Research paper

Quantitative classification of *Camellia japonica* and *Camellia rusticana* (Theaceae) based on leaf and flower morphology

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ABSTRACT

In Japan, *Camellia japonica* and *Camellia rusticana* are naturally distributed. Despite differences in their habitats and morphologies, they have been classified by various researchers as either varieties, sub-species, or species. The taxonomic position of *C. japonica* and *C. rusticana* remain unclear because morphological comparisons have been restricted to limited areas and quantitative data are scarce. *C. rusticana* grows in snowy places, unlike *C. japonica*. While *C. japonica* displays ornithophily, *C. rusticana* displays entomophily. Both species have adapted to different growing environments and pollinators, which have altered the morphology of flowers and leaves. We therefore quantitatively estimated the differentiation between these two taxa by comparing the morphologies of leaf hypodermis, flower form, petal color, and filament color in twenty populations. Our findings allowed us to differentiate these two species by the presence or absence of a leaf hypodermis. We also discovered an intermediate type of leaf hypodermis, which might also be caused by hybridization. Principal component analysis (PCA) indicated that the flower morphologies between these species were significantly different. The petal and filament colors were also significantly different. Our quantitative analysis suggests that speciation caused by differences in both pollinators and environment is one of the factors involved in this group. These findings in *C. japonica* and *C. rusticana* help to explain speciation processes for other species as well.

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1. Introduction

Plants adapt to various ecological niches (e.g., diverse habitats and pollination systems) through morphological diversification and speciation (Futuyma and Slatkin, 1983). For example, leaf morphology in the silversword alliance (Asteraceae), which consists of 25 species that all evolved from a single colonizing ancestor in Hawaii, has been shown to diverge in dissimilar habitats (Barrier et al., 1999). Adaptations to a particular pollination syndrome promote speciation by attracting new pollinators through traits such as flower color and scent. Research has shown that changes in flower morphology lead to speciation by shifting pollinator preferences from bees to hummingbirds or bats (Grant and Grant, 1965;

Castellanos et al., 2003; Wilson et al., 2007). Plants also hybridize, but because they are sessile, hybridization is less common than speciation, emphasizing the importance of pre-mating rather than post-mating isolation (Ando et al., 2001; Ramsey et al., 2003; Yasumoto and Yahara, 2006). Thus, identifying the factors that play a role in pre-mating isolation are critical to understanding plant speciation.

"Japan Sea elements" offer an opportunity to examine how ecological niches drive morphological divergence and speciation. Plant species unique to the region near the Sea of Japan, which are known as Japan Sea elements, are distributed according to levels of snowfall (Maekawa, 1977). Observations that species closely related to Japan Sea elements are distributed in the warm temperate zones of the Pacific regions have suggested that Japan Sea elements adapted to a past climate change and speciated from the temperate environments in Japan (Hotta, 1974; Sakai, 1982). In total, 212 taxa of Japan Sea elements and their relative species have been reported, including *Camellia japonica* L., and *Camellia rusticana* Honda (Sato, 2005; Honda, 1950) (Figs. 1 and 2). *C. japonica* is distributed along

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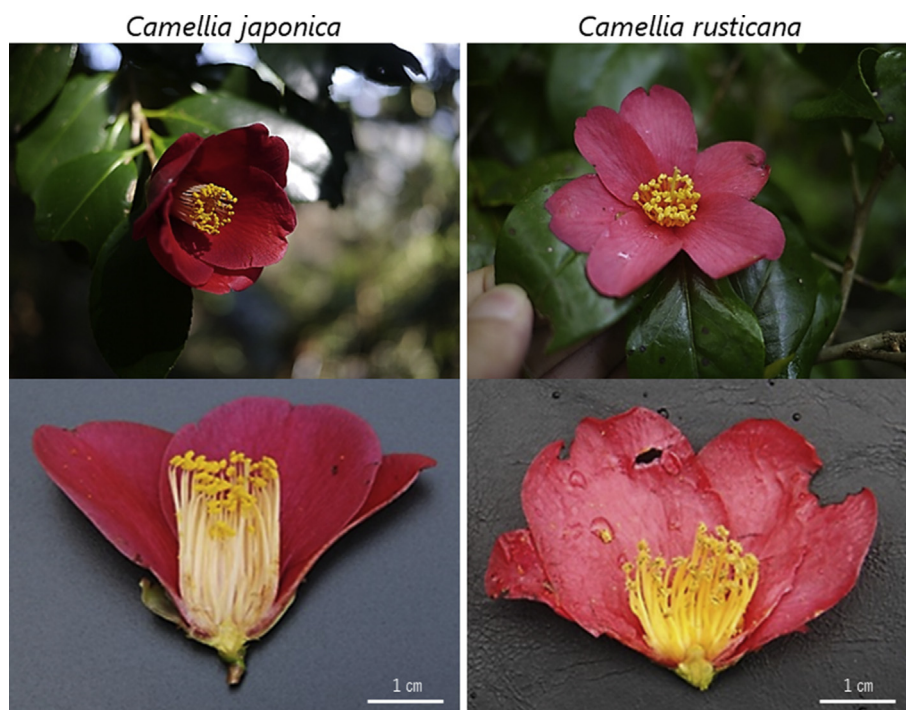[1] The authors contributed equally to this study.

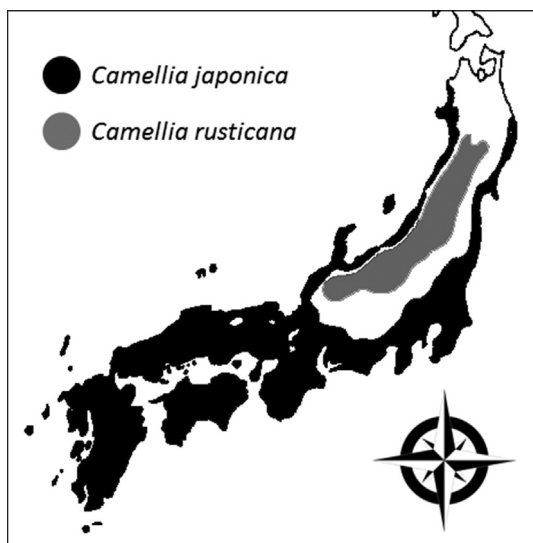**Fig. 1.** Photos of *Camellia japonica* and *C. rusticana*.

Fig. 2. Distribution of *Camellia* sect. *Camellia* in Japan. This figure was transcribed from Ishizawa (1988).

the coast of the Natsudomari peninsula in the Aomori Prefecture of eastern Japan (Horikawa, 1972). It is also found in southern Korea and in the coastal regions of mainland China (Nagamasu, 2006). *C. rusticana* ranges from the Shiga prefecture to the snowy regions on the Japan Sea side of the Akita prefecture (Ishizawa, 1988) (Fig. 2). Therefore, it is often considered that *C. rusticana* is adapted to snowy regions and has speciated from *C. japonica* (Tsuyama, 1988).

The taxonomic positions of *Camellia japonica* and *C. rusticana* remain controversial. Various researchers have classified them as either variety, subspecies, or species (Tsuyama, 1956; Kume et al., 1998; Ishizawa, 2005; Tateishi et al., 2007). According to Ishizawa (2003), *C. rusticana* was identified by Sugimoto (1936) as a

variant, and initially named *C. japonica* var. *decumbenus* Sugimoto. *C. rusticana* was recognized by Honda (1950) as an independent species, but Kitamura (1950) later recognized it as a subspecies, *C. japonica* subsp. *rusticana* Kitamura. Individuals with an intermediate form called 'yuki-bata-tsubaki' can be seen in areas where the ranges of the two species are adjacent (Hagiya and Ishizawa, 1961; Orikawa et al., 1998), and hybridization occurs naturally (Hagiya and Ishizawa, 1961; Ueno, 2009). FTIR spectroscopy and microsatellite data have indicated that *C. japonica* and *C. rusticana* cluster together in the same clade (Shen et al., 2008; Caser et al., 2010; Eguchi et al., 1991; Ueno, 2009; Vijayan et al., 2009). Furthermore, anthocyanin pigment distribution in the petals indicates that *C. rusticana* possibly speciated from a common ancestor earlier than *C. japonica* (Sakata et al., 1987). The phylogeny of the genus *Camellia* is similarly inconsistent (Min and Bartholomew 2007; Vijayan et al., 2009; Yang et al., 2013; Huang et al., 2014; Kim et al., 2017; Li et al., 2019); therefore, estimating the evolution of *Camellia* requires integrating morphological, geographical, and genetic data.

Camellia japonica and *C. rusticana* differ morphologically, ecologically, and physiologically. *C. japonica* occurs in places where the average temperature in January is higher than −0.6 °C and the minimum warmth index proposed by Kira (1945) is 85 (Ishizuka, 1947). Although areas within this temperature range occur extensively in the inland snowy regions of the Japan Sea side, to the north of Fukui Prefecture, *C. rusticana* rather than *C. japonica* grows in places where snow accumulation exceeds 150 cm (Ishizawa, 1978) (Fig. 2). Morphological differences between *C. japonica* and *C. rusticana* include the presence or absence of petiole hair, presence or absence of a layer of hypodermis tissue under the epidermis, shape of the corolla, size of the seeds, and ratio of the filament coalescence of flowers (Tsuyama, 1956; Ishizawa, 2005). *C. japonica* flowers between December and April and is mainly pollinated by birds, as insects are scarce in winter (Yumoto, 1988; Kunitake et al., 2004; Abe and Hasegawa, 2008; Abe et al., 2011). *C. rusticana* flowers in early spring, from April to June, and is

pollinated by insects such as flies, bees, and small beetles (Ishizawa, 1988). *C. japonica* and *C. rusticana* flowers have different traits, which may play a role in attracting different pollinator types (Ishizawa, 2005). The photosynthetic characteristics of *C. japonica* and *C. rusticana* also differ (Kume et al., 1998). The observable differences between these species suggest that speciation may have occurred through pre-mating isolation following morphological divergence of both flowers and leaves. However, little quantitative data is available on leaf and flower morphology or petal and filament color of these species.

In this study, we focused on the quantitative morphological differences between *Camellia japonica* and *C. rusticana*. First, we compared their leaf morphology to identify the differences in adaptation to the varied environments of their respective habitats. Second, we examined the degree of differentiation of the two species by quantitative comparison of flower form, petal color, and filament color.

2. Material and methods

2.1. Comparative analysis of leaf morphology

Previous studies that examined five leaf morphology traits of *Camellia japonica* and *C. rusticana* (blade length, blade width, petiole length, distance of the widest point from base, distance of the widest point from base/blade length) found no significant differences (Orikawa et al., 1998). However, *C. japonica* has only two layers of palisade tissue beneath the upper epidermis, whereas *C. rusticana* has one layer of hypodermis next to the epidermis, which is made of slightly larger cells, followed by two layers of palisade tissue (Shimada and Hisada, 1966). Therefore, in this study, we used differences in leaf hypodermis to examine how ecological conditions drive morphological divergence.

We examined 80 individuals of *Camellia japonica* (Mean = 10 individuals; SD ± 0.0) from eight populations and 120 individuals of *C. rusticana* (Mean = 10 individuals; SD ± 0.0) from 12 populations for the presence of hypodermal tissue (Table 1). All specimens were collected from April to June 2014 and in July 2015. Specimens collected within previously reported ranges for respective species were considered to belong to those species (i.e., specimens collected in *C. japonica* range were considered *C. japonica*)

(Ishizawa, 1988). At these sampling sites, we avoided areas adjacent to both species, where plants may have hybridized. Samples were taken from individuals more than 20 m apart. We also examined the leaves of 13 individuals from elevations between 20 and 220 m in Yoneyama, Niigata Prefecture, where hybridization is known to occur.

The hypodermal tissue was examined using the following procedure. Leaves were cut into 1-cm squares and were vertically sliced as thinly as possible using a razor blade. The thin slices were then observed under an optical microscope (50 ×) (CX41; Olympus, Tokyo, Japan) for the presence of hypodermal tissue.
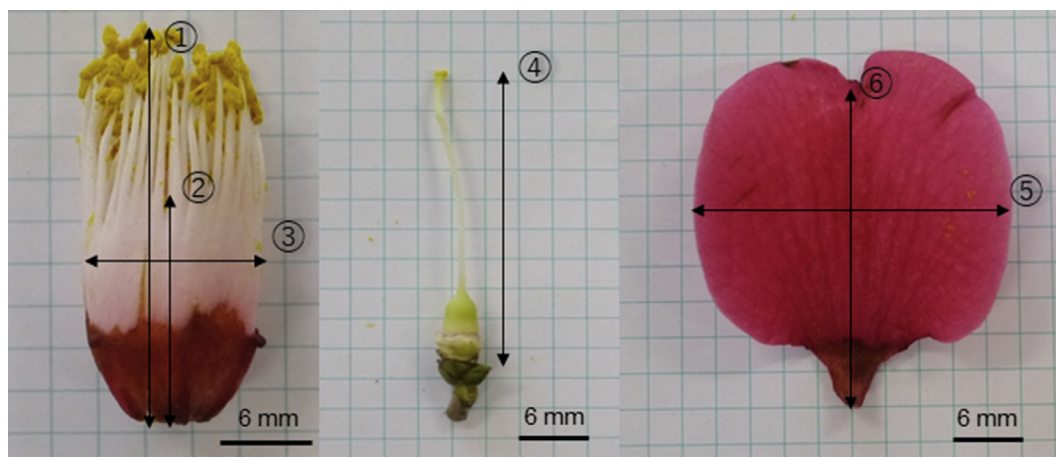
2.2. Comparative analysis of flower morphology

Camellia japonica and *Camellia rusticana* flowers differ in the shapes of the corolla and petal, rates of flower filament coalescence, lengths of stamen and pistil, and colors of flower filament (Ishizawa, 2005, 2010). In this study, we measured several quantifiable traits of *C. japonica* and *C. rusticana* flowers, including the length and breadth of the petal, aspect ratio of the petal, maximum length of the stamen, rate of flower filament coalescence (length of filament coalescence/length of filament), length of the pistil, and width of the stamen (Fig. 3). The morphological characters of flowers sometimes vary within a single individual plant, but these differences were not as large as the differences between respective individuals in the preliminary survey.

We compared the flower morphology of 124 individuals of *Camellia japonica* (Mean = 15.5 individuals; SD ± 8.9) from eight populations and 85 individuals of *C. rusticana* (Mean = 21.1 individuals; SD ± 11.1) from seven populations (Table 1; Fig. 4). We avoided sampling flowers in areas where both species occurred adjacent to each other. One flower per tree was collected from April 2014 to June 2014.

Flowers were dissected into each part as much as possible. A t-test was used to compare the flower parts of *Camellia japonica* and *C. rusticana*. Principal Components Analysis (PCA) was performed using the top three items (maximum length of stamen, rate of flower filament coalescence, and length of pistil) with the highest scores on the PC 1 axis obtained from initial analysis. Statistical analyses were performed in R 3.1.1 (R Development Core Team, 2014).

Table 1
Sample number and geographical locations of *Camellia japonica* and *C. rusticana*.

Pop No.	Species	Pop	Flower samples	Flower petal color samples	Filament color samples	Leaf samples	Latitude	Longitude	Elevation (m)
1	*C. rusticana*	Senpoku	0	0	0	10	39.6808	140.6654	423
2	*C. rusticana*	Osyu	5	4	3	10	39.1117	140.8546	472
3	*C. rusticana*	Haguro	0	0	0	10	38.7032	139.9831	379
4	*C. japonica*	Murakami	21	20	14	10	38.4921	139.5153	70
5	*C. rusticana*	Nishikawa	8	9	8	10	38.4553	140.0234	416
6	*C. rusticana*	Osado	27	20	20	10	38.2534	138.4235	8
7	*C. rusticana*	Yamagata	0	0	0	10	38.2453	140.1967	565
8	*C. japonica*	KunimiB	14	0	0	10	38.0496	138.4706	41
9	*C. rusticana*	KunimiA	28	25	21	10	38.0023	138.5060	579
10	*C. japonica*	Futami	13	20	0	10	37.9785	138.2571	23
11	*C. rusticana*	Shibata	8	8	8	10	37.9010	139.4810	1137
12	*C. japonica*	Niigata	27	14	7	10	37.7293	138.7921	16
13	*C. japonica*	Kashiwazaki	12	0	0	10	37.4542	138.6116	71
14	*C. rusticana*	Nagaoka	7	8	7	10	37.4101	139.1231	1429
15	*C. rusticana*	Tadami	28	19	20	10	37.3154	139.2672	584
16	*C. rusticana*	Tokamachi	0	0	0	10	37.1000	138.6173	313
17	*C. rusticana*	Joetsu	1	2	0	10	36.8573	137.8276	985
18	*C. rusticana*	Nagahama	0	0	0	10	35.6165	136.1718	295
19	*C. japonica*	Kitakyusyu	2	0	0	10	33.7100	130.5814	109
20	*C. japonica*	Kanoya	8	0	0	10	31.4900	130.8346	781
	Total		209	149	108	200			

pop = population. Population names are listed starting from the North.

Fig. 3. Measurement of flower morphology. 1, Maximum length of stamen; 2, Length of filament coalescence, 2/1; Rate of filament coalescence; 3, Stamen width; 4, Pistil length; 5, Petal width; 6, Petal length.
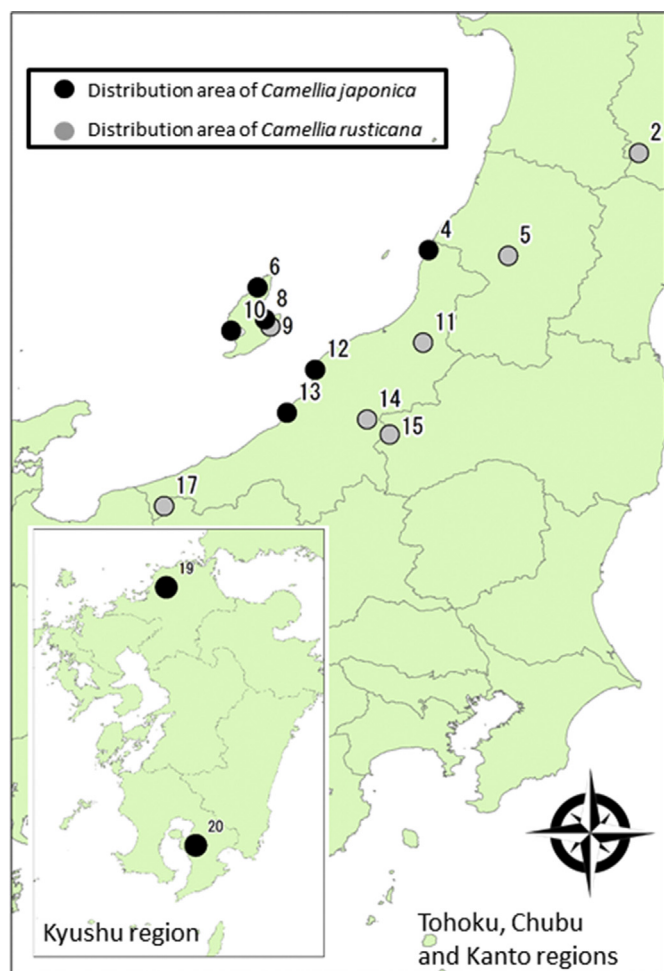
Fig. 4. Collection sites of floral materials of *Camellia* sect. *Camellia*. Number shows sampling location (Table 1).

2.3. Comparative analysis of petal and flower filament colors

Petal and flower-filament color were measured from 74 individuals of *Camellia japonica* (Mean = 18.5 individuals; SD ± 3.0) from four populations, and from 79 individuals of *C. rusticana* (Mean = 10.7 individuals; SD ± 8.3) from seven populations

(Table 1). All specimens were collected from April to June 2014. Measurements were taken on the day of collection or the next day to measure the flower parts before discoloration. All discolored flowers were excluded from the analyses. The value and chroma in the Munsell color space were measured to estimate petal color using a spectrocolorimeter (CM-2002; Konica Minolta, Tokyo, Japan). The colorimeter lights the petals, spectrally separates the light reflected from the petals, and converts the reflected light of each wavelength into numerical values. Wavelengths in the range of 400—700 nm were measured every 10 nm using the following conditions: 10° standard observer, illuminants DD65, and SCI setting, which means that the instrument's geometry and design allowed for the specular component to be included. The value and chroma in this system represent lightness and color purity, respectively. The value varies vertically along the color solid, from black (value 0) at the bottom, to white (value 10) at the top. Chroma, measured radially from the center of each color slice, represents the purity of a color related to saturation, with lower chroma being less pure and more washed out, as in pastels. Because the color of the flower filament could not be measured using the colorimeter due to its three-dimensional structure, we photographed flower filaments in a dark room (NIKON D100; Nikon, Tokyo, Japan) and processed the acquired images using ImageJ software (Abràmoff et al., 2004). During image processing, the anthers and petals attached to the flower filament were separated using ImageJ. The flower filament was extracted, and its color was transformed into the primary red (R), green (G), and blue (B) values (Motonaga et al., 1997, 2015).

3. Results

3.1. Comparative analysis of leaf morphology

Observation of the cross-sectional morphology of the leaves revealed three types of individuals (Fig. 5): 105 with hypodermal tissue, 91 without hypodermal tissue, and four with hypodermal tissue only above the cambium. The populations that had hypodermal tissue were predominantly found in the *Camellia rusticana* distribution area, whereas the populations without hypodermal tissue were mainly found in the *C. japonica* distribution area (Fig. 5). However, the presence of hypodermal tissue was not observed in the population collected from Nagahama (Shiga Prefecture) in the distribution area of *C. rusticana*. In Mt. Haguro, Yamagata Prefecture, which is a distribution area of *C. rusticana*, five out of 10
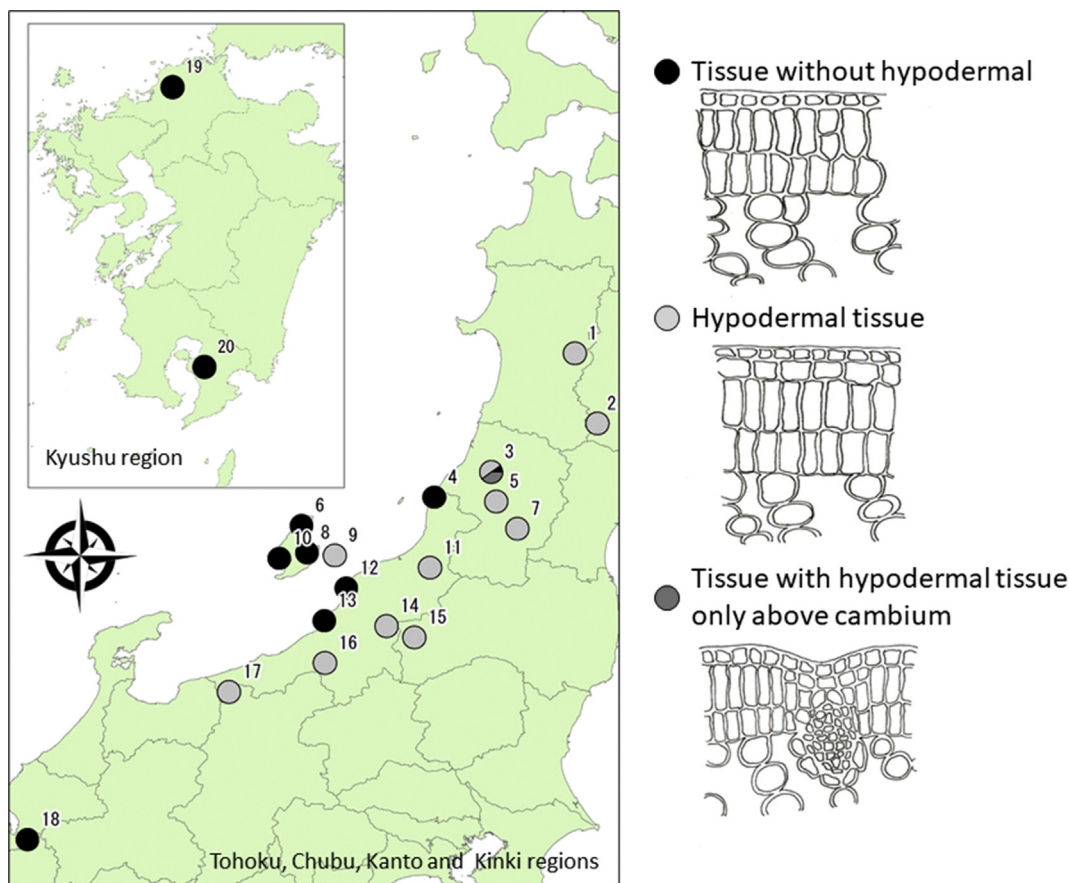
Fig. 5. Cross section of the morphology of leaves in the *Camellia* sect. *Camellia*. Number shows sampling location (Table 1).

individuals had hypodermal tissue, one did not have hypodermal tissue, and four had hypodermal tissue above the cambium (Table 1; population No. 3).

Of the thirteen individuals sampled from the hybridization zone (20—220 m), plants with hypodermal tissue were not observed below 50 m. Of the thirteen individuals sampled from the hybridization zone (20—220 m), plants with hypodermal tissue were not observed below 50 m. An intermediate form appeared in the two individuals at an elevation of approximately 50 m, and the presence of hypodermal tissue was observed in all individuals found at elevations higher than 50 m

3.2. Comparative analysis of flower morphology

Flower morphological traits of *Camellia japonica* and *C. rusticana* differed significantly (Table 2; Fig. 3). The length of stamen ($P < 0.01$), length of pistil ($P < 0.01$), width of stamen ($P < 0.01$), length of petal ($P < 0.01$), and width of petal ($P < 0.01$) were significantly larger in *C. japonica* than in *C. rusticana*. The rate of flower filament coalescence was significantly higher in *C. japonica* than in *C. rusticana* ($P < 0.01$). With respect to the petal

aspect ratio, *C. rusticana* had more elongated petals ($P < 0.01$). PCA divided the traits into two groups (Fig. 6). The cumulative contribution rate was about 87% on the PC 1 axis and about 96% on the PC 2 axis (Table 3). On the PC 1 axis, eigenvalues reached close to an upper −0.5, with the maximum length of the stamen, coalescence rate, and length of the pistil. Only one individual collected at Mt. Kunimi, Sado City, Niigata Prefecture, which is a distribution area of *C. rusticana*, showed values on the *C. japonica* side of the PC 1 axis (Table 1; population No. 9, Fig. 6). Other than this individual, most of the individuals were largely divided into two distributional areas, that of *C. japonica* or *C. rusticana*, by the PC 1 axis around 0.5 (Fig. 6).

3.3. Comparative analysis of petal and flower filament colors

Petal color analysis showed that the brightness and saturation of *Camellia japonica* and *C. rusticana* flowers differed significantly ($P < 0.01$; Fig. 7). *C. japonica* had darker and purer red petals than *C. rusticana*. The average Munsell value of *C. japonica* was 4.28, whereas that of *C. rusticana* was 4.50. The average Munsell chroma of *C. japonica* was 10.34, whereas that of *C. rusticana* was 9.96.

Table 2
Comparisons of flower morphology between *Camellia japonica* and *C. rusticana*.

Species	No.	Stamen length	Stamen width	Rate of filament coalescence	Pistil length	Petal width	Petal length	Aspect ratio of petals
Camellia japonica	124	34.59 (±4.53)	15.64 (±2.45)	0.62 (±0.07)	32.00 (±3.47)	35.8 (±5.92)	43.97 (±6.97)	1.24 (±0.17)
C. rusticana	85	15.64 (±4.56)	11.95 (±2.94)	0.34 (±0.07)	15.43 (±5.10)	23.95 (±4.25)	33.02 (±5.41)	1.40 (±0.21)

Collection sites are shown in Table 1. Significant differences were observed in all traits ($P < 0.01$; t-test).
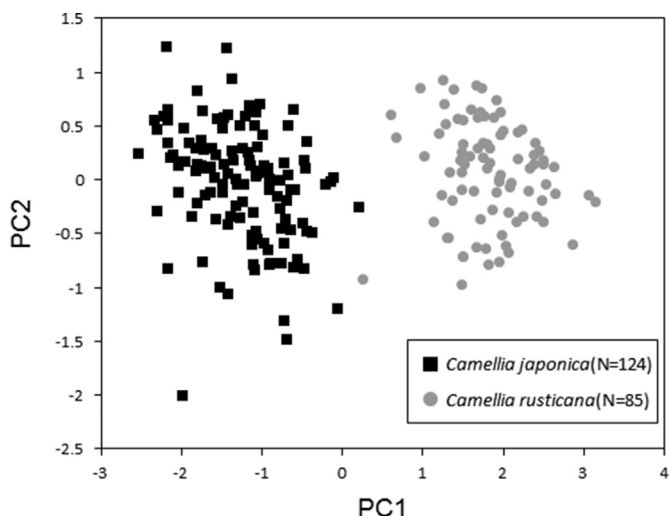
Fig. 6. Principal component analysis (PCA) on flower morphology of *Camellia japonica* and *C. rusticana*. PCA analysis was repeated using the top three items (stamen maximum length, rate of flower filament coalescence, and length of pistil) that had the highest score on the PC1 axis obtained from initial analysis.

Table 3
Synthetic variables based on principal component analysis on flower morphology of *Camellia japonica* and *C. rusticana*.

	PC1	PC2
Stamen length	−0.58	0.43
Pistil length	−0.59	0.37
Connate range	−0.56	−0.83
Cumulative proportion	0.87	0.96

Scatterplot of this PCA is shown in Fig. 6.

Average combined RGB values showed that *Camellia japonica* flower filaments were closer to white, whereas *C. rusticana* flower filaments were closer to yellow (Fig. 8). The average R, G, and B values for *C. japonica* flower filaments were 184, 141, and 51, respectively; for *C. rusticana*, R, G, and B values were 210, 184, and 157, respectively. The largest difference in RGB values was observed in the B values ($P < 0.01$).
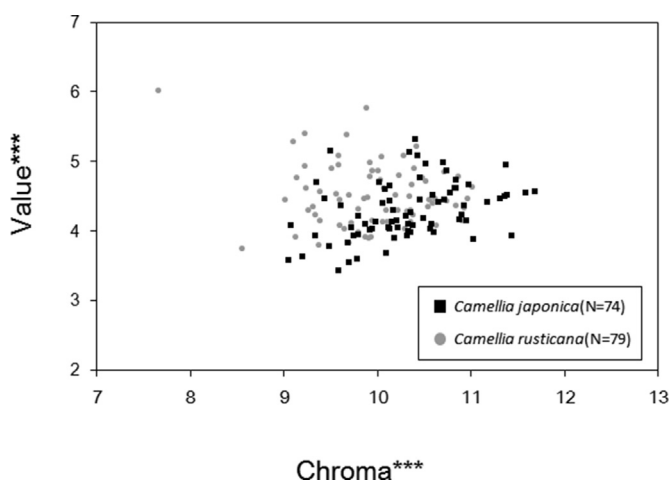
Fig. 7. Comparison of petal color of *Camellia japonica* and *C. rusticana*. Brightness and saturation were determined for the petal color using a colorimeter (CM-2002; Konica Minolta, Tokyo, Japan). The measurement wavelength is from 400 to 700 nm at 10 nm pitch. "Chroma" indicates saturation and "Value" indicates lightness. ***; $P < 0.01$ (t-test).

4. Discussion

### 4.1. Comparison of leaf morphology

In this study, we observed hypodermal tissue in leaves of plants sampled from *Camellia rusticana* distribution areas but not in leaves of those sampled from *C. japonica* distribution areas (Fig. 5). Our findings support those from previous studies that distinguish these species based on their distribution areas (Ishizawa, 1988) and leaf morphology (Shimada and Hisada, 1966; Lu et al., 2012). However, leaf morphology of plants in Mt. Haguro, Yamagata Prefecture (Table 1; population No. 3) and Nagahama City, Shiga Prefecture (Table 1; population No. 18), which are distribution areas of *C. rusticana*, were inconsistent with findings from a previous study (Ishizawa, 1988).

Previous research indicated that various forms of *Camellia japonica*, *C. rusticana*, and their hybrids are distributed at lower elevations; in contrast, at higher elevations, only populations of *C. rusticana* and its hybrids are found (Orikawa et al., 1998). Although *C. rusticana* is distributed at high elevations, the boundaries of its distribution are likely found at low to mid-elevations that comprise hybrid-dominant zones. In this study, differences in leaf morphology indicate that *C. rusticana* is distributed from 295 to 1429 m (average 631 m), *Camellia japonica* is distributed from 8 to 781 m (average 140 m), and *C. rusticana* grows in snowy areas at higher elevations (Table 1). Elevation and snow depth are not necessarily positively correlated; however, owing to atmospheric pressure, higher elevations are more likely to have greater levels of snow.

We discovered an intermediate leaf type for the first time. Specifically, of thirteen individuals sampled at an elevation of 20−220 m, two individuals sampled at approximately 50 m had an intermediate. These findings suggest that plants without hypodermal tissue are *Camellia japonica*, those with hypodermal tissue are *C. rusticana*, and those with the intermediate type are hybrids. There are some areas in Shiga Prefecture, where the distribution of both species overlaps. The sampling site in Tsubakizaka, Nagahama city (No. 18) was near the south boundary and located at a mid-elevation of 295 m, which is the lowest elevation for *C. rusticana* in this study. Therefore, hybridization appears to occur under natural conditions. We collected leaves from around a shrine in Mt. Haguro, Yamagata Prefecture, where leaf morphology could be predicted by the distribution area. Although hybridization may occur under natural conditions, there is a high probability that hybridization also occurs due to artificial planting near the shrine.

Previous studies have indicated that the presence or absence of hypodermal tissue reflects a difference in the physiological characteristics of plants living in snowy environments. For example, *Camellia rusticana* is distributed in areas that receive annual snowfall of 150 cm or more and are snow-covered for 90−200 days (Ishizawa, 1978). Photosynthetic characteristics of *C. japonica* and *C. rusticana* have suggested that *C. rusticana* must covered by snow in winter (Kume and Tanaka, 1996). In addition, Kume et al. (1998) also showed that under experimental conditions, *C. rusticana* maintains physiological activity under snow for over 360 days. These findings lead us to speculate that the presence of hypodermal tissue may be related to the maintenance of photosynthetic properties and physiological activity under snow, and that differences in environmental conditions have driven physiological adaptations that caused speciation. In our study, however, we have found only circumstantial evidence of the correspondence between the distribution and leaf morphology of *C. japonica* and *C. rusticana*. Additional work is required to confirm this relationship and elucidate the mechanisms that underlie this evolutionary process.
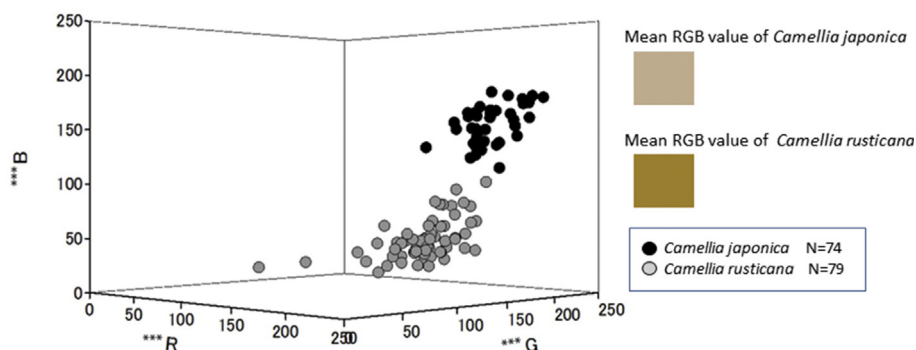
Fig. 8. Distribution filament color of *Camellia japonica* and *C. rusticana* in the RGB color space. RGB values of filament color were analyzed using camera (NIKON D100; Nikon, Tokyo, Japan) and Image J (Abràmoff et al., 2004). ***; $P < 0.01$ (y-test). i: average R, G, B values of *C. japonica* (184, 141 and 51, respectively); ii: average R, G, B values of *C. rusticana* (210, 184, and 157, respectively).

4.2. Comparison of flower morphology

Analysis of flower morphology revealed that *Camellia japonica* has larger petals than *C. rusticana* (Table 2; $P < 0.01$). While the flower filament of *C. japonica* has a high coalescence rate and a tubular shape, that of *C. rusticana* was found to be spread ($P < 0.01$). This is consistent with the findings of Ishizawa (2005). PCA showed that flower morphology was divided into two groups at approximately 0.5 on the PC 1 axis (Fig. 6). This is consistent with the distribution area of *C. japonica* and *C. rusticana* reported in a previous study (Ishizawa, 1988). Hakoda and Akihama (1988) used 44 morphological traits of flowers and leaves from *Camellia sasanqua* (three native individuals and 61 individuals from the line varieties), *Camellia oleifera* (three individuals from three varieties), and *C. japonica* (three native individuals, and three individuals from three varieties) for PCA, and demonstrated that these three strains could be distinguished by these traits. This study also suggested that discrimination between species based on flower morphology is quantitatively possible.

Petals serve as landing guides for pollinators (Faegri and Van der Pijl, 1979). In some cases, altered flower morphology results in speciation by changing pollinator preference (Grant and Grant, 1965; Zung et al., 2015). As such, altered flower morphology in *Camellia japonica* and *C. rusticana* may have resulted in a pollinator shift (e.g., from birds to insects) that drove speciation.

Adaptations to different environments may have changed pollination systems in *Camellia japonica* and *C. rusticana*. The main pollinators of *C. japonica* are birds; the plant flowers in winter when the availability of fruits and insects in temperate forests is limited (Yumoto, 1988; Kunitake et al., 2004; Abe and Hasegawa, 2008; Abe et al., 2011). In contrast, *C. rusticana* is pollinated by small insects in the early spring, which marks the beginning of the life cycles of many insects (Ishizawa, 1988). Flower morphology may also have played a role in pollinator shifts. *C. japonica* has a larger petal than *C. rusticana*. Small birds, such as Japanese white-eyes (*Zosterops japonicus*), are known to grip the petals to drink the nectar. Thus, we speculate increases in petal size allowed birds to begin visiting the *C. japonica* flowers. Furthermore, pollination syndromes are associated with particular pollinator types (Faegri and Van der Pijl, 1979). As viewed from these categories, the coalescence of the flower filament of *C. japonica* is thought to be a structure that can hold a larger amount of nectar, and this phenomenon may be related to the bird pollination syndrome (Hainsworth and Wolf, 1976; Bolten and Feinsinger, 1978). In fact, in a preliminary study, it has also been shown that the amount of nectar is considerably higher in *C. japonica* than in *C. rusticana*. We therefore speculate

that the flower morphology of *C. japonica* is more suitable for bird pollination than that of *C. rusticana*.

4.3. Comparison of petal and flower filament colors

The petals of *Camellia japonica* are more red, while the petals of *C. rusticana* are slightly lighter ($P < 0.01$) (Fig. 7), although petal colors widely overlap between the two species (89.3% of the entire area). *C. rusticana* contains more of the petal pigment 3-galactoside than the other species of the genus *Camellia* (Sakata et al., 1986, 1987). This pigment may account for the difference between *C. japonica* and *C. rusticana* petal color. Red is commonly associated with bird pollination syndrome (Faegri and Van der Pijl, 1979; Rodríguez-Gironés and Santamaría, 2004), suggesting that *C. japonica* flowers are adapted for bird pollination.

Pollinators are known to be attracted not only by petal colors, but also by color contrasts within the entire flower (Hempel de Ibarra and Vorobyev, 2009; Sletvold et al., 2016). Flower filaments of *Camellia japonica* and *C. rusticana* showed a large difference in the RGB values, with combined RGB values indicating that *C. japonica* flower filaments are white and *C. rusticana* flower filaments are yellow ($P < 0.01$; Fig. 8). The contrast between the red petals and white filaments of *C. japonica* flowers may make it easier for birds to find larger flowers from a distance, and also helps birds, which drink nectar from an unstable platform, identify where nectar is present (Cronk and Ojeda, 2008). Contrasting colors within flowers are also an important signal for insects (Hempel de Ibarra and Vorobyev, 2009; Sletvold et al., 2016). However, for insects such as flies, bees, and small beetles, the contrast of flower colors of *Camellia*, whose flower is many times larger than their body sizes, may be less important.

4.4. Speciation of Camellia based on morphological study

In this study, *Camellia japonica* and *C. rusticana* were quantitatively distinguished by the presence or absence of a hypodermis, flower morphology, and petal and filament colors.

The differentiation of flower traits is one of the barriers to genetic exchange for plants that rely on pollinators for pollen dispersal. A previous study demonstrated that in *Petunia* pollinator type depends on flower color (Hoballah et al., 2007). Another study reported that in *Campanula* the size of the corolla depends on pollinator type (Inoue and Amano 1986). In this study, we showed that flower traits of *Camellia japonica* and *C. rusticana* are clearly distinct. These distinct flower traits suggest that differences in pollinator type let to reproductive isolation and speciation of *C. japonica* and *C. rusticana*. Differences in leaf morphology

indicate that environmental conditions drove physiological adaptations that may also have caused speciation. Pre-mating mechanisms include physiological or ecological barriers to fertilization (Mayr, 1970). Taken together, the differences between these species mean pre-mating isolation, which could promote geographical and ecological reproductive isolation and consequently speciation.

Our findings in *Camellia japonica* and *C. rusticana* may represent speciation processes for other species as well. Sakata et al. (1987) reported that the distribution of anthocyanin pigments in the petals suggests that *C. rusticana* probably diverged from the common ancestor earlier than *C. japonica*. Isozyme and nrDNA ITS sequences of *C. japonica* and *C. rusticana* suggest something similar (Eguchi et al., 1991; Vijayan et al., 2009). There is a high probability that *C. japonica* and *C. rusticana* are not closely related enough to be considered sister species. However, as previously stated, the phylogeny within the genus *Camellia* is very complicated (Vijayan et al., 2009; Yang et al., 2013; Huang et al., 2014; Kim et al., 2017: Li et al., 2019). Thus, some of these specimens may represent hybrids. Accurate predictions of *Camellia* evolution require integration of molecular phylogenetic results with geographic morphological information. In addition, it is necessary to examine the evolutionary background that might have led to speciation by elucidating the phylogenetic relationships and physiological and ecological characteristics of all plants in the genus *Camellia*. Importantly, researchers should examine speciation based on pollination syndrome shifts.

Analysis of leaf morphology identified an intermediate type between *Camellia japonica* and *C. rusticana* in two regions (population No. 3 and population No. 18). Because both species grow adjacent to each other, introgressive hybridization may occur in which genetically differentiated taxa are secondarily contacted and crossed. However, since phenology does not overlap between the two plants, with *C. japonica* usually blooming in winter and *C. rusticana* usually blooming in early spring, we speculate that there are only limited opportunities for these two species to hybridize under natural conditions.

In the future, we need to examine the historical dynamics leading to speciation in the genus *Camellia,* by matching genetic classification and morphology, clarifying the influence of introgressive hybridization of the two species, and performing phylogenetic analysis of the entire *Camellia* genus.

5. Conclusions

In this study, we compared leaf morphology of *Camellia japonica* and *C. rusticana* to identify diverse adaptive strategies to different environments. We also examined the degree of speciation of these species by quantitative comparative analysis of flower form, petal color, and filament color. We were able to distinguish species by the presence or absence of leaf hypodermis. PCA indicated that the flower morphologies of these species were significantly different. We also found that petal and filament colors were significantly different. These findings suggest that pre-mating isolation caused by differences in pollinator type and environmental conditions promote speciation in *Camellia*.

Author contributions

Harue Abe: Conception and design of the study. Drafting of the article. Critical revision of the article for important intellectual content. Final approval of the article. **Hiroki Miura**: Collection and assembly of data. Drafting of the article. **All authors**: Analysis and interpretation of data.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript. All the authors agreed to submit this manuscript.

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