinations. Although a second cross-pollination was performed on May 27, for 4 of 30 flowers, a second cross-pollination could not be

performed because flowering had finished. The crossing bags were removed on May 16 and July 24 in Maze and Irihiro. To prevent natural fall, a 0.5-mm mesh net was used, and the fruiting process was observed. To investigate maturation, the fruit set rate was recorded three times for each population (May 16, August 27, and October 11 for *C. japonica*; July 24, August 25, and September 25 for *C. rusticana*).

Numbers of fruits and seeds per fruit

In 2018, from mid-September to mid-October (September 14 in Maze, October 25 in Murakami, September 20 in Irihiro, October 3 in Ninouji, and October 12 in Itoigawa), three to four people walked around the study plot for a few hours and searched for ramets with fruits. When a ramet had multiple fruits, all fruits were sampled. In 2019, fruits were sampled from late August to early October (August 27 in Maze, September 6 in Murakami, September 25 and October 11 in Irihiro, October 3 in Ninouji, and October 3 in Itoigawa). For the two *C. japonica* populations, three 10 \times 10 m plots were established for each population and all fruits in the plots were sampled. For *C. rusticana*, fruits were collected as in 2018 because of difficulty establishing 10 \times 10 m plots and collecting all fruits. For all fruits collected, the number of seeds per fruit was investigated. To compare the number of seeds per fruit between species and populations, the Wilcoxon rank-sum test and Steel-Dwass test, respectively, were performed in R ver. 3.3.3 (R Core Team 2015).

Results

Survey site

The average slope angle of the plots for Murakami, Maze, and Ninouji was approximately 40°, whereas the average slope angle of Itoigawa and Irihiro was more moderate (Table 1). The average stem lengths and basal diameters of *C. japonica* and *C. rusticana* were 293.16 ± 196.43 cm/ 47.39 ± 45.89 mm and 94.51 ± 45.17 cm/ 23.21 ± 10.76 mm, respectively. The basal diameter of *C. japonica* was significantly larger than that of *C. rusticana* (Kruskal-Wallis test, $F = 109.18$; $p < 0.001$). *C. rusticana* had a very high ramet density; all plots included more than 300 ramets (Table 1) and some quadrats included more than 10 ramets.

Clonal structure

The 14 EST-SSR markers showed considerable degrees of polymorphism; the probability of identity (PI), calculated by all ramets subjected to clonal analysis, was $3.8\text{E}-15$, suggesting that highly accurate clonal analysis was possible. The clonal analysis revealed clonal propagation in one population of *C. japonica* (Maze) and all populations of *C. rusticana* (Fig. 1). The numbers of genets consisting of ≥ 2 ramets were 10 and 9 in the Irihiro and Ninouji populations, respectively; these genets were of various sizes. However, in the Itoigawa population, most (70 of 76) ramets showed the same genotype, which indicated that a very large genet was occupied. The sedimentation pressure and slope snow pressure increased in the order of

Table 2
Results of artificial crossing.

	Pollination treatment	Number of flowers	Fruit set (%)	Number of fruit	Number of eaten fruits
<i>C. japonica</i>	Self-pollination	30	-	0	-
	Cross-pollination	30	63	19	12
	Natural pollination	44	18	8	8
<i>C. rusticana</i>	Self-pollination	30	3	1	0
	Cross-pollination	30(26)	67(73)	20(19)	18
	Natural pollination	38	21	8	7

Values in parentheses are numbers of pollinations performed twice.

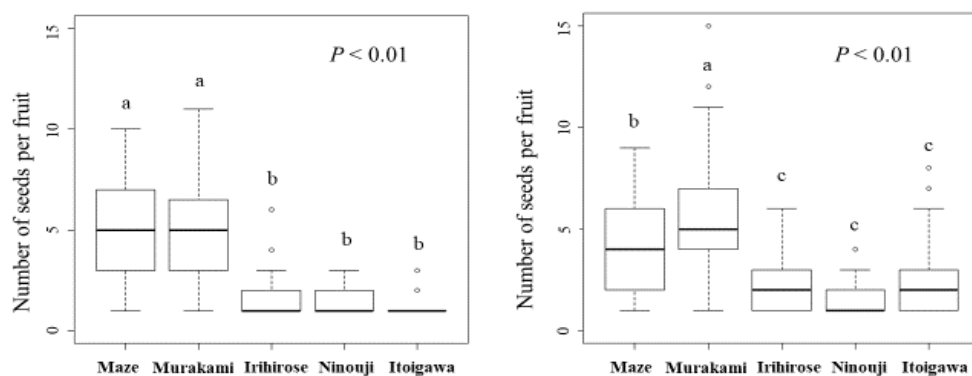


Figure 2
Number of seeds per fruit in each population. Left, 2018; right, 2019. Line within box, median; top and bottom edges of box, 25th and 75th percentiles; ends of whiskers, minima and maxima of non-outliers; circles, outliers. Same letters above box plots indicate no significant difference according to Steel–Dwass test ($p > 0.01$).



Figure 3
Fruits eaten by rodents.

Irihiro, Ninouji, and Itoigawa, where a large amount of snow falls in winter. Irihiro, which has the greatest snow cover, had the highest snow pressure, despite its shallow slope (Table 1).

Artificial crossing test

After the first observation of fruit set, self-pollination did not produce fruit in *C. japonica*; the fruit set rates of cross-pollination and natural pollination were 63 % (19 flowers from 19 ramets) and 18 % (8 flowers from 5 ramets), respectively (Table 2). In *C. rusticana*, the fruit set rates of self-pollination, cross-pollination, and natural pollination were 3 % (1 flower from 1 ramet), 67 % (20 flowers from 13 ramets) and 21 % (8 flowers from 4 ramets), respectively (Table 2). After the second observation of fruit set, 3 of the 19 nets for *C. japonica* and 1 of the 20 nets for *C. rusticana* were torn by bites, and the fruits were eaten by rodents or lost. After the third observation of fruit set, 12 of 19 nets (63 %) for *C. japonica* and 18 of 20 nets (90 %) for *C. rusticana* were torn by bites, and the fruits were eaten by rodents or lost (Fig. 3).

Numbers of fruits and seeds per fruit

Fruit sampling in 2018 yielded 177 fruits from 53 maternal ramets (3.34 ± 2.07 per maternal ramet) in *C. japonica* and 55 fruits from 53 maternal ramets (1.06 ± 0.30 per maternal ramet) in *C. rusticana* (Table 3). These fruits included 886 and 88 seeds in *C. japonica* and *C. rusticana*, respectively. In 2019, fruit sampling yielded 288 fruits from 84 maternal ramets (4.22 ± 4.25 per maternal ramet) in *C. japonica* and 112 fruits from 96 maternal ramets (1.17 ± 0.86 per maternal ramet) in *C. rusticana* (Table 3). These fruits included 1,476, and 239 seeds in *C. japonica* and *C. rusticana*, respectively. In both years, the number of seeds per fruit was significantly greater in *C. japonica* (5.01 ± 2.25 in 2018; 5.13 ± 2.36 in 2019) than *C. rusticana* (1.60 ± 0.99 in 2018; 2.13 ± 1.48 in 2019) (Table 3, Figure 2). *C. japonica* produced a large number of fruits, but it was difficult to find fruits of *C. rusticana* (Table 3).

distributed only in areas with little snow in Niigata Prefecture, close to the Sea of Japan (Ishizawa et al. 1990), disturbances other than snow pressure may lead to rare clonal propagation. Vigorous clonal propagation was detected in all three populations of *C. rusticana*. In some tree species, such as *Cryptomeria japonica*, *Prunus padus*, and *Ilex leucoclada*, layering (a type of clonal propagation) has been reported (Ogasawara et al., 1988; Torimaru and Tomaru 2005; Taira et al., 1997). In *P. padus*, drooping branches typically contact the ground surface and are covered by defoliation and soil; adventitious roots develop at those positions and branches subsequently grow upward in a manner independent of the parent stem. In *Cryptomeria japonica*, layering similar to that of *P. padus* has been reported, as has layering from seedlings (i.e., seedling-layering) (Taira 1994). Seedling-layering occurs when seedlings fall as a result of sedimentation pressure and snow slope pressure, roots develop from this ground contact, and the branches and the tip of the stem grow upward. Shrub species (e.g., *I. leucoclada* and *C. rusticana*) propagate by both seedlings and in a clonal manner (e.g., seedling-layering in *Cryptomeria japonica*).

Taira et al. (1997) reported that a *Cryptomeria japonica* population distributed at >1,750 m in the Northern Alps consisted of a single clone derived from layerings (seedlings could not occur because of inbreeding depression). Although the population distributed around 1,650 m, consisted of several genets, all newly established seedlings died within 1 year. In the *Cryptomeria japonica* population at the high altitude, the clones that experienced difficulty rooting gradually died out because of repeated clonal propagation or were isolated by the severe environment, finally leading to a single clone (Taira 2011). The main factors affecting clonal propagation are snow pressure and tree size (years from establishment) in *Cryptomeria japonica* (Kimura et al., 2013). The *C. rusticana* in the Itoigawa population, where the sedimentation pressure and snow slope pressure were lowest (Table 1), had a very large genet size. Clones with poor rooting gradually died out, whereas the clones with strong rooting expanded their area of occupation

Table 3
Results of fruits and seeds obtained in 2018 and 2019.

	Number of trees		Number of fruits		Number of Seeds per fruit		Total number of seeds	
	2018	2019	2018	2019	2018	2019	2018	2019
<i>C. japonica</i>	53	84	177	288	5.01 ± 2.25^a	5.13 ± 2.36^a	767	1476
<i>C. rusticana</i>	53	96	55	112	1.60 ± 0.99^b	2.13 ± 1.48^b	88	239

*Wilcoxon rank-sum test was performed for each year. Letters indicate no significant differences ($p > 0.01$).

Discussion

Clonal structure

One genet consisting of two ramets in the Maze population of *C. japonica* had shallow root connections. Therefore, *C. japonica* does not regularly propagate clonally. Because *C. japonica* is

via the process of repeated clonal propagation. In contrast, *C. rusticana* in the Irihiro population, where the sedimentation pressure and snow slope pressure were highest, had the smallest average basal diameter (Table 1), suggesting that genets were small because of the short time elapsed since its establishment.

Self-incompatibility and number of seeds per fruit of *C. rusticana*

The artificial crossing test of wild populations of *C. rusticana* and *C. japonica* showed that the fruit set rates of cross-pollination, self-pollination, and natural pollination were similar in the two species. The fruit set rate of *C. rusticana* under self-pollination was only 3 %, indicating that *C. rusticana* has high self-incompatibility, similar to findings in *C. japonica*. The fruit set rates obtained by cross-pollination and self-pollination were similar to those using a *Camellia* cultivar planted at the Agricultural and Forestry Technology Development Center in Nagasaki Prefecture (self-pollination, 5.0 %; cross-pollination, 47.5 %; Furumura et al., 2018).

We observed that the number of fruits was lower in *C. rusticana* populations than in *C. japonica* populations, supporting the results of previous studies (Ishizawa 2005, 2009). Despite our use of nets, 90 % and 63 % of the fruits produced by *C. rusticana* and *C. japonica* in the artificial crossing tests were eaten by rodents or lost, respectively. The seeds of *C. japonica* are consumed and hoarded by the Japanese wood mouse, *Apodemus speciosus* (Abe et al. 2006). The related *A. argenteus* inhabits the high-altitude zone where the *C. rusticana* grows. The *A. argenteus* is believed to be absent from lower-elevation areas where *C. japonica* grows, we note that *A. argenteus* is more arboreal and *A. speciosus* is more terrestrial. Therefore, the fruits of *C. rusticana* may be more susceptible to serious damage by *A. argenteus*. Given the similar fruit set rates by natural pollination of *C. rusticana* and *C. japonica* and the possibility of dispersal by rodents before fruit falls, re-evaluation of the amount of seeds in the two species (excluding the effect of rodents) is needed.

C. japonica and *C. rusticana* have three ovarian locules (rarely four) per fruit. The numbers of ovules in each ovarian locule are three in *C. japonica* (a total of nine) and three to five in *C. rusticana* (Ishizawa 2005, Tsuyama 1964). Therefore, *C. rusticana* can produce more seeds per fruit than *C. japonica*. However, Ishizawa (2005, 2009) reported that the number of seeds per fruit was larger in *C. japonica* (average, 4.47) than in *C. rusticana* (average, 1.27) based on observation of 382 fruits in *C. rusticana* (Aga Town in Niigata Prefecture and Sakaemura in Nagano Prefecture) and 57 fruits in *C. japonica* (Murakami City and Sado City in Niigata Prefecture). The results of our 2-year observation also showed that the number of seeds per fruit was significantly greater in *C. japonica* than *C. rusticana*. Here, we aimed to investigate differences in fruit set and the number of seeds per fruit under cross-pollination and natural pollination. Unfortunately, rodent consumption meant that we were unable to obtain these data.

For most plants, limitations of both pollen and resources are important constraints on reproduction (Knight et al., 2005; Asikainen et al., 2005). A plant is considered pollen limited if additional pollen increases fruit or seed production (Schemske, 1980; Willson and Schemske, 1980; Bierzychudek, 1981; Burd, 1994; Larson and Barrett, 2000), whereas a plant receiving sufficient pollen is considered resource limited if the addition of resources increases fruit or seed production (Zimmerman and Aide, 1989). In the artificial pollination tests in this

study, we did not address pollen and resource limitation due to the inability to collect data regarding the number of seeds per fruit, although we had fruit set observations. In natural stands, we did not assess total flower number, which makes it difficult to discuss pollen and resource limitations, despite observations of fruit set and the number of seeds per fruit. Investigating pollen and resource limitation in *C. rusticana* and *C. japonica* requires additional artificial pollination tests and observations of natural stands.

Finally, plants in low-light environments have been shown to divert resources away from reproductive tissues to parts that increase light capture, such as stems and leaves (McConaughay and Coleman 1999). *C. rusticana* may be investing more resources in clonal propagation rather than seed production to increase resource capture in highly shaded understories. In addition, self-incompatibility in *C. rusticana* may play a role in producing high-quality seeds despite low overall seed production.

Conclusion

Clonal propagation was more vigorous in *C. rusticana* than in *C. japonica*. *C. rusticana* may be investing more resources in clonal propagation rather than seed production to increase resource capture in highly shaded understories. In *C. rusticana*, which inhabits regions with heavy snowfall, the genet size is typically expanded by clonal propagation because branches and stems are grounded by snow. *C. rusticana* can maintain a genet for an extended duration, even in a severe environment such as a high-altitude area with considerable snow, because this species can propagate clonally if one genet survives. Yet, sexual reproduction (*i.e.*, seed reproduction) has advantages for dispersal to distant suitable habitats. Self-incompatibility in *C. rusticana* may play a role in producing high quality seeds despite low overall seed production.

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