

# Quantitative morphological analysis of populations in a hybrid zone of *Epimedium diphylum* and *E. sempervirens* var. *rugosum* (Berberidaceae)

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**Background and aims** – The populations of *Epimedium* in the western part of Chugoku district on the Japanese island of Honshu are morphologically diverse: most consist of individuals showing wide variation in morphological characteristics, and the character composition varies with population. We explored the morphological variations between *Epimedium* populations in Chugoku district to test the hypothesis that these variations are the result of natural hybridization between *E. diphylum* and *E. sempervirens* var. *rugosum*, and to elucidate to what extent natural hybridization between these two species has occurred in this region.

**Methods** – We measured the length of three flower parts using a digital caliper and counted the number of teeth per leaflet. The leaf architecture was also categorized. In addition, we quantified flower colour and leaflet apex shape using a spectrophotometer and principal component analyses of elliptic Fourier descriptions, respectively. From these measurements, we calculated Anderson's hybrid index for each individual.

**Key results** – The populations in Chugoku district showed wide variation in the morphological characters examined. In particular, the populations located near the center of the hybrid zone tended to show greater variation than those located at the periphery. The variation observed across the populations showed similar patterns among the morphological characters examined. No correlations were found among the examined characters.

**Conclusions** – The wide variation in morphological characters likely resulted from natural hybridization between *E. diphylum* and *E. sempervirens* var. *rugosum*. Hybridization between the two species occurred intensively where the distributions of the species overlap, and bi-directional introgressions of these species are advancing throughout the region. To clarify the factors that produced the hybrid zone, it will be necessary to compare fitness between hybrids and their parental species throughout the parental species' distribution areas and the hybrid zone.

**Key words** – Berberidaceae, *Epimedium*, hybridization, hybrid zone, morphological variations.

## INTRODUCTION

*Epimedium* L. is a genus of the family Berberidaceae that is distributed from Japan to Algeria with enormous gaps (Stearn 2002). Over fifty species of *Epimedium* have been described; however, the center of diversity is in China (Stearn 2002). In Japan, four species and five varieties are recognized (Suzuki 2006). Until the 1960s, the taxonomy of Japanese *Epimedium* was confusing; there was considerable disagreement between taxonomists as to species delimitation (Shimizu 1960, Kitamura & Murata 1961). One reason for this is that Japanese *Epimedium* species often form interspecific hybrids,

and some hybrid derivatives had been identified as independent taxa. *Epimedium trifoliatobinatum* (Koidz.) Koidz. is a clear example of this. In particular, among *Epimedium* occurring in Chugoku district on the Japanese island of Honshu (fig. 1), extensive morphological diversity has been present for a long time, and several independent taxa have been described.

*Epimedium sempervirens* Nakai ex F.Maek. var. *rugosum* (Nakai) K.Suzuki is distributed mainly in the region bordering the Sea of Japan in western Honshu while *E. diphylum* Lodd. is distributed widely throughout western Honshu, Shikoku, and Kyushu (fig. 1). As expected from its

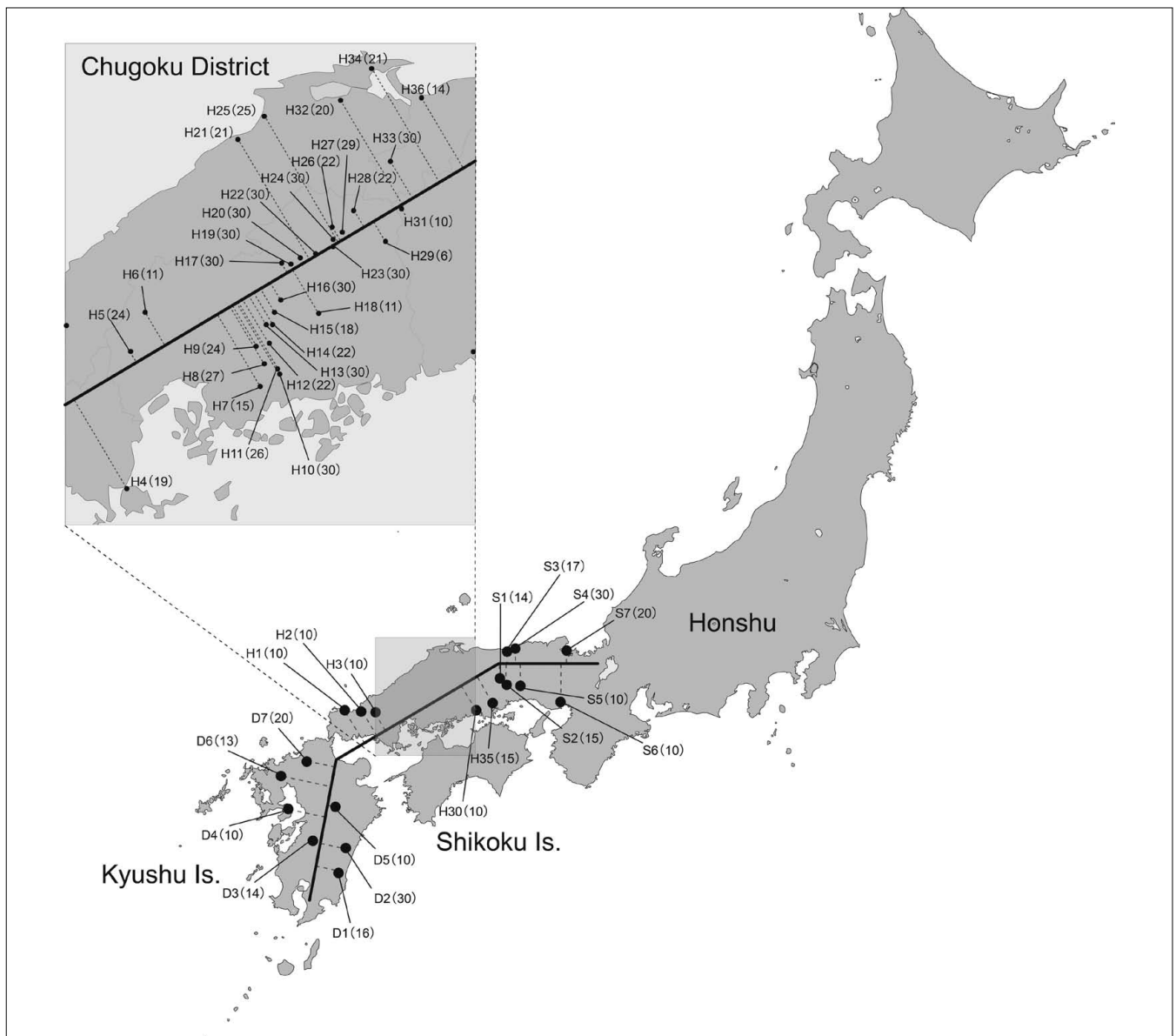
spur length, *E. sempervirens* var. *rugosum* is pollinated by nectar-foraging bees such as *Bombus diversus* and *Tetralonia nipponiensis* and pollen-foraging bees such as *Andrena* and *Lasioglossum* species, whereas *E. diphyllum* is pollinated only by pollen-foraging bees (Suzuki 1984). These two species have no post-mating reproductive barrier; cross-pollination between the species results in normal seed production compared to intraspecific pollination (Suzuki 1983).

Kurosaki (1985) examined the morphological variation in six characters (flower colour, spur length, inner sepal length, number of teeth per leaflet, leaflet apex shape, and leaf architecture) among 78 populations of *Epimedium* in Chugoku district, and suggested that *E. sempervirens* var. *rugosum* and *E. diphyllum* form a wide hybrid zone in the region.

One piece of evidence for natural hybridization between two species is morphological intermediacy in hybrid individ-

uals. Before molecular or biochemical markers could be utilized easily, morphological analysis was a major method for examining natural hybridization. To date, many morphological analyses have been conducted in putative hybrid zones of plant species (e.g., Chemiawsky & Bayer 1988, González-Rodríguez & Oyama 2005, Ito 2009). The advantage of morphological analyses is the technical simplicity of measuring characters. However, quantitative analyses are often difficult for morphological characters that differ between the parental species. For example, although leaf shape characters are frequently used as diagnostic tools to identify plant species, it is often difficult to convert them to quantitative values (see further).

In *E. diphyllum* and *E. sempervirens* var. *rugosum*, some morphological characters are difficult to treat quantitatively although they are distinctive between the two species. For



**Figure 1** – Map showing locations of the fifty populations of *E. diphyllum* (D1–D7), *E. sempervirens* var. *rugosum* (S1–S7), and putative hybrids (H1–H36) used in this study. The number in parentheses indicates the number of samples examined for each population.

example, the leaflet apex is one diagnostic character used to distinguish the two species: the apex is blunt in *E. diphyllum*, whereas it is acuminate in *E. sempervirens* var. *rugosum* (Suzuki 2006). Also, the flowers in *E. diphyllum* are white, whereas those in *E. sempervirens* var. *rugosum* are reddish purple. However, these characters are difficult to convert to continuous variables, even though putative hybrids between these species show seemingly intermediate forms for these characters. Kurosaki (1985) treated these characters as an arbitrary discontinuous class in the putative hybrid zone of the two species, even though such treatment seems to be inappropriate.

In this study, in addition to the simple measurement of morphological characters that can be treated straightforwardly as quantitative traits, the shape of the leaflet apex was examined in putative hybrids and parental species using principal component analysis (PCA) of elliptic Fourier descriptions (EFDs). Flower colour was also analyzed as spectral data using an optical spectrometer. We treated these two characters as continuous quantitative variables. Using these morphological characters, we calculated morphological, or Anderson's (1949) hybrid indices for populations in the putative hybrid zone of *E. diphyllum* and *E. sempervirens* var. *rugosum* within Chugoku district, with the goal of describing morphological diversity among the populations within the hybrid zone in detail and to elucidate to what extent natural hybridization between the two species occurred in this region. Examining how the hybridization between *E. diphyllum* and *E. sempervirens* var. *rugosum* generated the morphological variations in Chugoku district, we aim to clarify that the taxonomical confusion in *Epimedium* in this region was caused by their interspecific hybridizations.

## MATERIALS AND METHODS

### Study organisms

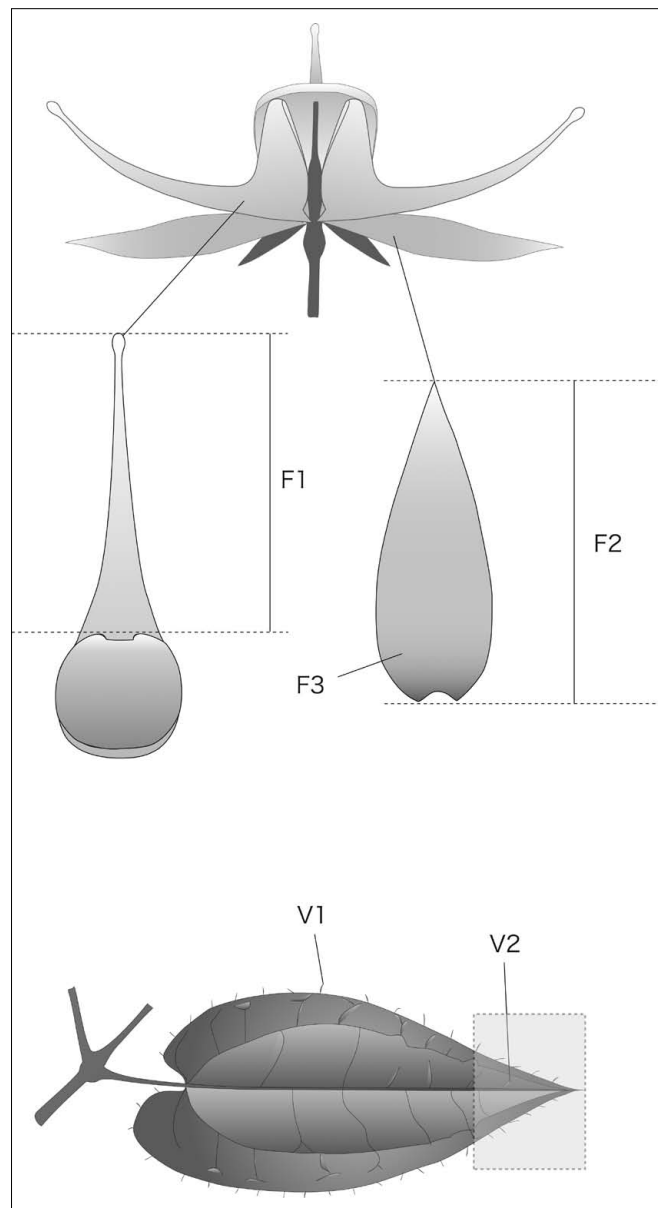
*Epimedium diphyllum* and *E. sempervirens* var. *rugosum* are perennial herbs occurring at the edge or understory of temperate forests. These two species are conspicuously different in their morphology. Although the flowers of *E. sempervirens* var. *rugosum* are reddish purple with four petals and a long spur (15–25 mm), those of *E. diphyllum* have white spurless petals. The inner sepals of *E. sempervirens* var. *rugosum* are approximately 2 cm long while those of *E. diphyllum* are approximately 1 cm long. The leaves of *E. sempervirens* var. *rugosum* are nine- or three-foliolate, while those of *E. diphyllum* are normally two-foliolate. The two species also differ in their leaflet morphology; the apexes of the leaflets are blunt and acuminate in *E. diphyllum* and *E. sempervirens* var. *rugosum*, respectively (Suzuki 2006).

### Population sampling

A total of fifty populations were sampled from Kyushu Island and Chugoku district (fig. 1). Considering their distributions and morphologies, populations D1–D7 and S1–S7 were sampled as typical ones of *E. diphyllum* and *E. sempervirens* var. *rugosum*, respectively. The remaining 36 populations (H1–H36) were sampled as putative hybrid populations. No other *Epimedium* species occurs in the area from which we

sampled, except on Kyushu Island; although *E. grandiflorum* occurs parapatrically with *E. diphyllum* on Kyushu Island, no hybridizing population between these two species was found in this study. We did not sample *E. diphyllum* populations from Shikoku Island because few morphologically typical populations were found, and most of the *E. diphyllum*-like plants in Shikoku were probably hybrids between *E. diphyllum* and other *Epimedium* taxa such as *E. trifoliatobinatum* or *E. grandiflorum* (Suzuki 1990).

Ten to thirty individuals per population were sampled at random and transplanted. The individuals were cultivated in plastic pots filled with soil and sand in a nursery at the experimental garden of the Faculty of Science, Tohoku University. Morphological measurements were made using these cultivated plants between 2001 and 2002.



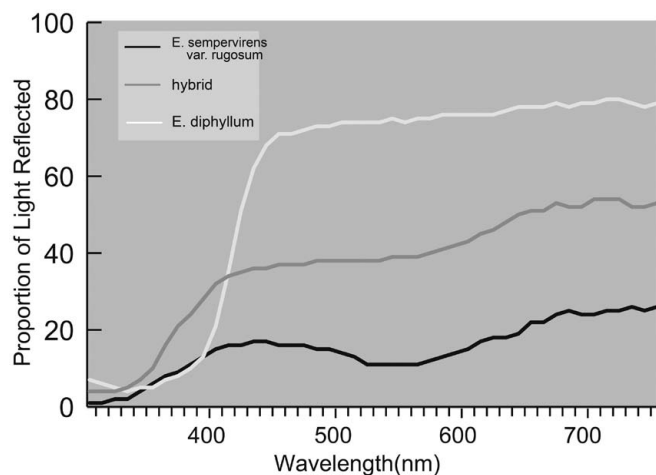
**Figure 2** – The flower and leaflet characters measured. Spur length (F1), inner sepal length (F2), flower colour (F3), number of teeth per leaflet (V1), and leaflet apex shape in one-fifth of the leaflet tips.

### Morphological character measurement and quantification

As in Kurosaki (1985), six morphological characters, spur length, inner sepal length, flower colour, number of teeth per leaflet, leaflet apex shape, and leaf architecture, were examined (fig. 2). Different from Kurosaki (1985), all six characters were examined for each individual, enabling calculations of Anderson's hybrid index for each individual. In the present study, we quantified flower colour and leaflet apex shape using a spectrophotometer and PCA of the EFDs, respectively, whereas Kurosaki (1985) analyzed these characters qualitatively.

Flower spur length and inner sepal length were measured using a digital caliper and one flower randomly selected from each individual at flowering time. These flowers were selected from the middle position in inflorescences. The number of teeth per leaflet was counted using the largest mature leaflet from each individual in summer. Flower colour was quantified as spectral data using an Ocean Optics USB 2000 spectrophotometer (Dunedin, Florida). This device measures the proportion of light that is reflected off an object at specific wavelengths between 300 and 700 nm by standardization against fully reflecting (white) and fully absorbing (black) reference standards. Figure 3 shows the changes in percent reflectance of flowers with a wavelength for typical *E. diphyllum*, *E. sempervirens* var. *rugosum*, and a putative hybrid individual with an intermediate flower colour.

To quantify leaflet apex shape, an image of the largest and most outer mature lateral leaflet on each individual was acquired using a scanner (EPSON-GT-F500, Epson, Tokyo, Japan) at a resolution of 300 dpi. The leaflet apex contour coordinates extracted by image analysis were described using 77 coefficients of normalized EFDs (Kuhl & Giardina 1982). One-fifth of each leaflet tip was subjected to image analysis (fig. 2). The digitally captured shapes were first converted into full colour bitmap images, then, using the software package SHAPE (Iwata & Ukai 2002), the image contours expressed as a chain-code were described by EFD coefficients and examined using PCA.



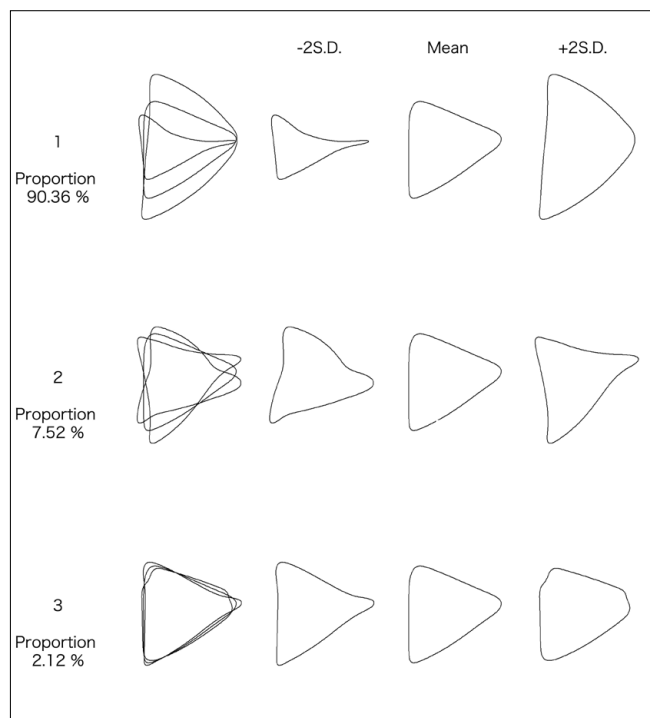
**Figure 3** – Changes in percent reflectance of flowers exhibiting a wavelength typical of *E. diphyllum*, *E. sempervirens* var. *rugosum*, and a hybrid individual with an intermediate flower colour.

The leaf architecture for each individual was determined using cauline leaves in summer. When multiple leaves with racemes were found in a single individual, one leaf was selected at random and leaf architecture was typified.

Correlations among the five variables (other than leaf architecture) were assessed for 90 individuals pooled from three putative hybrid populations (H13, H17, and H24).

### Morphological hybrid index

Anderson's (1949) morphological hybrid index was calculated for each individual. The representative characters of *E. diphyllum* were assigned a rank of 1 while those of *E. sempervirens* var. *rugosum* were assigned a rank of 5. Intermediate characters were assigned a rank of 2, 3, or 4. Rank 2 indicates an *E. diphyllum*-like character while rank 4 indicates an *E. sempervirens* var. *rugosum*-like character. In this manner, spur lengths of 0, 0–5.5, 5.5–11.0, 11.0–16.5, and > 16.5 mm were classified as ranks 1–5, respectively, while inner sepal lengths of < 9, 9–10, 10–11, 11–12, and > 12 mm were classified as ranks 1–5, respectively. Per leaflet, 0–10, 10–20, 20–40, 40–50, and > 50 teeth were classified as ranks 1–5, respectively. For flower colour, a percent reflectance at 600 nm of 5–20, 20–35, 35–50, 50–65, and > 65 was classified as rank 1–5, respectively. In our analysis of leaflet apex shape, fig. 4 shows the reconstructed leaflet apex contours and the influence of the principal components on the contours. Because the value of the first principal component



**Figure 4** – Reconstructed leaflet apex contours and the contributions (%) of the three most influential principal components to shape variation in the leaflet apex images. Numbers 1–3 correspond to the first three principal components, respectively. Each column shows a case where the score equaled -2 standard deviation (s.d.) units, the mean, or +2 s.d. units; the left column shows overlaid drawings for all three cases.

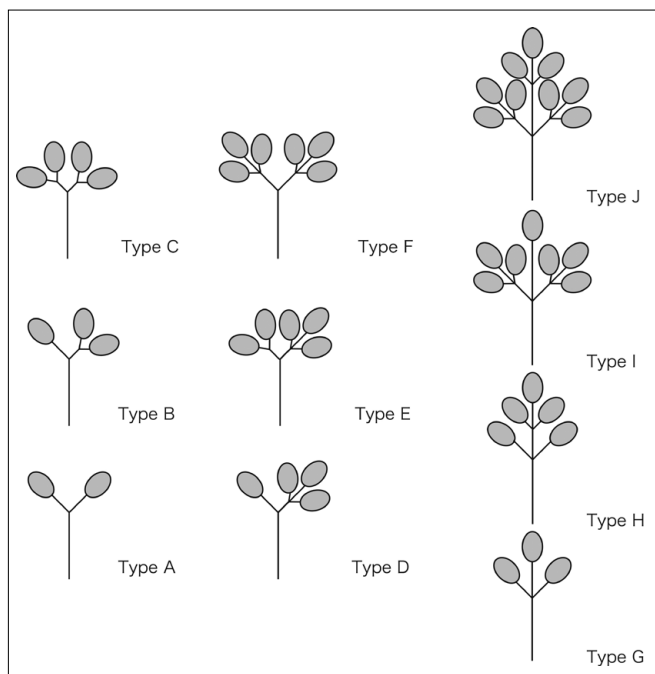


Figure 5 – Patterns of leaf architecture found in this study.

(PC1) compared to the total variance was high (90.36%), only PC1 was used as a parameter for leaflet apex shape, and values of 1.000–0.001, 0.001–0.000, 0.000 to -0.001, -0.001 to -0.002, and -0.002 to -1.000 were classified as ranks 1–5, respectively. For leaf architecture (fig. 5), because types A, B, and C were found in typical populations of *E. diphyllum* while types G, H, I, and J were found in typical populations of *E. sempervirens* var. *rugosum*, the former types were classified as rank 1 and the latter types as rank 5. Types D, E, and F were found only in populations in the putative hybrid zone, and all were classified as rank 3.

Anderson’s morphological hybrid index was calculated for each individual by averaging the ranks of the six characters; that is, a hybrid index score of 1 means that the individual was morphologically typical of *E. diphyllum* while a hybrid index score of 5 means that the individual was morphologically typical of *E. sempervirens* var. *rugosum*. These rank scores were categorized into five classes: 1 ( $1 \leq H < 1.5$ ), 2 ( $1.5 \leq H < 2$ ), 3 ( $2.5 \leq H < 3.5$ ), 4 ( $3.5 \leq H < 4.5$ ), and 5 ( $4.5 \leq H \leq 5$ ).

### RESULTS

Fig. 6A shows the variation in spur length among the populations sampled. In typical *E. diphyllum* populations, the flowers had no spurs, whereas spur length was > 15 mm in typical *E. sempervirens* var. *rugosum*. In populations in the putative hybrid zone, the variations in spur length were quite large; the mean length within a population ranged from 0 mm (populations H1, H7, H8, H10 – H12, H14, H15, H18, H19, H23, and H29–H31) to 22 mm (populations H5 and H33). Intrapopulation variation, if any, in spur length also varied among the populations in the putative hybrid zone, and was small in some populations (e.g. H9, H25, and H27) and large in others (e.g. H13 and H17). Putative hybrid populations such as

Table 1 – Correlations among the examined morphological characters.

Values are Kendall rank correlation coefficients. None of the values were significant.

	F1	F2	F3	V1	V2
Spur length (F1)	1				
Inner sepal length (F2)	0.398	1			
Flower colour (F3)	0.391	0.175	1		
Number of teeth per leaflet (V1)	0.308	0.109	0.350	1	
Leaflet apex shape (V2)	-0.254	-0.168	-0.262	-0.232	1

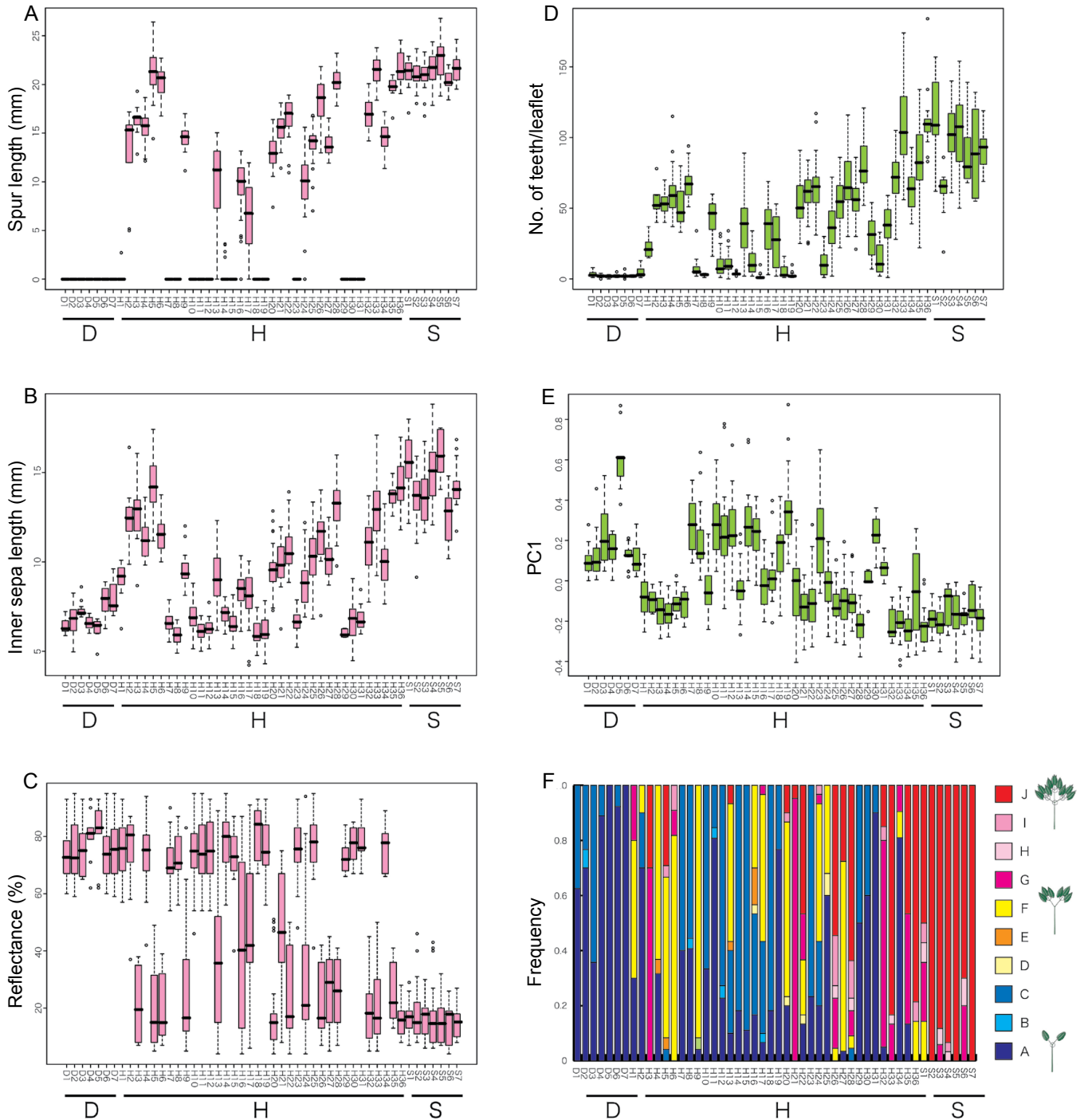
H33, H35, and H36, which were located close together in a distribution typical of *E. sempervirens* var. *rugosum*, tended to show spur lengths similar to those of typical *E. sempervirens* var. *rugosum*, and a reverse pattern was found in the populations adjacent to the distribution of *E. diphyllum* (populations H1 and H2). However, the populations located in the western part of the putative hybrid zone (H5 and H6) also had long spurs (mean: 21–22 mm), comparable to those of typical *E. sempervirens* var. *rugosum*, and the reverse pattern was found in the eastern part of the region (H29–H31).

Compared to spur length, inner sepal length varied widely in both the typical *E. diphyllum* (mean: 6.25–7.92 mm) and *E. sempervirens* var. *rugosum* (mean: 12.64–16.00 mm) populations, although the difference between the two species was distinct (fig. 6B). In the putative hybrid zone, a pattern similar to that for spur length variation within a population was found in inner sepal length; some populations showed little variation while others showed relatively large variation. The geographical pattern of variation in inner sepal length within the putative hybrid zone was similar to that for spur length.

The number of teeth per leaflet was approximately zero in typical *E. diphyllum* populations and varied greatly in typical *E. sempervirens* var. *rugosum* populations (mean: 66–113). As a whole, the patterns of intra- and interpopulation variation in the putative hybrid zone were similar to those for spur length and inner sepal length (fig. 6D).

Fig. 6C shows the variation in percent reflectance of the flowers. The percent reflectance among flowers was nearly 70–90 in typical *E. diphyllum*, whereas it was nearly 10–20 in typical *E. sempervirens* var. *rugosum*. The patterns of inter- and intrapopulation variation were similar to those for the three characters examined above.

Fig. 6E shows the variation in PC1 of the EFDs for leaflet apex shape. Although the differences in PC1 were distinct between *E. diphyllum* and *E. sempervirens* var. *rugosum*, they varied widely among the populations sampled. As found for the other characters measured, the variation in PC1 was small for some populations in the hybrid zone but relatively large for others. Also, for leaflet apex shape (PC1), a gradu-



**Figure 6** – Interpopulation variation in six morphological characters. A, spur length; B, flower inner sepal length; C, flower colour; D, number of teeth per leaflet; E, PC1 of the EFDs for leaflet apex shape; F, leaf architecture.

al change in pattern from west to east was not found; some populations in the eastern portion of the putative hybrid zone showed a low value and vice versa in the western portion.

There was no significant correlation ( $P < 0.05$ ) between the five characters measured above for the three populations examined (H13, H17, and H24) (table 1).

Fig. 6F shows the leaf architecture type for each population sampled. In typical *E. diphyllum* populations, types A, B,

and C were found, whereas types F, G, H, I, and J were found in typical populations of *E. sempervirens* var. *rugosum*. The populations in the putative hybrid zone usually contained several leaf architectures. Also, a gradual change in leaf architecture was not detected in the hybrid zone; some populations in the western part of the hybrid zone (populations H3 and H21) showed types G and J, which are representative of *E. sempervirens* var. *rugosum*, while some populations in the

eastern part of the putative hybrid zone (populations H29, H30, and H31) showed types A and C, which are representative of *E. diphyllum*.

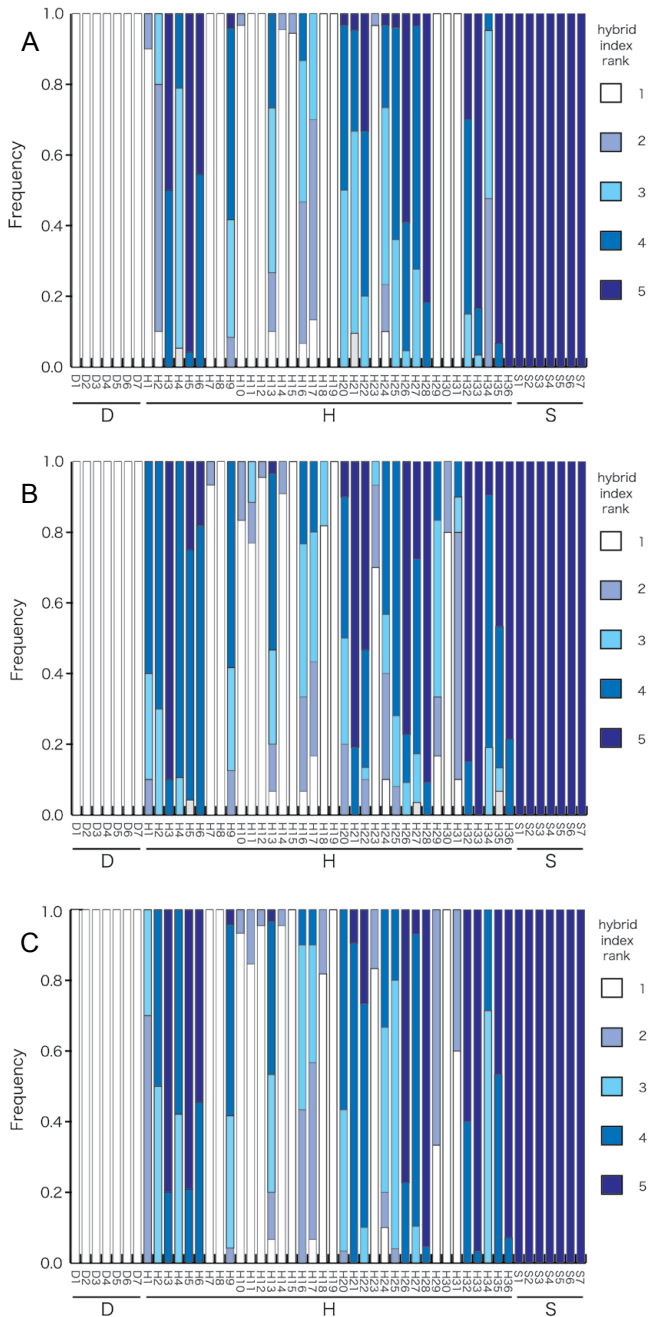
Fig. 7 shows the population breakdown for Anderson’s morphological hybrid index ranks for the flower characters (spur length, inner sepal length, and percent reflectance of flowers), leaf characters (number of teeth per leaflet, leaflet apex shape, and leaf architecture), and a combination of the two. Only rank 1 was found in the typical populations of *E. diphyllum* while only rank 5 was found in those of *E. sem-*

*pervirens* var. *rugosum*. In the populations near the center of the hybrid zone, rank 3, which indicates intermediate morphology, was frequently found, together with the other ranks. Some populations (populations H13, H17, and H24) contained four ranks for the flower and leaf characters, and most of the populations in the putative hybrid zones contained more than two ranks. The geographic pattern of Anderson’s morphological hybrid index scores was somewhat complex; some populations in the western part of the hybrid zone had a relatively large mean score, while the reverse trend was found in the eastern part of the hybrid zone.

### DISCUSSION

There was considerable morphological variation among the populations analyzed in this study. As a whole, a general trend along the geographical gradient was found for all characters examined in the putative hybrid zone: the populations were highly morphologically diverse around the center of the putative hybrid zone, while the populations in the western and southern parts of the putative hybrid zone were morphologically *E. diphyllum*-like and *E. sempervirens* var. *rugosum*-like, respectively. However, this trend was not always straightforward; some populations in the western part of the putative hybrid zone contained morphologically *E. sempervirens* var. *rugosum*-like individuals together with intermediate ones, while the reverse pattern was found in the eastern part of the putative hybrid zone. This is probably because the past distribution of the two parental species was mosaic-like around the contact zone. It is probable that the distribution of *E. sempervirens* var. *rugosum* once extended from the Sea of Japan in Chugoku district and contacted and formed hybrid zones with *E. diphyllum*, which was once extant on the Inland Sea side of Chugoku district. Similarly, the distribution of *E. diphyllum* expanded from the Inland Sea side of eastern Chugoku district to form hybrid populations with *E. sempervirens* var. *rugosum*, although typical populations of *E. diphyllum* are not extant in Chugoku district at present due to introgression of the genes of *E. sempervirens* var. *rugosum*.

In the putative hybrid zone, there were populations having parental species-like flowers. For example, populations H7, H8, H10–H12, and H29–H31 had spurless white flowers like those of *E. diphyllum*. However, a few of them (e.g. H29 and H31) contained individuals having leaflets with a substantial number of teeth, which is indicative of *E. sempervirens* var. *rugosum*. The opposite pattern was found in some populations; that is, individuals had relatively large spurs but leaflets with a relatively small number of teeth (e.g. populations H2, H3, and H4). In these populations, individuals had a combination of flower and leaf characters resembling both parental species. On the other hand, some populations (e.g. populations H13, H16, H22, and H24–H27) around the center of the putative hybrid zone contained individuals with morphologically intermediate flowers and intermediate leaves. These results are in accordance with the fact that there was no correlation among the characters examined. Morphological character coherence (i.e. strong correlation among parental morphological characters) has been rarely observed in plant populations (Rieseberg & Ellstrand 1993). Only very strong selection can maintain linkage disequilibrium at un-



**Figure 7** – Population breakdown of Anderson’s hybrid index ranks. A, flower characters; B, leaf characters; C, combination of flower and leaf characters.

linked loci. In addition, a single episode of recombination was sufficient to randomize several characters in experimental populations of *Gossypium barbadense*, *G. hirsutum*, and their hybrids (Goodman 1966). Considering this, the absence of morphological character coherence in the hybrid zone is probably the rule rather than the exception.

Extreme or transgressive characters in hybrid individuals have been reported in studies of hybrid plant populations (Rieseberg et al. 1999). For example, morphological (41.5%) and ecophysiological (25%) characters were transgressive in *Helianthus anomalus* (Schwarzbach et al. 2001). However, in the populations examined in this study, we could not find such a character state. This may be because we used only six characters that were distinct between the parental species to calculate Anderson's hybrid index; if more morphological characters were examined, such characters may be found. Rieseberg & Ellstrand (1993) suggested that transgressive characters were the rule rather than the exception, especially for later generation hybrids, which are likely contained in the putative hybrid populations examined in this study.

Lastly, some populations in the putative hybrid zone showed small variations in flower and leaf characters while others showed large variations. In particular, around the center of the putative hybrid zone, the variations in character were often larger than in the peripheral areas of the putative hybrid zone. This suggests that introgression occurred intensively only around the center of the putative hybrid zone, though parental species gene flow also extended to the opposite side of the species distribution. Another explanation is that strong selection on the characters caused low morphological variation in the peripheral area of the putative hybrid zone while extensive gene flow occurred across the range. Around the center of the putative hybrid zone, the fitness of morphologically intermediate individuals may be higher than or equivalent to that of the parental species. The bound superiority model (Moore & Price 1993) may be applicable to the latter case. To clarify the factors responsible for creating the hybrid zone, it will be necessary to compare fitness between the hybrids and parental species throughout the parental species' distribution areas and the hybrid zone.

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