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The intersectional hybrid between *Weigela hortensis* and *W. maximowiczii* (Caprifoliaceae)

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Morphologically intermediate plants between *Weigela hortensis* (Siebold & Zucc.) K.Koch and *W. maximowiczii* (S.Moore) Rehder have been found in Miyagi and Yamagata Pref., northern Japan. Quantitative character analyses of flowers, pollen stainability and molecular analyses indicated that the intermediate plants were hybrids of those two species. This is the first record of an intersectional hybrid with *W. maximowiczii* (sect. *Weigelastrum*) as one of the parent species. The morphological differences among hybrid individuals imply the possibility of backcrosses or formation of second or later generations of hybrids, although those may be quite rare because of a low frequency of viable pollen grains. Causes of hybridization between two distantly-related species in *Weigela* are discussed. © 2002 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2002, **138**, 369–380.

ADDITIONAL KEYWORDS: *Bombus* spp. – internal transcribed spacer regions – pollen stainability – principal component analysis – Tohoku district – *Weigelastrum* – Wilson's test for hybridity.

INTRODUCTION

Weigela Thunb. (Caprifoliaceae) is endemic to East Asia and approximately 12 species are now recognized in the genus. These species are classified into four sections: section *Weigela* Thunb. which includes most species of the genus; section *Calysphyrum* (Bunge) Bailey which consists of three continental species; and two other monomorphic sections, which are section *Calyptrostigma* (Koehne) Rehder and section *Weigelastrum* (Nakai) Rehder (Hara, 1983; Ohba, 1993). From a molecular phylogenetic study using ITS sequences, section *Weigela* and section *Calysphyrum* formed a monophyletic group (the core group of *Weigela*: Kim & Kim, 1999) although monophyly of each section was obscure. Monophyly of *Weigela*, however, was not supported because North American *Diervilla* Mill. showed close phylogenetic affinity with section *Calyptrostigma* (*W. middendorffiana* (Carrière) K.Koch). The phylogenetic position of section *Weigelastrum* (*W. maximowiczii* (S.Moore) Rehder), as studied by different analysis procedures, was not stable and the relationship between the core group and the other two monomorphic sections was unclear (Kim & Kim, 1999). From the molecular information, at least it is obvious that section *Calyptrostigma* (*W. middendorffiana*) and section *Weigelastrum* (*W. maximowiczii*) are distantly related to other species of *Weigela* as the genus is presently circumscribed.

Although interspecific hybridizations of the genus have been known in some natural conditions, all natural hybrids recognized so far were formed between the species of the core group of *Weigela* (Hara, 1983; Ohba, 1993; Kim & Kim, 1999). Here we report a new hybrid of the genus between *Weigela hortensis* (Siebold & Zucc.) K.Koch and *W. maximowiczii*. This is the first record of hybridization between distantly-related species classified in different sections within the genus. Low frequency of intermediate individuals in populations may indicate a sporadic occurrence of

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hybridization in this combination. Our finding of the hybrid at two different localities suggests extensive hybridization may occur between the two species, at least in the Tohoku district.

MATERIAL AND METHODS

Plant material

Morphologically intermediate plants were found at two localities, the Yamagata Prefecture side of Futakuchi Pass (38°16′ N, 140°28′ E; Yamagata-shi, Yamagata Pref.) and at the eastern foot of Mt. Izumigatake (38°25′ N, 140°43′ E; Sendai-shi, Miyagi Pref.), both of which are in the Tohoku district of northern Japan (Fig. 1). In both localities, dozens of individuals of the putative parent species, *W. hortensis* and *W. maximowiczii*, grow together along the forest margin. Samples for the analyses described below were collected from both localities. Flowers were fixed in 70% ethanol until analyses of morphological traits and pollen stainability were made. Fresh leaves were dried using appropriate amounts of silica gel for DNA isolations. All voucher specimens were deposited in the Herbarium, Biological Institute, Graduate School of Science, Tohoku University (TUS).

Analyses of quantitative characters of flowers

Four intermediate plants (all three samples from Futakuchi and one from Izumigatake) were used for measurements of flowers. The other plant from Izumigatake was excluded from the measurements because of the lack of sufficient flowers on it. Putative parent species were also sampled for comparison. Ten plants of *W. hortensis* were collected at both Futakuchi and Izumigatake. For *W. maximowiczii,* ten individuals were sampled at Futakuchi and three from Izumigatake. Three flowers were examined from each individual of intermediate plants and from *W. hortensis,* and two from *W. maximowiczii*.

For the analysis, we chose the following eight characters based on preliminary observations of flowers (Figs 2, 3): length and width of corolla tube (COL and COW, respectively), length from the base to the adnate point of the dorsal stamen to the corolla (APL), length of the upper-left and lower-central lobes of the corolla (ULL and LLL, respectively), length of dorsal stamen and pistil (STL and PSL, respectively), and the length of the calyx (CAL). All characters were measured with a digital caliper.

Wilson's test for hybridity (Wilson, 1992) was conducted using the above eight characters. Morphological intermediacy was determined by comparing the ranges of measurements in intermediate plants with those of both putative parent species. If the range of a given measurement of intermediate plants was completely included in the combined range of both putative parent species (from the smallest measurement of smaller species to the largest of larger species), the character was determined as an intermediate. Characters determined as intermediate were counted and the degree of hybridization was assessed by a one-sided sign test of intermediate *vs* non-intermediate characters (Wilson, 1992).

Besides the direct comparisons of measurements, principal component analysis (PCA) was applied to

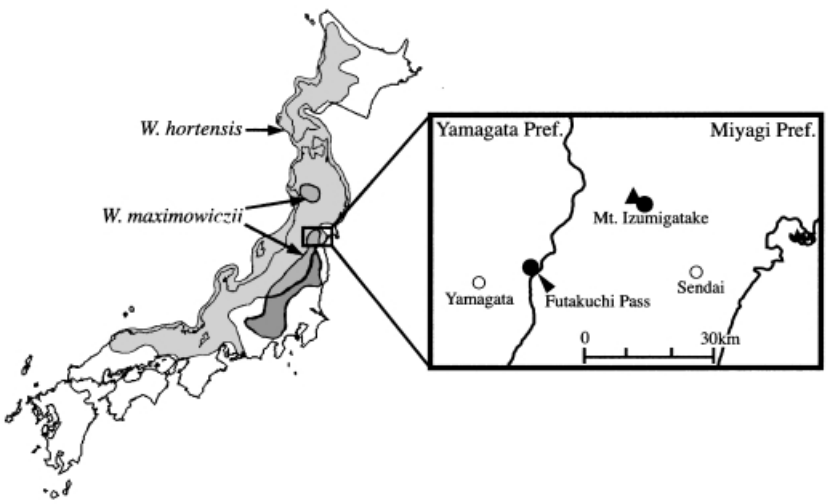
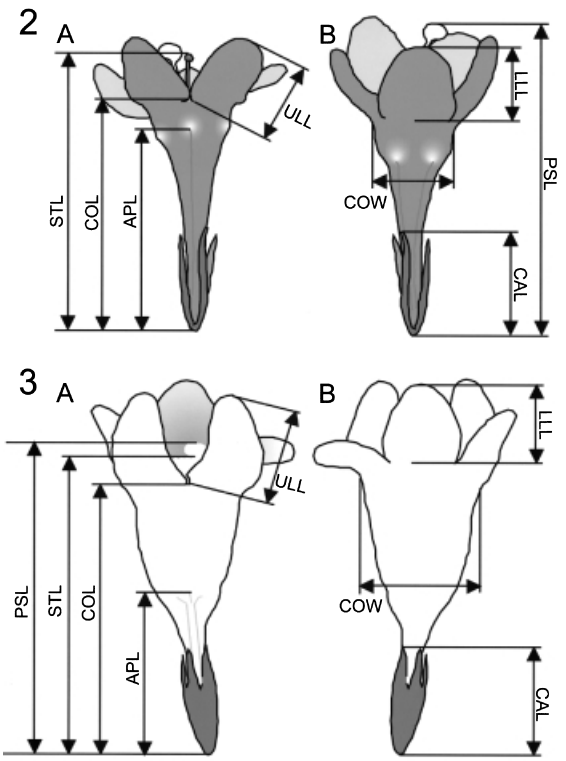


Figure 1. Sampling localities of intermediate plants with distribution ranges of putative parent species based on Hara (1983). The lightly shaded areas indicate the distribution range of *Weigela hortensis* and the dark shaded areas indicate that of *W. maximowiczii*.



Figures 2, 3. Schematic diagrams of flowers of two *Weigela* species showing positions of characters measured. Fig. 2: *W. hortensis*; Fig. 3: *W. maximowiczii*. A, a view from the dorsal side of a flower. B, a view from the ventral side of a flower.

summarize the overall tendencies of character variations. PCA was computed with EXCEL Statistics 2000 (Social Survey Research Information Co. Ltd, Tokyo). To minimize the differences in the magnitude of measurements, all values were log-transformed before analysis.

Pollen stainability

To confirm pollen viability of intermediate plants, we examined the stainability of pollen grains by lactophenol/cotton blue solution. Fixed young flowers full of pollen grains in their anthers were used for examinations. Anthers were cut from the flowers, soaked in a drop of lactophenol/cotton blue solution on a slide under a cover-slip. Stained pollen grains were observed and counted under a light microscope. At least 500 pollen grains were counted and the ratios of well-stained pollen grains with normal shape and size compared to those from both putative parent species were determined.

Molecular analyses

PCR–RFLP analyses for the internal transcribed spacer (ITS) regions of 18–26S nuclear ribosomal DNA were conducted to clarify the hybrid nature of the intermediate plants. Kim & Kim (1999) have shown that there are some nucleotide substitutions, identifiable by use of restriction enzymes, which can differentiate between putative parent species. We used two *Mva*I sites (CCWGG) in ITS1 and a *Sma*I site (CCCGGG) in ITS2 as markers; the ITS of *W. hortensis* has all the cleavage sites but *W. maximowiczii* does not (Fig. 4). One *Mva*I site gain is an apomorphy of the core group of *Weigela* and the other site loss is an autapomorphy of *W. maximowiczii*. A loss of the *Sma*I site is a synapomorphy of *W. maximowiczii*, *W. middendorffiana* and *Diervilla* (Kim & Kim, 1999). Although autapomorphies of *W. hortensis* should strictly be used for genetic confirmations of parentage, restriction enzymes which digest at autapomorphic substitution sites of *W. hortensis* (e.g. *Hae*III or *Msp*I) are not appropriate for PCR–RFLP analyses because apomorphic fragments are too short to detect with agarose gel electrophoresis.

Samples used for the molecular analysis are listed in Table 1. Total DNA was isolated from 200–300 mg of dried leaves by the modified 2XCTAB method (Hasebe & Iwatsuki, 1990). The PCR reaction mixtures contained 100–200 ng of total DNA, 1 μM of each primer, 200 mM of each deoxynucleotide, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 5% (v/v) DMSO, and 1.25 units of recombinant Taq DNA polymerase (TaKaRa Co. Ltd, Japan).

We used primers described in White *et al*. (1990) and Kim & Kim (1999) to amplify the ITS region including the 5.8S rRNA gene. The thermal cycle profile for amplifications was: one cycle of 94 °C (2 min), 45 °C (2 min), 60 °C (3 min) and 30 cycles of 94 °C (1.5 min), 45 °C (2 min), 60 °C (3 min) followed by 15 min of extension profile at 72 °C. The same profile was previously used by Yokoyama *et al*. (2000). The amplification cycle was performed by the QTP-1 thermal cycler (Nihon Genetics, Japan).

Based on the data of Kim & Kim (1999), there is a single substitution between sequences of the two samples of *W. hortensis*. Thus, we preliminarily sequenced ITS regions from selected samples of the *Weigela* used in this study to confirm whether PCR–RFLPs can effectively demonstrate hybridization (Table 1). The PCR products were separated on 1% low-melting-point agarose gels and the appropriate size of DNA bands were purified. Cycle sequencing reactions were conducted using about 80–100 ng of purified PCR products and the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (Applied

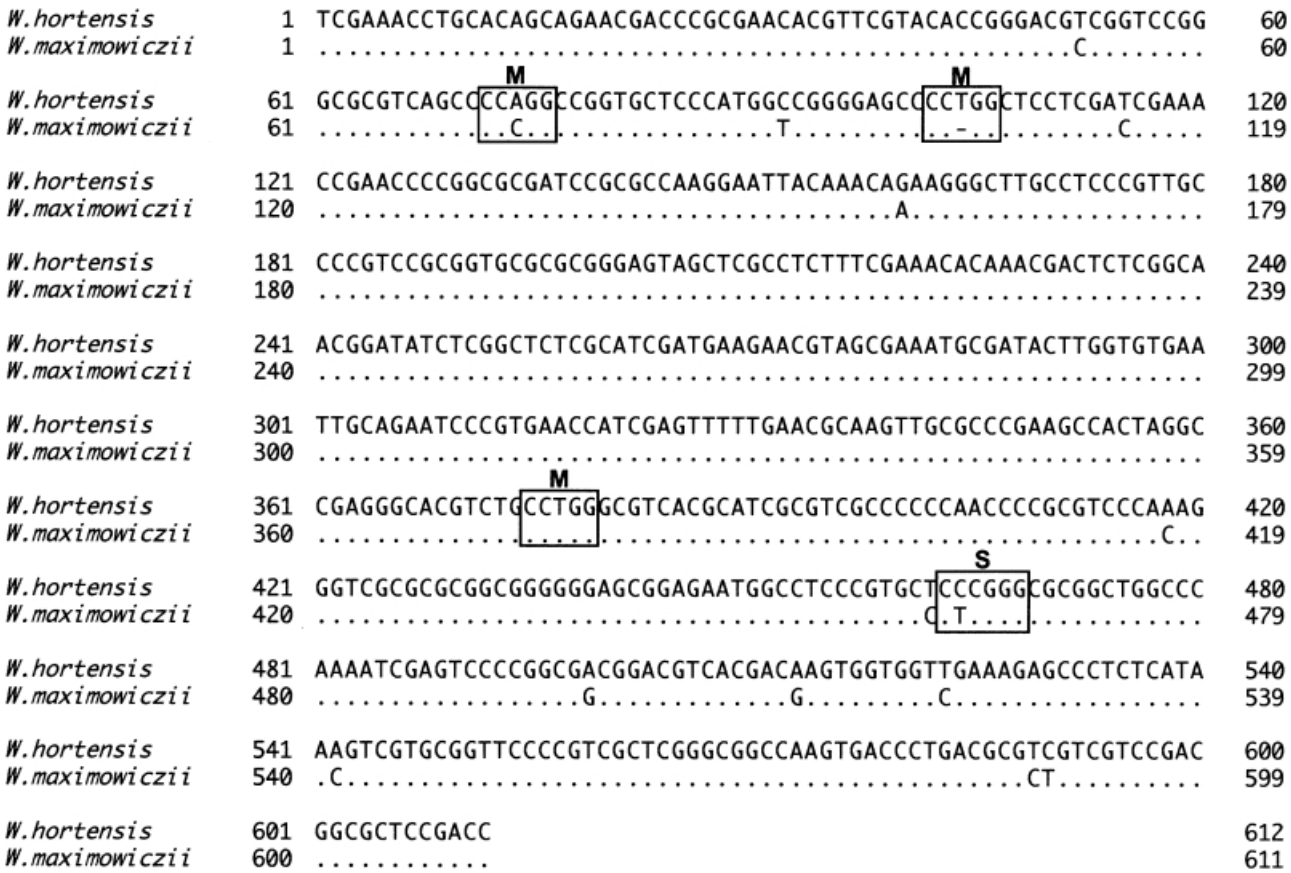


Figure 4. Expected restriction sites for molecular characteristics of ITS regions by PCR–RFLPs. Sequence data from Kim & Kim (1999). M: restriction site of *Mva*I; S: restriction site of *Sma*I.

Table 1. Samples of *Weigela* used for the molecular analyses. The numbers in parentheses indicate individuals sequenced

Species	Locality	*N*
W. hortensis	Futakuchi	5 (1)
	Izumigatake	2 (1)
	Miyagi: Sendai, Aoba-ku, Aramaki	6 (1)
W. maximowiczii	Futakuchi	4 (1)
	Izumigatake	1
Intermediate plants	Futakuchi	3 (1)
	Izumigatake	2

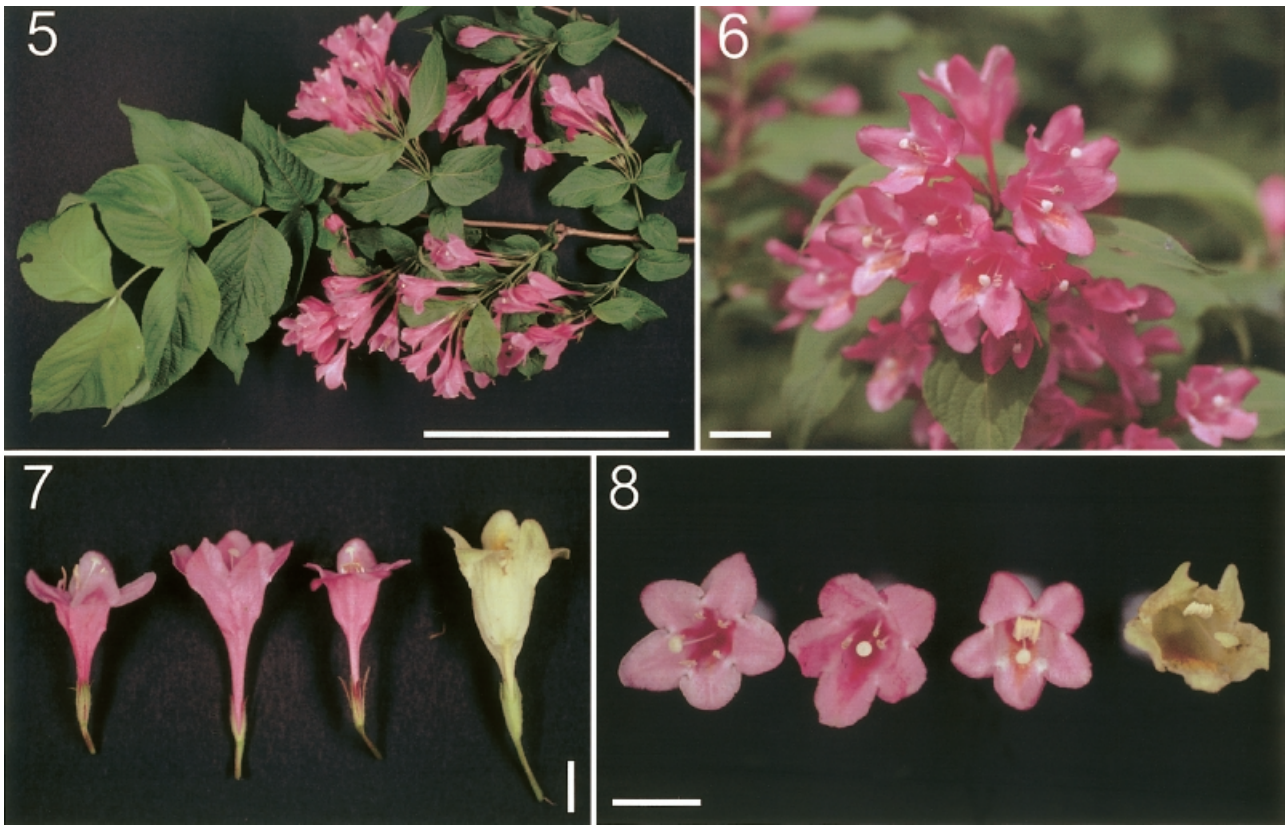
Biosystems) following manufacturer's instructions. DNA sequences were obtained using the 373A DNA Sequencer (Applied Biosystems).

PCR–RFLP analyses were carried out after checking the sequence results. Amplified products were digested by each of the above restriction enzymes at either 30 °C (*Sma*I) or 37 °C (*Mva*I) for more than an hour. Digested DNAs were separated on 1% agarose gels (run with appropriate size-markers) and the size of each band was determined.

RESULTS

OBSERVATIONS

We found three intermediate plants at Futakuchi and two at Izumigatake. The intermediate plants were



Figures 5–8. Morphological features of intermediate plants. Fig. 5. A flowering branch of an intermediate plant from Futakuchi. Scale bar = 10 cm. Fig. 6. Flowers of an intermediate plant from Izumigatake Scale bar = 1 cm; Figs 7, 8. Dorsal (7) and front (8) views of flowers of intermediate plants from Futakuchi (middle two from different individuals) and their putative parent species, *W. hortensis* (left) and *W. maximowiczii* (right). Scale bar = 1 cm.

1.5–2 m high. Each branch of the previous year had developed a main shoot from the top and had several lateral branches with flowers (Fig. 5). Leaf characters were intermediate. Leaves were pubescent on both sides, similar to those of *W. maximowiczii*, while leaf size (6.1–9.8 cm long and 2.4–5.1 cm in width for the largest leaf of a branch, $N = 10$ (all five plants, two branches per individual)) and overall form (width/length ratio was 0.39–0.53) were more like *W. hortensis* (6.3–11.6 cm long and 2.4–5.3 cm in width, width/length ratio: 0.36–0.53, $N = 20$ (10 plants, two branches per individual)) than *W. maximowiczii* (4.0–6.8 cm long and 1.8–3.8 cm in width, width/length ratio: 0.42–0.58, $N = 20$ (10 plants, two branches per individual)). The length of petiole was between that of the two putative parent species (1.6–3.7 mm in intermediate plants, 3.5–6.1 mm in *W. hortensis*, and 0–1.1 mm in *W. maximowiczii*).

Inflorescence habit was quite similar to that in *W. hortensis*. An inflorescence was developed at each leaf axil and at the apex of the lateral branch. A single inflorescence consisted of two to three flowers (Fig. 5; Hara, 1983). In contrast to this, only a single flower was present at each leaf axil and no flowers occurred at branch apices in *W. maximowiczii* (Hara, 1983). At first glance, the rose-coloured flowers looked like those of *W. hortensis*. However, zygomorphic corollas with strong orange or reddish-pink colouration from the lower-central lobes to the throat, and more-or-less connivent anthers on the upper half of the corollas implied genetic relationship with *W. maximowiczii* (Fig. 6). The calyx lobes of intermediate plants were completely split as in *W. hortensis* or connate to form very short (1–1.5 mm) tubes as in *W. maximowiczii*. Floral characteristics of an individual from Futakuchi were more similar to *W. hortensis* than other intermediate plants, especially in flower colouration and anther form and position (Figs 7, 8). This implies the possible occurrence of backcrossing, or formation of the second or later generations of the hybrids.

Analyses of quantitative characters of flowers

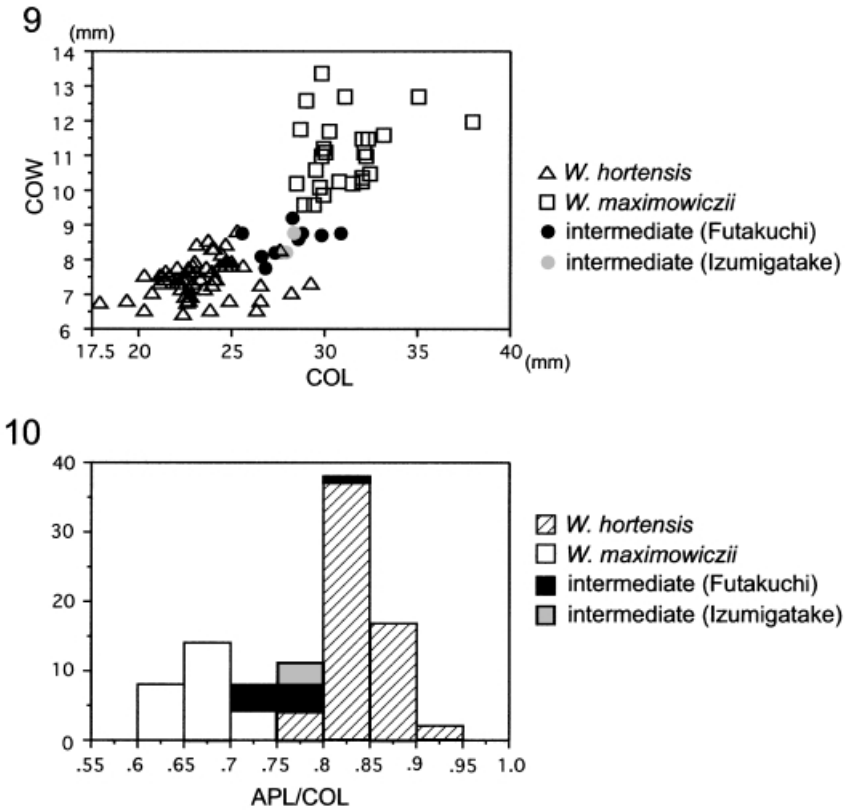
A total of 12 flowers were measured from four different individuals of intermediate plants (three individuals from Futakuchi and one from Izumigatake). Measurements of 60 flowers from 20 plants of *W. hortensis* and 26 flowers from 13 individual plants of *W. maximowiczii* were also used for comparisons. Averages, standard deviations, and ranges of measurements are listed in Table 2. Average measurements of all characters were larger for *Weigela maximowiczii* than for *W. hortensis*. In particular, COL and COW were quite different and the ranges of COW did not overlap between the two species. The measurements of COL and COW for the intermediate plants occupied the medial range between the two species (Fig. 9). One of the significant differences between flowers of *W. hortensis* and *W. maximowiczii* is the proportion of throat to total corolla length. *W. maximowiczii* has a relatively larger proportion of throat than *W. hortensis*. The ratio of APL to COL is a useful index. The values of APL/COL were 0.79–0.92 (average 0.84) in *W. hortensis* and 0.61–0.71 (average: 0.67) in *W. maximowiczii*. The values for intermediate plants fell between these (0.72–0.80 (average: 0.76) in Futakuchi and 0.75–0.78 (average: 0.77) in Izumigatake; Fig. 10). Some average values of measurements from intermediate plants were beyond those of the putative parent species. The average values of APL for the intermediate plants were larger than for both putative parent species (Table 2). This might be the result of relatively large flowers, as in *W. maximowiczii*, and a relatively small proportion of throat, as in *W. hortensis*. The average value of LLL for an Izumigatake plant was larger than that of *W. maximowiczii*, and the average value of CAL was smaller than that of *W. hortensis*. These might be due to a small sample size (Table 2). Nonetheless the ranges of all eight characters were within the combined ranges of both putative parent species.

All measurements of the intermediate plants were intermediate between those of the putative parent species (Table 2). Based on Wilson's test for hybridity, this result was evaluated with the one-sided sign test, which yielded $P = 0.004$. Thus, the null hypothesis of the origin of the intermediate plants (primary divergence from ancestral species and not hybrid origin) can be rejected: the result of the test indicates the hybrid origin of intermediate plants.

Table 2. Results of measurements of quantitative floral characters in intermediate plants and their putative parent species, *W. hortensis* and *W. maximowiczii*. Values are given as means ± SE, with ranges indicated. For explanation of characters see Figures 2 and 3

Character (mm)	*W. hortensis* ($N = 60$)*	Intermediate plants (Futakuchi: $N = 9$)	Intermediate plants (Izumigatake: $N = 3$)	*W. maximowiczii* ($N = 26$)
COL	23.3 ± 2.00 (17.9–29.3)	28.0 ± 1.66 (25.5–30.8)	28.0 ± 0.31 (27.7–28.3)	31.5 ± 2.04 (28.4–37.9)
COW	7.4 ± 0.54 (6.4–8.8)	8.6 ± 0.44 (7.8–9.2)	8.4 ± 0.35 (8.2–8.8)	11.1 ± 1.02 (9.6–13.4)
APL	19.6 ± 1.85 (14.9–24.7)	21.3 ± 1.88 (19.1–24.8)	21.6 ± 0.55 (21.0–22.1)	20.6 ± 1.61 (18.3–24.9)
ULL	7.2 ± 0.91 (5.7–9.6)	6.8 ± 1.00 (5.7–8.2)	8.1 ± 0.70 (7.4–8.8)	8.3 ± 0.94 (5.1–9.9)
LLL	7.5 ± 0.83 (6.0–9.9)	7.3 ± 0.90 (6.2–8.4)	9.0 ± 0.66 (8.3–9.6)	8.8 ± 0.63 (7.2–9.8)
STL	27.4 ± 2.31 (22.0–34.0)	30.5 ± 1.64 (28.3–33.2)	30.7 ± 0.32 (30.5–31.1)	33.4 ± 1.66 (30.9–37.9)
PSL	30.1 ± 2.90 (24.0–36.6)	32.4 ± 1.51 (30.4–34.3)	32.8 ± 0.79 (32.2–33.7)	35.5 ± 1.92 (32.3–40.6)
CAL	8.0 ± 1.57 (4.9–11.2)	8.2 ± 0.63 (7.4–9.1)	6.1 ± 0.49 (5.8–6.7)	10.6 ± 1.50 (7.8–14)

*N indicates the number of flowers measured (three flowers were chosen from each individual of intermediate plants and *W. hortensis*, and two from each individual of *W. maximowiczii*, see Material and Methods).

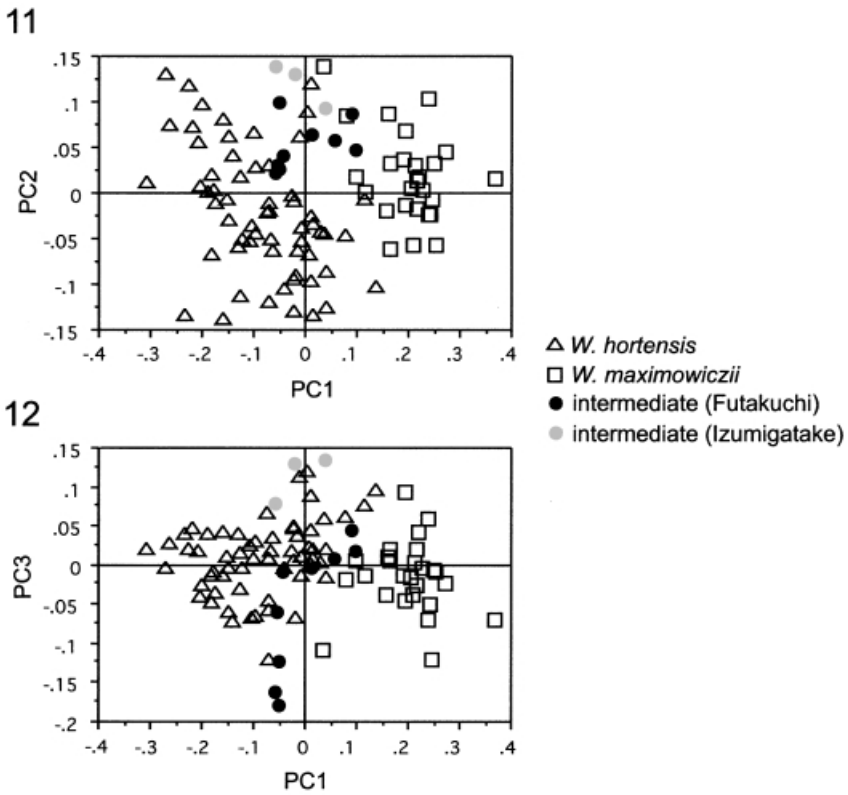


Figures 9, 10. Comparative measurements of intermediate plants and their putative parent species. Fig. 9. A plot of corolla length (COL) and corolla width (COW) of intermediate plants and their putative parent species. Each symbol in the plot represents a single flower (three flowers taken from each individual of an intermediate plant and *W. hortensis*, and two from each individual of *W. maximowiczii*, see Material and Methods). Fig. 10. A histogram showing distribution of the ratio of the length from the base to the adnate point of dorsal stamen to corolla (APL) to corolla length (COL).

The variances accounted for the principal components (PC) 1 to 3 were 66.5, 14.2, and 9.6%, respectively. Principal component loadings on the first three components are listed in Table 3. PC1 well represented the total size of flowers. PC2 accounted for the length of corolla (include APL) and reproductive parts. PC3 mainly expressed the length of lobes and calyx. The plots of samples on PC1 and 2, or PC1 and 3 are shown in Figures 11 and 12, respectively. PC1 made an important contribution to dividing samples of putative parent species into two groups. *Weigela hortensis* and *W. maximowiczii* appeared mainly in the negative and positive field on the PC1 axis, respectively. Intermediate plants were plotted around the boundary of the two putative parent species. In contrast, PC2 and PC3 did not separate the putative parent species, although the plotted area of *W. hortensis* was extended deeper into the negative field than that of *W. maximowiczii* on the PC2 axis (Fig. 11). All samples of intermediate plants appeared in the positive field on the PC2 axis, representing a relatively large flower and a shorter calyx length. On the PC3 axis, intermediate plants showed a wider distribution range than the putative parent species (Fig. 12). This was mainly caused by the variation in calyx lobe length (ULL and LLL); samples from Izumigatake and those of a single plant from Futakuchi have larger and smaller lobes, respec-

Table 3. Principal component loadings on the first three components. For explanation of characters see Figure 2

	Principal component		
Character	1	2	3
COL	0.853	0.471	–0.119
COW	0.871	0.229	–0.147
APL	0.547	0.436	0.099
ULL	0.751	–0.214	0.579
LLL	0.719	–0.110	0.603
STL	0.878	0.372	–0.049
PSL	0.878	0.290	0.008
CAL	0.819	–0.513	–0.238



Figures 11, 12. Plots of PC1/PC2 (Fig. 11) and PC1/PC3 (Fig. 12) for intermediate plants and their putative parent species using eight characters shown in Figs 2 and 3. Each symbol in the plot represents a single flower as in Figure 9.

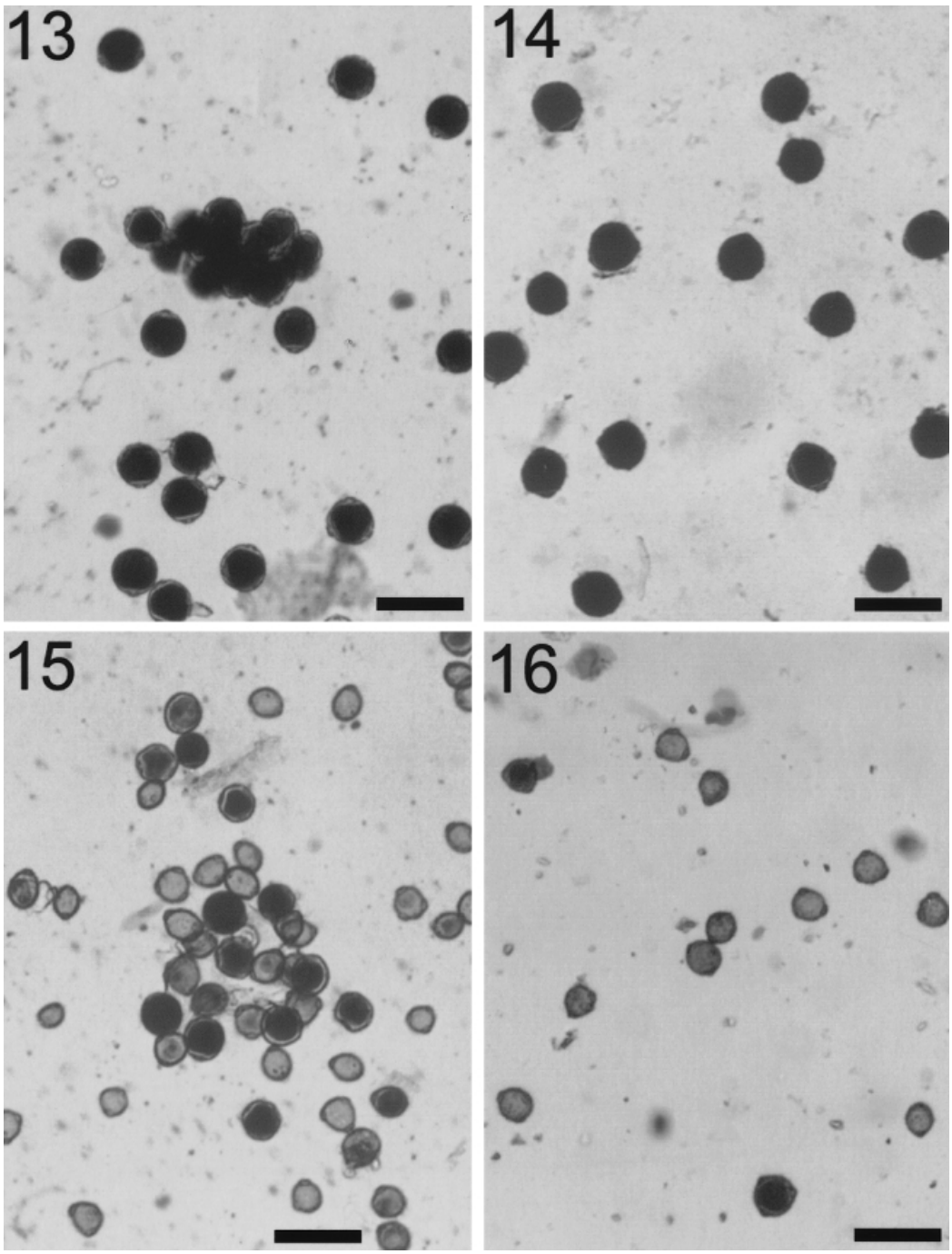
tively, than most of the flowers from both putative parent species. Thus in this analysis, we found that PC1 summarized accurately the morphological differences between flowers in both putative parent species and in samples of intermediate plants that showed values around their boundaries. However, the contribution of lower PCs to an overview of morphological differences was poor, mainly because of the insufficient sample size. Further studies of the floral morphometry of *W. hortensis* and *W. maximowiczii* over their distribution range are necessary to confirm the result.

Pollen stainability

Pollen grains of *W. hortensis* and *W. maximowiczii* were round and stained by cotton blue (Figs 13, 14). Most pollen grains of intermediate plants, however, shrank and were very small, and were not stained by cotton blue (Figs 15, 16). The stainabilities of five intermediate plants compared with their putative parents are listed in Table 4. Low stainabilities of all intermediate plants suggest their hybrid origin.

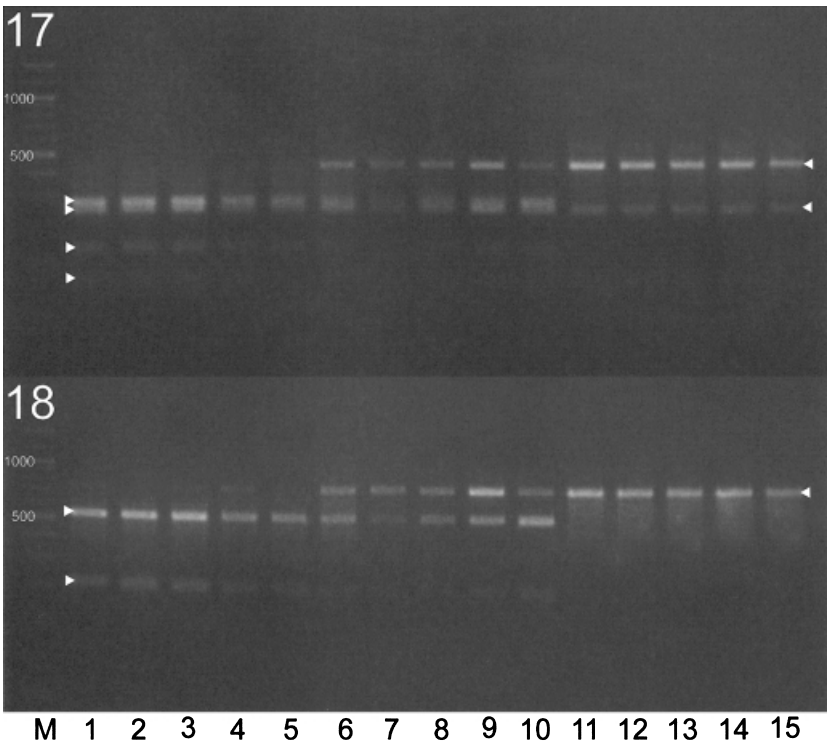
Molecular analyses

Based on sequence data of Kim & Kim (1999), the amplified ITS region of *W. hortensis* has three *Mva*I sites and one *Sma*I site, while *W. maximowiczii* has only one of the three *Mva*I sites and no *Sma*I sites (Fig. 4). Preliminary examination of sequences of selected samples in this study revealed that the ITS region of *W. hortensis* had exactly the same sequence as *W. hortensis* 1 of Kim & Kim (1999). Although sequences of *W. maximowiczii* had a single nucleotide substitution in ITS2 compared with their data, this did not influence the expected digestion patterns. Thus, we could anticipate the presence of the above digestion sites for the samples used in this study (sequences determined in this study will appear in EMBL/GenBank/DDBJ under the accession numbers of AB055080–AB055087). In an intermediate plant from Futakuchi, the sequence results processed by an autosequencer were on the whole identical to those of *W. hortensis*. There were, however, weak but evident signals which might imply the presence of the ITS of *W. maximowiczii*. Thus, we conducted PCR–RFLPs to obtain further evidence of the hybrid nature of intermediate plants.



Figures 13–16. Pollen stainabilities of intermediate plants and their putative parent species. Fig. 13. *W. hortensis* (Futakuchi). Fig. 14. *W. maximowiczii* (Izumigatake). Fig. 15. Intermediate plant (Futakuchi). Fig. 16. Intermediate plant (Izumigatake). Scale bar = 100 μm.

Digestion patterns of all samples of *W. hortensis* and *W. maximowiczii* examined in this study agreed with the expected patterns. All intermediate plants showed combined patterns of both putative parent species (Figs 17, 18; Table 5). These results clearly indicate the hybrid nature of all intermediate plants. As outlined above, a *Mva*I site used as a marker of *W. hortensis* is a synapomorphy of the core group of *Weigela*. Thus, the results of PCR–RFLPs alone do not exclusively show genetic evidence of parentage of *W.*



Figures 17, 18. PCR–RFLP profiles of intermediate plants and their putative parent species. Fig. 17. *Mva*I; Fig. 18. *Sma*I. Arrowheads indicate expected fragments of both putative parent species. M: size markers; 1–5: *W. hortensis*; 6–10: intermediate plants from Futakuchi (6, 9, 10) and Izumigatake (7, 8); 11–15: *W. maximowiczii*.

Table 4. Pollen stainabilities of intermediate plants and their putative parent species

Species	Locality	*N*	Stainability
Intermediate plants	Futakuchi	3	19.4–24.2%
	Izumigatake	2	19.6, 30.3%
W. hortensis	Futakuchi	6	93.5–99.0%
	Izumigatake	2	98.5, 99.4%
W. maximowiczii	Futakuchi	3	98.0–99.0%
	Izumigatake	1	91.6%

Table 5. Results of PCR–RFLPs of ITS regions. H indicates an expected digestion pattern of *W. hortensis* and M indicates one of *W. maximowiczii*. The numbers in parentheses indicate the number of individuals examined

Species	Locality	*Mva*I	*Sma*I
W. hortensis	Futakuchi (5)	H	H
	Izumigatake (2)	H	H
	Aramaki (6)	H	H
W. maximowiczii	Futakuchi (4)	M	M
	Izumigatake (1)	M	M
Intermediate plants	Futakuchi (3)	H/M	H/M
	Izumigatake (2)	H/M	H/M

hortensis. Although only a single sample was sequenced, the result clearly showed the existence of ITS of *W. hortensis* in an intermediate plant. Therefore, we consider the result as evidence of hybridization between *W. hortensis* and *W. maximowiczii*.

DISCUSSION

All results in this study clearly indicate that the intermediate plants we found are hybrids between two distantly-related species in *Weigela*. Although interspecific hybridizations have been recorded in the genus, all hybrids recognized so far were formed only between species of the core group of *Weigela* (Hara, 1983; Ohba, 1993; Kim & Kim, 1999). Accordingly, this is also the first record of intersectional hybridization in which *W. maximowiczii* (sect. *Weigelastrum*) is one of the parent species. *W. maximowiczii* has quite different morphological features from the species belonging to the core group of *Weigela*, and was formerly considered as a distinct genus (*Weigelastrum* Nakai:

Nakai, 1936). Molecular phylogenetic analyses also indicate a distinctness in the lineage of *W. maximowiczii* form the core group of *Weigela*, if the North American *Diervilla* is retained as a genus (Kim & Kim, 1999). The degrees of sequence divergence of ITS regions between *W. maximowiczii* and species of the core group of *Weigela* are, however, low compared with those among congeneric species of other related genera (percent pairwise sequence divergence excluding 5.8S rDNA regions: 1.8–3.1% (mean: 2.2) (Kim & Kim, 1999), compared with 0–6.5% (mean: 4.0) of *Sambucus* spp. (Eriksson & Donoghue, 1997), and 0–13.6% (mean: 7.3) (ITS1) or 0–11.9% (mean: 6.1) (ITS2) of *Viburnum* spp. (Donoghue & Baldwin, 1993; Baldwin *et al*., 1995)). Although sequence divergence of ITS does not always reflect the degree of overall genetic differentiation or post-mating reproductive isolation among species, the molecular data suggest that *W. maximowiczii* is less divergent genetically than considered from morphological characteristics. Thus, hybridization with other species of *Weigela* may have occurred more easily than expected.

At least from our observations in Miyagi and Yamagata Pref., *W. hortensis* and *W. mazimowiczii* occur sympatrically on the margin of beech (*Fagus crenata* Blume) or oak (*Quercus crispula* Blume) forests at *c*. 700–1000 m altitude. Although *W. maximowiczii* blooms earlier than *W. hortensis* in these areas, flowering times of both species largely overlap. Furthermore, snow remaining on the northern face of the slopes untill late spring delays flowering time of plants there. Many individuals of *W. maximowiczii* grow on the northern slopes, especially in Futakuchi, causing co-flowering with *W. hortensis*. Thus, hybridization could occur over a wide range of the Tohoku and northern Kanto districts where both species are potentially distributed sympatrically. The frequency of hybridization between the two species might be confirmed by an extensive survey of regions where both species grow together. Moreover, the distribution range of *W. maximowiczii* is expanded to the southern Kanto and Chubu districts in central Japan (Hara, 1983; Ohba, 1993) where another *Weigela* species, *W. decora* (Nakai) Nakai, rather than *W. hortensis* is distributed. The findings of this study imply the possibility of intersectional hybridization of other combinations of species. Further investigations are clearly needed.

Important pollinators of *Weigela* spp. are apoid bees, especially a long-tongued andrenid bee *Andrena halictoides* Smith, small carpenter bees *Ceratina* spp., and bumblebees *Bombus* spp. (Matsuura *et al.,* 1974; Yamauchi *et al*., 1976; Inoue *et al*., 1990; Kato *et al*., 1990; Kato *et al.,* 1993; Chang, 1997; Iwata, 1997). In Miyagi and Yamagata Pref., *A. helictoides* and *Bombus* spp. are the main pollinators of *W. hortensis*, with the latter being the most important (M. Nakajima & J. Yokoyama, unpublished observations). *Bombus* spp. are also important for the pollination of *W. maximowiczii* (J. Yokoyama & A. Yokoyama, unpublished observations). We observed queens of *Bombus diversus* and workers of *B. beaticola* Tkalcu on flowers of both species and their hybrids in Futakuchi. Flowers of *W. maximowiczii* have basic characters of nototribically-pollinated gullet flowers (Faegri & van der Piji, 1966), and the pollinators of primary importance are the queens of the large, long-tongued bumblebees such as *B. diversus*. *W. hortensis* has more actinomorphic flowers than *W. maximowiczii* with radially placed anthers. All visiting bumblebees can potentially contact reproductive organs of *W. hortensis*. Thus, the queens of *B. diversus* probably contribute to interspecies pollen transfer between *W. hortensis* and *W. maximowiczii*. The workers of *B. beaticola*, on the other hand, seem not to be suitable as pollinators of *W. maximowiczii* when they visit its flowers nototribically, because their body size is too small to contact the reproductive organs. In our observations, however, the workers of *B. beaticola* visited *W. maximowiczii* flowers for pollen collection by inverting themselves and by scratching anthers. They can also contact stigmas at the same time. Thus, *W. maximowiczii* is pollinated sternotribically by the pollen-collecting workers of *B. beaticola*. The behaviour of *B. beaticola* may be more important for interspecies pollen transfer between *W. hortensis* and *W. maximowiczii* than the visits of *B. diversus*. The pistils of *W. hortensis* are exserted and pendent from the openings of mature corollas and bumblebees usually land on flowers such that stigmas are under their bodies. Thus, *W. hortensis* is generally pollinated sternotribically by bumblebees. These facts suggest that *B. beaticola* can transfer pollen bidirectionally while *B. diversus* usually transfers pollen from *W. hortensis* to *W. maximowiczii*. Although the relative importance of the *Bombus* species for hybridization needs further confirmation by extensive observations and experiments, we know at least that two bumblebee species play an important role for the interspecies pollen transfer between *W. hortensis* and *W. maximowiczii*.

Low stainability of pollen grains of hybrids found in this study indicates that extensive introgressions between the parent species are less probable. Some viable pollen grains, however, introduce the possibility of backcrosses or production of second or later generations of hybrids themselves. Floral features seem to be segregated among hybrid individuals implying this possibility, as does the relatively long lifetime of the plants. Although hybridization of *W. hortensis* and *W. maximowiczii* may be a rare event at present, its effects on the evolution of both species should not be neglected. Further observa-

tions and experiments including artificial crosses are needed to confirm the evolutionary significance of the hybridization.

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