NOTES AND COMMENTS

Genetic structure of a hybrid zone between two violets, *Viola rossii* Hemsl. and *V. bissetii* Maxim.: dominance of F_1 individuals in a narrow contact range

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Abstract

The genetic composition of a hybrid zone can provide insight into the evolution of diversification in plants. We carried out morphological and amplified fragment length polymorphism analyses to investigate the genetic composition of a hybrid zone between two violets, *Viola bissetii* Hemsl. and *Viola rossii* Maxim. Our aim was to clarify the formation and maintenance of hybrids between these *Viola* species. We found that most hybrid individuals (*V. bissetii* × *V. rossii*) were of the F_1 generation, with a few of the F_2 generation. We found no backcrosses. The scarcity of post-F_1 hybrids indicates that a species barrier is established between the parental species. The F_1-dominated hybrid zone occupied only a narrow, intermediate ecotone between the parental habitats, suggesting that selection by environmental factors against hybrids may help to maintain the current conditions in this hybrid zone.

Keywords: amplified fragment length polymorphism, F_1-dominated hybrid zone, interspecific hybridization, leaf index, Violaceae.

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Introduction

Hybridization is a common and important phenomenon in angiosperms (Ellstrand *et al*. 1996; Mallet 2005), and a hybrid zone can occur in regions where the distributions of closely related species or genetically differentiated populations come into contact. Hybrid zones often form in disturbed areas, partly because prezygotic ecological isolation between the parental species often breaks down in such habitats (Rieseberg & Carney 1998). Alternatively a hybrid zone can form in an environmental ecotone between parental habitats (Campbell & Waser 2007).

The genetic composition of individuals in such a hybrid zone reflects the intensity of reproductive isolation and gene flow (Broyles 2002; Ma *et al*. 2010). In general, the occurrence of many types of hybrids within a hybrid zone, including abundant post-F_1 hybrids (F_2 individuals, backcrosses, etc.), is indicative of a high rate of gene flow (Arnold *et al*. 1992; Nason *et al*. 1992). Among Louisiana

irises, for example, the hybrid zone is dominated by post-F_1 hybrids and no F_1 individuals have been found in the wild (Hodges *et al*. 1996), indicating that a hybrid zone can occur even when the crossing rate between parental species is very low.

Hybrid zones dominated by F_1 individuals have also been recorded. In such hybrid zones, F_1 individuals may dominate temporarily, either because of the breakdown of post-F_1 generations or as a result of cross-incompatibility between the parental species and F_1 individuals. In these cases, the species barrier between parental species is maintained. If negative selection against F_1 hybrids exists, the hybrids (F_1 individuals) will eventually disappear unless they propagate vegetatively. Or they may continue to be produced and occupy a specific habitat range owing to their superior adaptation to that habitat compared with the other hybrid classes (parental species, F_2 individuals, backcrosses, etc.). Milne *et al*. (2003) have called a habitat range occupied mainly by fertile F_1 individuals an F_1-dominated hybrid zone (F_1DZ), but few such zones have been documented (Kyhos *et al*. 1981; Milne *et al*.

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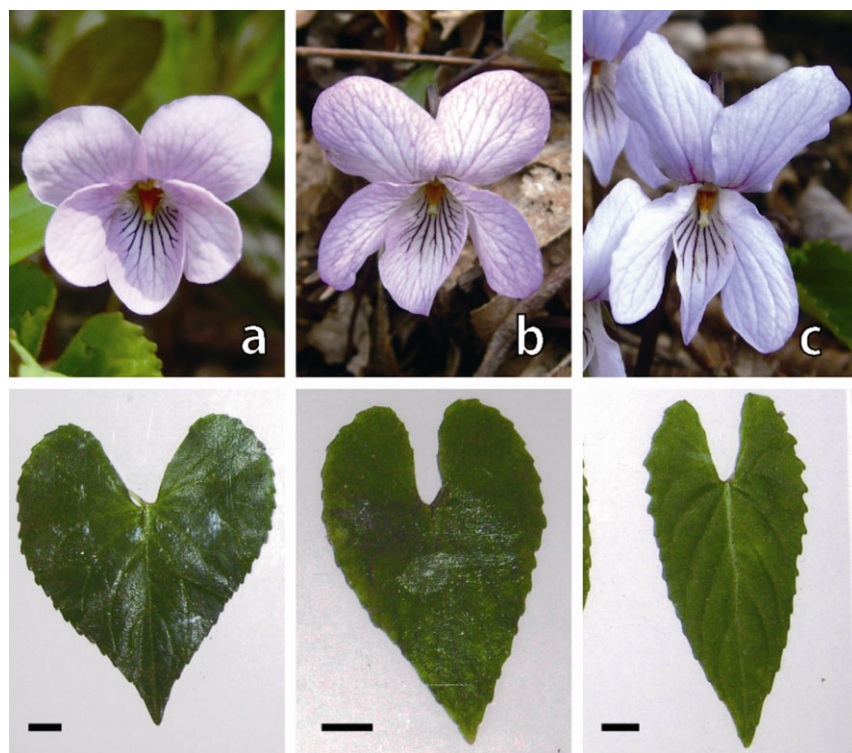Email: ny_thitb2_nmk2@yahoo.co.jp

Fig. 1 Flower and fully opened leaf morphologies of the parental *Viola* species (*V. rossii* and *V. bissetii*) and their hybrid (F_1) on Mount Ougi: (a) *V. rossii*; (b) hybrid (F_1); (c) *V. bissetii*. The morphological characters and colors of the flowers and leaves of the hybrids are intermediate between those of the parental species. The scale in each leaf photograph is 1 cm.

2003; Zha *et al*. 2010). In an F_1DZ, fertile F_1 individuals are produced in sufficient numbers to occupy most or all of the available habitats within the hybrid zone to the exclusion of the parental species and post-F_1 individuals. Because these F_1 populations occur only in certain types of habitat, reproductive barriers in such hybrid zones are highly habitat-dependent (exogenous, habitat-related factors select against post-F_1 individuals; Milne *et al*. 2003). However, situations have also been reported in which F_1 individuals do not show any preference for a specific environment but in which they are selected for by endogenous factors (Kameyama & Kudo 2011). In contrast, hybrid zones occupied exclusively by sterile F_1 individuals, suggestive of repeated outcrossing between the parental species, have not been documented to our knowledge. Note, however, that even sterile F_1 individuals might be able to increase their numbers through vegetative propagation. In fact, Kameyama *et al*. (2008) reported that long-term clonal perpetuation of F_1 individuals contributes to the maintenance of hybrid zones of species of the plant genus *Phyllodoce*. Because hybrid zones are formed and maintained in so many different ways and because they have important evolutionary consequences, studies of the genetic structures of hybrid zones can provide insight into ecological and evolutionary interactions between parental species and their hybrids.

The frequency of hybridization can differ among families and genera (Ellstrand *et al*. 1996; Jiggins & Mallet

2000), and hybridization may play various roles in the evolutionary diversification of taxa. By studying current conditions in hybrid zones of diversified and hybridized taxa, it is possible to gain insight into the evolutionary significance of hybridization. In Japan, the genus *Viola* (Violaceae) is highly diversified, and species are distributed in various environments, from the seashore to alpine habitats. Among the 55 *Viola* species that occur in Japan, 14 are endemic to Japan (Akiyama 2011). Although many sympatric pairs of *Viola* species form hybrids in natural habitats in Japan, hybrid individuals are infrequently observed in the wild (Hama 2002), and their genetic composition (e.g., F_1 and F_2 hybrids and backcrosses) and the degree to which they hybridize in the wild are not well known.

The aim of this study was to clarify the genetic composition of a hybrid zone between two *Viola* species and to gain insight thereby into the evolutionary significance of hybridization. We focused on a hybrid zone between *Viola rossii* Hemsl. and *Viola bissetii* Maxim. Their putative hybrid *Viola bissetii* × *V. rossii* (Fig. 1) has been recorded within a narrow contact range at several inland sites where the two parental species co-occur (Hama 2002). Although it is possible that the number of these hybrids increases through vegetative propagation, the genetic compositions of such hybrid zones are unknown. Because *V. bissetii* × *V. rossii* is morphologically intermediate between the parent species and it does not display the

Fig. 2 Distributions of the parental species and their hybrids along the main ridge of on Mount Ougi and on the northern and southern slopes of the ridge (the lower southern slope is not shown). The contours show elevations (contour interval, 50 m). The summit (triangle) is at 1138 m.
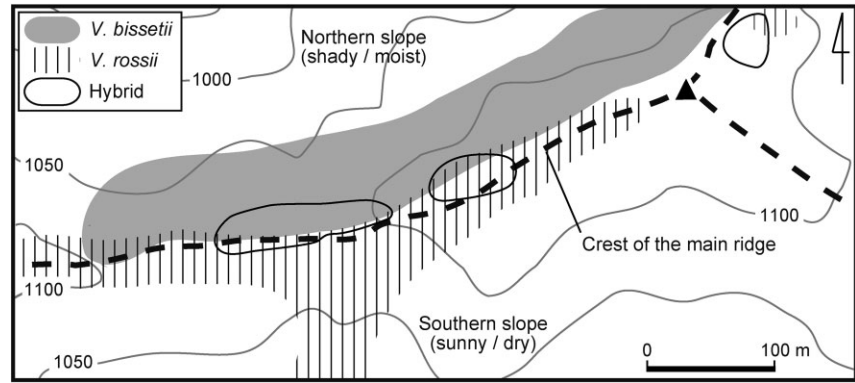
morphological variability normally associated with segregating post-F_1 hybrids (Hama 2002; Y. Nagano, pers. obs.), hybrid zones between this *Viola* species pair may be dominated by F_1 individuals.

In this study, we examined the genetic composition of a *V. bissetii* × *V. rossii* hybrid zone to determine the mechanisms of the hybrid zone formation and maintenance in a narrow contact range. First, we confirmed that *V. bissetii* × *V. rossii* (individuals having leaf and flower morphologies intermediate between those of the parent species) is a hybrid of *V. rossii* and *V. bissetii* and used morphological and amplified fragment length polymorphism (AFLP) analyses to estimate the genetic composition of the hybrid zone. Next, we investigated how the hybrid zone formed and how it is maintained by examining the small-scale distribution of the parental species and hybrids. On the basis of our results, we then discuss the evolutionary significance of hybridization between these *Viola* species.

Materials and methods

Species

Viola rossii Hemsl. and *V. bissetii* Maxim. (both species belong to section *Vaginatae* of Violaceae; Fig. 1) are distributed parapatrically in inland Japan and on the Pacific Ocean side, respectively. *Viola rossii* is also found in northeastern China and the Korean Peninsula, whereas *V. bissetii* is endemic to Japan. Both species have the same number of chromosomes ($2n = 12$, Hama 2002). Their putative hybrid *V. bissetii* × *V. rossii* (Fig. 1), which was first recorded as a form of *V. rossii* (*Viola rossii* Hemsl. f. *longifolia* T. Hashim. et Seriz.: Hashimoto & Serizawa 1971), has since been recorded at several inland sites where the two parental species co-occur (Hama 2002). *Viola* plants are known to be pollinated by Diptera (e.g., *Bombylius major*) or Hymenoptera (e.g., *Tetralonia nipponensis*) species in Japan (Hama 2002), and they frequently reproduce by producing cleistogamous flowers.

Viola bissetii and *V. bissetii* × *V. rossii* can also spread by vegetative propagation (Hama 2002).

Study area, sampling, and morphology

We conducted our study on Mount Ougi (35°64′N, 139°01′E; elevation, 1138 m a.s.l.) in central Japan. The parental species are distributed parapatrically on Mount Ougi, with only a narrow contact zone along the crest of the main ridge. *Viola rossii* is distributed on the sunny, dry southern slope of the ridge (Fig. 2), where desiccation-resistant trees such as *Pinus densiflora* grow. In contrast, *V. bissetii* is distributed on the shady, moist northern slope, where hygrophilous trees such as *Fagus crenata* grow. The parental species co-occur only within a narrow range on the northern side of the ridge crest, and the hybrids are also distributed there (hybrid zone width, about 20 m: Fig. 2). Although a patch of *V. bissetii* was found along a stream on the lower southern slope (a relatively moist environment, 700 and 780 m a.s.l., not shown in Fig. 2) within 10 m of a *V. rossii* patch, no hybrids were found there.

In April and July 2009, we collected one fully opened leaf sample from each of 57 plants selected haphazardly at least 1 m apart along the whole mountain ridge (all within about 500 m), where both parental species co-occur and over 500 hybrids grow (Fig. 2). We also collected leaf samples from an additional 17 plants, selected haphazardly, on the lower southern slope (< 800 m a.s.l, not shown in Fig. 2), where both parental species, but no hybrids, grow. The leaf samples were used for measurement of leaf traits and AFLP analysis.

Because the leaf shape of the hybrids is intermediate between the leaf shapes of *V. rossii* and *V. bissetii* (Igari 2004; Hama 2002: Fig. 1), we calculated the Leaf Index (defined as the leaf length/width ratio) of the 74 samples collected to examine whether leaf shape might be a useful criterion for discriminating the parental species and hybrids.

The AFLP analysis

Fresh leaf samples were dried and preserved in silica gel, and total genomic DNA was extracted from each with a DNeasy Plant Mini Kit (QIAGEN Inc.).

The AFLP analysis was performed according to the method of Vos *et al.* (1995) with some modifications. Genomic DNA was digested with the restriction enzymes *Eco*RI and *Mse*I at 37°C for 1.5 h. Double-stranded adaptors were ligated to the ends of the digested DNA fragments at 20°C, overnight. Pre-selective polymerase chain reaction (PCR) amplifications were then performed for 20 cycles, using a primer pair with one additional nucleotide on each restriction enzyme (*Mse*I-C/*Eco*RI-A), with the following cycle profile: a 30 s DNA denaturing step at 94°C, a 1 min annealing step at 56°C, and a 1 min extension step at 72°C. Selective amplifications were then conducted with three fluorescence-labeled primer combinations: *Eco*RI-ACA/*Mse*I-CCA; *Eco*RI-ACG/*Mse*I-CAT; *Eco*RI-ATC/*Mse*I-CCG. Selective amplifications were performed for 30 cycles with the following cycle profile: a 30 s DNA denaturing step at 94°C, a 20 s annealing step, and a 2 min extension step at 72°C. The annealing temperature of the first cycle was 66°C, then for the next 10 cycles it was reduced by 1°C each cycle, and finally it was maintained at 56°C for the remaining 19 cycles. A Dice TP600 PCR Thermal Cycler (Takara Bio, Shiga, Japan) was used with the AFLP Amplification Core Mix (Applied Biosystems, Foster City, CA, USA) for both the pre-selective and selective amplifications. The AFLP fragments were detected with an ABI Prism 3130 automated sequencer (Applied Biosystems) and Gene Mapper software version 4.0 (Applied Biosystems).

First, from AFLP results, we used the 90% criterion to select polymorphic bands, selecting only those bands possessed by 10–90% of individuals. Then, we conducted a principal coordinate analysis (PCO) with R version 2.15.1 software (R Development Core Team 2012) to investigate the genetic relationship among the samples.

We used a Bayesian statistical method, developed by Anderson and Thompson (2002), to identify the hybrids that do not require pure samples of the parental species.

Using NewHybrids version 1.1 software (Anderson & Thompson 2002), we identified the hybrid classes of the samples by setting the number of samples to 50 000 after a burn-in of 50 000, and then computing the posterior probability that a sample belonged to each of six hybrid classes: the two parental species, the F_1 and F_2 generations, and backcrosses to each parental species.

We also used a maximum likelihood statistical method in the HINDEX software package (Buerkle 2005) and AFLPdat R-script software (Ehrich 2006) to estimate the molecular hybrid index (HI) and the clonality of the hybrid individuals, respectively. The AFLP genotype data of individuals identified as a parental species with high posterior probability (> 99%) by the NewHybrids software were used as reference data for pure parental species in the HINDEX analysis.

Results

Morphology

In the study area, section *Vaginatae* individuals could easily be classified into one of three types, namely, *V. rossii*-like, *V. bissetii*-like, or *V. bissetii × V. rossii*-like, on the basis of three morphological characters: flower color, the presence of petal trichomes, and leaf shape. Most morphological characters of *V. bissetii × rossii*-like individuals were intermediate between those of the parental species (Fig. 1, Table 1). Within each of the *V. rossii*-like, *V. bissetii*-like, and *V. bissetii × V. rossii*-like groups, however, we observed little morphological or ecological variation.

The AFLP analysis

A total of 155 polymorphic bands were identified by using the three primer combinations. Among the bands, 23 were specific to *V. rossii* and 34 were specific to *V. bissetii*. A PCO plot revealed that three clusters of individuals, and each cluster corresponded to one of the morphologically identified parent species or to the hybrids (Fig. 3).

The Bayesian statistical results (Table 2) indicated that most of the hybrid samples (17/21 samples) were F_1

Table 1 Morphological and ecological characters of the two parental species and their hybrids on Mount Ougi

Character	*Viola rossii*	*Viola bissetii*	Hybrids
Flower color†	Light pink - magenta	Light violet or white	Light pink
Trichomes on petals†	Present	Absent	Present or absent
Vegetative propagation†	Unusual	Usual	Usual
Flowering season‡	Early May	Early to late April	Late April to early May
Habitat†	Sunny, dry	Shady, moist	Intermediate

†These characters match those recorded by Igari (2004) and Hama (2002). ‡The parental species flowered at the same time (late April to early May in 2009) in the hybrid zone.
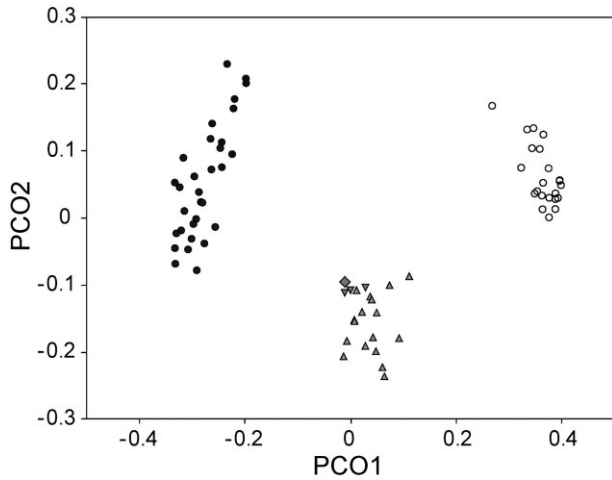
Fig. 3 Results of the principle coordinate analysis, based on 155 AFLP markers, of individuals from the hybrid zone on on Mount Ougi. The proportion of total variance explained by PCO1 and PCO2 was 30.2% and 5.3%, respectively. Hybrid classes were identified by using NewHybrids software. ○, *V. rossii*; ●, *V. bissetii*; ▲, F₁; ◆, F₂; ▽, uncertain.

Table 2 Genetic composition of *Viola* taxa on Mount Ougi

Hybrid class	Location	N	Hybrid index
Viola rossii	S	7	—
	H	15	—
Viola bissetii	S	10	—
	H	21	—
F₁	H	17	0.36–0.59
F₂	H	1	0.38
Uncertain (F₁ or F₂)	H	3	0.38–0.45
BCr	—	0	—
BCb	—	0	—
Total		74	

The habitat locations are the southern slope (S, no hybrids) and the mountain ridge (H, with hybrids). Uncertain (F₁ or F₂) refers to those individuals with low posterior probabilities both to F₁ and F₂ (<70%). Hybrid indices were estimated by using HINDEX software. BCr: backcross to *V. rossii*, BCb: backcross to *V. bissetii*.

hybrids between *V. rossii* and *V. bissetii*, and only one hybrid sample was classified as F₂ with high posterior probability (>90%). Another three hybrid samples were classified as F₁ or F₂ with low posterior probabilities (<70%: uncertain samples, Table 2). No individuals were inferred to be backcrosses to either parental species. These classifications are consistent with the morphological taxonomy and the PCO clusters (Fig. 3). The 21 hybrid samples had hybrid index values ranging from 0.36 to 0.59 (Table 2), suggesting again that they are not backcrosses.

Rigorous clonal distinction was difficult because the AFLP error rate was not monitored. However, even when

we assumed a high error rate (10%) the AFLPdat results suggested that the analyzed hybrid individuals were not clones, whereas three samples from the *V. bissetii* parent were identified as clones.

Leaf shape

The Leaf Index results were consistent with the hybrid class assignments based on the AFLP analyses (Table 3). The Leaf Index distributions of the two parental species did not overlap, and the hybrid index values were intermediate between those of the parental species.

Discussion

Although hybrid zones often form in disturbed areas, in the present study area the hybrid zone was not a disturbed area but a natural mountain ridge. In the study area, *V. rossii* is distributed on the southern, sunny, and dry slope of the ridge, whereas *V. bissetii* is distributed on the northern, shady, and moist slope (Fig. 2), in keeping with well-known differences in their habitat preference (Hama 2002). The hybrids are distributed along the ridge crest, within a narrow range between the parental habitats (Fig. 2). Thus, the hybrid zone seems to be in the environmental ecotone between the parents' preferred habitats. As we did not evaluate the environmental conditions (e.g., soil or sunlight conditions), additional investigations are necessary to further clarify the role of environmental gradient in the maintenance of the hybrid zone.

The NewHybrids analysis showed that most of the hybrids were F₁ generation hybrids (Table 2). The absence of backcrosses in this study suggests that at present little or no introgression occurs with the parental species, and indicates that a species barrier is established between the parental species.

The dominance of F₁ individuals only in the hybrid zone can be explained by three, not mutually exclusive factors: (i) frequent interspecific hybridization, (ii) narrow-range dispersal of pollen and seeds, and (iii) ecological superiority of F₁ individuals. For (i), abundant F₁s indicated frequent reproduction in two possible modes: seedling recruitment and clonal propagation. However, because we observed no clones among hybrid samples, the latter is rejected. Therefore, frequent recruitment of F₁-hybrid seedlings generated by interspecific hybridization can explain the abundant F₁s. With regard to (ii), the pollen dispersal distance by pollinators and the seed dispersal distance by ants are both limited in *Viola* (Beattie & Lyons 1975; Beattie 1976; Culver & Beattie 1978; Ohkawara & Higashi 1994). For example, pollen of *Lonicera japonica* is dispersed by the solitary bee, *Tetralonia nipponensis*, which is also one of the main pollinators of *Viola* species, over a relatively short distance (<5 m), although the bees some-

Table 3 Hybrid class, morphological taxonomy, and Leaf Index (mean, median, minimum, maximum, and range) of samples from the parental *Viola* species and their hybrids on Mount Ougi

Hybrid class	Morphological taxonomy	Mean ± SE	Median	Range	Minimum	Maximum	N
V. rossii	*V. rossii*	1.121 ± 0.019	1.151	0.339	0.941	1.280	22
F_1	Hybrid	1.507 ± 0.025	1.502	0.350	1.331	1.681	17
F_2	Hybrid	1.753 ± 0.000	—	—	—	—	1
Uncertain	Hybrid	1.601 ± 0.034	1.628	0.109	1.532	1.641	3
V. bissetii	*V. bissetii*	2.067 ± 0.046	2.099	1.130	1.303	2.433	31

times fly longer distances (>10 m) (Miyake & Yahara 1998). Thus, limitations in dispersal may also contribute to the dominance of F_1 individuals within only a narrow range. As for (iii), F_1 individuals will persist if they outcompete other genotypes (i.e., both parental species and post-F_1 individuals) in a certain habitat. In the study area, the hybrids were distributed only in the narrow environmental ecotone between the parents' preferred habitats. This distribution clearly points to possible ecological superiority of F_1 individuals in this narrow contact range. However, to further clarify whether the F_1 generation has ecological superiority in the hybrid zone, it will be necessary to carry out reciprocal transplant experiments using crossed seeds.

Our results indicate that the hybrid zone between *V. rossii* and *V. bissetii* in the study area is an F_1DZ, because F_1 individuals dominate a part of the hybrid zone. The lack of post-F_1 hybrids in this F_1DZ may be due to exogenous and/or endogenous selection. Milne *et al.* (2003) indicated that the same parental species might form multigeneration hybrid zones when they meet where other habitat conditions occur, suggesting that exogenous selection plays an important role in the formation of an F_1DZ. Alternatively, Kameyama *et al.* (2008) and Kameyama and Kudo (2011) suggested that negative endogenous selection may prevent the establishment of post-F_1 hybrids in F_1DZs of *Phyllodoce*. The relative importance of endogenous and exogenous selection for the maintenance of a hybrid zone remains unclear in *Viola*.

The existence of a F_1DZ has important implications with regard to speciation (formation and maintenance of isolating barriers), and the evolution of plant mating systems (Milne *et al.* 2003; Kameyama *et al.* 2008; Zha *et al.* 2010; Kameyama & Kudo 2011). In *Rhododendron* (Milne *et al.* 2003; Zha *et al.* 2010), the genetic composition of hybrid zones differs among sites, indicating that habitat-mediated selection occurs as a result of environmental differences, and an F_1DZ can prevent interspecific gene flow. In *Phyllodoce* (Kameyama *et al.* 2008; Kameyama & Kudo 2011), clonally propagated F_1 individuals dominate patches in alpine plant communities, and the existence of such F_1 dominated patches may affect reproduction in nearby patches of the parental species (Kameyama & Kudo 2011). These studies found that environmental conditions are one factor that can promote the diversity of hybrid zones, and F_1DZs promote new interactions between the parental species and the F_1 generation. Comparative ecological investigations of interspecific crossing in other *Viola* hybrid zones will provide additional insight into the evolutionary significance of hybridization in *Viola*.

References

Akiyama S. (2011) Violaceae. In: Kato M. & Ebihara A. (eds). *National Museum of Nature and Science Book Series No. 11. Endemic Plants of Japan*. Tokai University Press, Kanagawa, pp. 93–94 (in Japanese).

Anderson E. C. & Thompson E. A. (2002) A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160**: 1217–1229.

Arnold M. L., Robinson J. J., Buckner C. M. & Bennet B. D. (1992) Pollen dispersal and interspecific gene flow in Louisiana irises. *Heredity* **68**: 399–404.

Beattie A. J. (1976) Plant dispersion, pollination and gene flow in *Viola*. *Oecologia* **25**: 291–300.

Beattie A. J. & Lyons N. (1975) Seed dispersal in *Viola* (Violaceae): adaptations and strategies. *American Journals of Botany* **62**: 714–722.

Broyles S. B. (2002) Hybrid bridges to gene flow: a case study in milkweeds (*Asclepias*). *Evolution* **56**: 1943–1953.

Buerkle C. A. (2005) Maximum-likelihood estimation of a hybrid index based on molecular markers. *Molecular Ecology Notes* **5**: 684–687.

Campbell D. R. & Waser N. M. (2007) Evolutionary dynamics of an *Ipomopsis* hybrid zone: confronting models with lifetime fitness data. *American Naturalist* **169**: 298–310.

Culver D. C. & Beattie A. J. (1978) Myrmecochory in *Viola*: dynamics of seed–ant interactions in some West Virginia species. *Journals of Ecology* **66**: 53–72.

Ehrich D. (2006) AFLPdat: a collection of R functions for convenient handling of AFLP data. *Molecular Ecological Notes* **6**: 603–604.

Ellstrand N. C., Whitkus R. & Rieseberg L. H. (1996) Distribution of spontaneous plant hybrids. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 5090–5093.

Hama E. (2002) *The Wild Violets of Japan in Color*. Seibundo-shinkosha, Tokyo (in Japanese).

Hashimoto T. & Serizawa S. (1971) A long-leaved form of *Viola rossii*. *Journal of Japanese Botany* **46**: 36–37.

Hodges S. A., Burke J. M. & Arnold M. L. (1996) Natural formation of *Iris* hybrids: experimental evidence on the establishment of hybrid zones. *Evolution* **50**: 2504–2509.

Igari M. (2004) *Violets of Japan*. Yama-to-keikoku-sha, Tokyo (in Japanese).

Jiggins C. D. & Mallet J. (2000) Bimodal hybrid zones and speciation. *Trends in Ecology & Evolution* **15**: 250–255.

Kameyama Y., Kasagi T. & Kudo G. (2008) A hybrid zone dominated by fertile F$_1$s of two alpine shrub species, *Phyllodoce caerulea* and *Phyllodoce aleutica*, along a snowmelt gradient. *Journal of Evolutionary Biology* **21**: 588–597.

Kameyama Y. & Kudo G. (2011) Clarification of the genetic component of hybrids between *Phyllodoce caerulea* and *Phyllodoce aleutica* (Ericaceae) in Hokkaido, northern Japan. *Plant Species Biology* **26**: 93–98.

Kyhos D. W., Clark C. & Thompson W. C. (1981) The hybrid nature of *Encelia laciniata* (Compositae: Heliantheae) and control of population composition by post-dispersal selection. *Systematic Botany* **6**: 399–411.

Ma Y. P., Milne R. I., Zhang C. Q. & Yang J. B. (2010) Unusual patterns of hybridization involving a narrow endemic *Rhododendron* species (Ericaceae) in Yunnan, China. *American Journal of Botany* **97**: 1749–1757.

Mallet J. (2005) Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* **20**: 229–237.

Milne R. I., Terzioglu S. & Abbott R. J. (2003) A hybrid zone dominated by fertile F$_1$s: maintenance of species barriers in *Rhododendron*. *Molecular Ecology* **12**: 2719–2729.

Miyake T. & Yahara T. (1998) Why does the flower of *Lonicera japonica* open at dusk? *Canadian Journal of Botany* **76**: 1806–1811.

Nason J. D., Ellstrand N. C. & Arnold M. L. (1992) Patterns of hybridization and introgression in populations of oaks, manzanitas, and irises. *American Journal of Botany* **79**: 101–111.

Ohkawara K. & Higashi S. (1994) Relative importance of ballistic and ant dispersal in two diplochorous *Viola* species (Violaceae). *Oecologia* **100**: 135–140.

R Development Core Team (2012) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. [Cited 3 May 2014.] Available from URL: http://www.R-project.org

Rieseberg L. H. & Carney S. E. (1998) Tansley review 102, Plant hybridization. *New Phytologist* **140**: 599–624.

Vos P., Hogers R., Bleeker M., Reijans M., Vandelee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M. & Zabeau M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Zha H. G., Milne R. I. & Sun H. (2010) Asymmetric hybridization in *Rhododendron agastum*: a hybrid taxon comprising mainly F$_1$s in Yunnan, China. *Annals of Botany* **105**: 89–100.