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Genetic variation and differentiation of floral morphology in wild *Primula sieboldii* evaluated by image analysis data and SSR markers

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Primula sieboldii (E. Morren), the ancestor of the Japanese garden *Primula*, is in danger of extinction in the wild. Genetic diversity is a key component for conservation efforts associated with population management. Genetic diversity in visible traits and several molecular makers were evaluated, respectively. Since it is difficult to determine the degree to which genetic and environmental differences contribute to observed phenotypic variation in natural habitats. A common-garden approach is used. The contribution of genetic and environmental differences to variation in petal shape and area of *Primula sieboldii* were evaluated. Samples from 108 genets gathered from five natural populations in three regions of Japan were analyzed in a common-garden experiment and also analyzed using eight microsatellite markers. From the results of quantitative evaluation based on image analysis, broad genetic variation in petal traits within populations and low level of population differentiation was found. For all petal shapes, Q_{st} was smaller than F_{st}, suggesting that wild populations might be under moderate selective pressure for a specific phenotype. For petal area, Q_{st} was nearly equal to F_{st}, suggesting that population differentiation has been caused mainly by genetic drift.

Key Words: broad-sense heritability, Q_{st}, F_{st}.

Introduction

Many ancestors and other closely related species of crops and garden plants are in danger of extinction in the wild, and conservation of these genetic resources has become an urgent issue (Prance 1997). *Primula sieboldii* (E. Morren), which is a perennial clonal herb that occurs in a range of moist habitats from the understory of deciduous forests to well managed grasslands, is distributed in Japan which from Hokkaido to Kyushu, on the Korean Peninsula, in northern China and in eastern Siberia (Yamazaki 1993). *Primula sieboldii* has been bred as a traditional garden herb for about 300 years from Edo era in Japan (Torii 1985), and there are more than 300 cultivars with various petal colors and shapes that originated by crossbreeding between wild *P. sieboldii* individuals in Japan (Honjo *et al.* 2008b in press). Recently, overexploitation and habitat destruction are threatening wild *Primula* populations with extinction. *P. sieboldii* now listed as "near threatened" in Japan (Environment Agency of Japan 2007). Maintenance of the genetic diversity of wild populations is a key for conservation efforts associated with population management and/or efficient use.

Previously, we evaluated genetic variation based on cpDNA (Honjo *et al.* 2004) and microsatellite (SSR) markers (Honjo *et al.* 2008a) to determine the conservation unit and delineate the ancestral region of cultivated *P. sieboldii*. Genetic differentiation among populations corresponded to the geographic distance between populations, and genetic variation within populations was related to the size of the populations.

Wild populations of *P. sieboldii* show a wide range of variation in floral morphology, such as shape, area, and color (Fig. 1). In a previous study (Yoshioka *et al.* 2007b), petals were photographed in the field and petal variation was examined by image analysis. Significant differences in major shape parameters among populations were found, although the proportion of variance was relatively low compared with the variance among genets within populations. Yoshioka *et al.* (2007b) also found an association between the divergence in subtle changes of petal shape and both geography and genetic markers and estimated that the population divergence in petal shapes of *P. sieboldii* results, in part, from the founder effect and isolation-by-distance effect. There was no association between divergence in major changes of petal shape and geographical and genetic distance. However, Yoshioka *et al.* (2007b) were unable to separate genetic control and direct environmental plasticity as the cause of phenotypic variation because measurements were made in situ. Estimating the genetic diversity related to floral morphology in a remnant wild population requires that wild individuals from each habitat be grown in a common garden. Furthermore, floral characteristics are important markers that can be used to visually assess genetic diversity in wild populations because individual genets can be discerned

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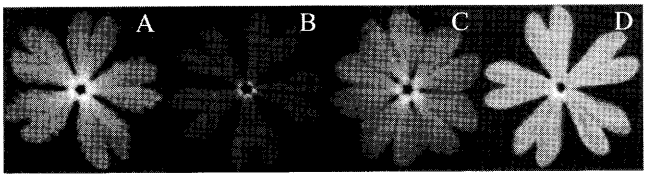

Fig. 1. Variation of petal shape in wild *Primula sieboldii* from three regions. A Saitama region; B Fukushima region; C, D Nagano region

based on flower shapes and other floral morphological characters (Washitani *et al.* 1991). Of course although the visual judgment have been effective in evaluation of genetic diversity in each wild population, it was not possible to compare the diversity from several samples that were influenced by different environmental conditions among population. If the inheritance of floral characters is examined in a common-garden experiment, it is possible to determine the degree to which genetic and environmental differences contribute to the observed phenotypic variation in each habitat. This enables the reliability of human visual assessment of genetic variability in the field.

Q_{st} values, which partitions the quantitative genetic variation of petal traits in a manner analogous to F_{st} for molecular markers, could help clarify how the genetic differentiation among populations arose and how genetic variation in petal morphology has been generated and maintained. There are three possible patterns for the relationship between Q_{st} and F_{st} (Merilä and Crnokrak 2001, McKay and Latta 2002): (1) when $Q_{st} > F_{st}$, populations are differentiated by directional natural selection acting on different phenotypes among the populations; (2) when $Q_{st} = F_{st}$, population differentiation arises mainly through genetic drift; or (3) when $Q_{st} < F_{st}$, natural selection favors the same phenotype in different populations.

The purpose of this study was to evaluate how much the variation of floral morphology in *P. sieboldii* is controlled by genetic factors and how much genetic variation in floral morphology remains in wild remnant populations using genets gathered from five wild populations in Japan. Petal shape and area were quantitatively analyzed by computer image analysis. Finally, the genetic differentiation of floral morphology within the species in Japan was estimated by comparing Q_{st} of floral morphology to F_{st}.

Materials and Methods

Samples

In *Primula sieboldii*, each genet is composed of various numbers of physiologically independent ramets, which propagate clonally by means of short rhizomes. In 2000, 108 genets were collected from five wild populations in three regions of Japan (Table 1): lowland area in southern Hokkaido (northern island in Japan, annual amount of precipitation 1410 mm, annual mean temperature 7.3 deg C in Hidaka area), Saitama (a lowland area in central Japan, annual amount of precipitation 1243 mm, annual mean temperature 14.6 deg C in Saitama Prefecture), and Nagano (a highland area in central Japan, annual amount of precipitation 1198 mm, annual mean temperature 7.9 deg C in Karuizawa area). Essentially only one ramet from each genet was sampled to prevent injury to population and ramets were propagated in the garden of the University of Tsukuba for three years. In the Hokkaido and Nagano 1 populations, 24 and 27 genets were randomly sampled from populations with more than 100 genets. Nearly all genets in the Nagano 2 and Nagano 3 populations were sampled (26 and 13, respectively). For the Saitama population, 18 genets were grown from seeds that were studied in a previous experiment (Washitani and Kabaya 1988).

In a common-garden experiment, 433 ramets were grown from the 108 genets in a greenhouse at the University of Tsukuba (140°10′N, 36°11′E, annual amount of precipitation 1287 mm, annual mean temperature 14 deg C) to evaluate the genetic variation in petal area and shape. Clonally propagated shoots were planted into 12-cm pot in Metro-Mix 350 (HYPONeX, JAPAN, CORP.) in January 2003. Four ramets from each genet were used as replications when available; otherwise two or three ramets were used.

Quantitative evaluation of floral traits

In 2003 both petal area and shape in each flower was investigated by image analysis according to the methods of Yoshioka *et al.* (2004). That is, each flower was separated into its five petals and photographed alongside a scale marker (9 mm × 9 mm) by a digital camera (COOLPIX-850, Nikon, Tokyo). Petal shape and petal area were evaluated quantitatively using the image analysis software SHAPE 1.2 (Iwata and Ukai 2002). For petal shape, 40 standardized elliptic Fourier coefficients were obtained and principal-components (PC) analysis performed based on a variance–

Table 1. Locations of populations in Japan and total and sampled number of *Primula sieboldii*

Population	Location	Latitude	Longitude	Altitude	Total no. of genets	No. of genets sampled
Hokkaido	Southern Hokkaido	142°01′N	42°31′E	30 m	140	24
Saitama	Central Honshu, Saitama	139°36′N	35°50′E	10 m	>300	18
Nagano 1	Central Honshu, Nagano	138°31′N	36°12′E	960 m	166	27
Nagano 2	Central Honshu, Nagano	138°38′N	36°19′E	940 m	28	26
Nagano 3	Central Honshu, Nagano	138°31′N	36°21′E	1120 m	16	13

covariance matrix to summarize the information contained in the coefficients. These PC scores were used as parameters of petal shape. Variation in petal shape was divided into symmetrical and asymmetrical variation using SHAPE. We only targeted symmetrical variation, because asymmetrical variation in the petal shape of *P. sieboldii* primarily results from environmental effects (Yoshioka *et al.* 2004).

DNA extraction and genotyping of microsatellite markers

Genomic DNA of each plant was extracted from frozen leaves using a modified CTAB method (Murray and Thompson 1980). We have already developed many microsatellite markers (unpublished), but no information on linkage between markers and several floral traits is available. So genotypes of 108 genets were determined using eight pairs of microsatellite PCR primers (Table 2). PCR condition for each primer was according to the protocol described by Honjo *et al.* (in press). The PCR products were run on a 3100 Genetic Analyzer with GeneScan (Applied Biosystems, Foster City, USA).

Data analysis

Nested ANOVA was used to estimate the contribution of variance among populations, among genets, and among ramets within a genet to the total variance in petal shape and petal area. The phenotypic variation among ramets within a genet was considered to represent environmental variation, because all ramets within a genet should have an identical genotype. Therefore, variance among genets and populations was ascribed to genotypic variance. Broad-sense heritability (h^2) was estimated by dividing the genotypic variance by the total variance (Falconer 1981).

Using Bartlett's test for equality of variance, whether the variation among genets within populations was identical among populations was examined. If the petal traits differed significantly among populations, each pair of populations was compared by Tukey-Kramer multiple-comparison test.

The degree of population differentiation of petal traits in wild populations was estimated using the Q_{st} parameter (Spitze 1993), which was calculated as:

$$Q_{st}=\sigma^2_{g(b)}/(\sigma^2_{g(b)}+2\sigma^2_{g(w)}) \quad (1),$$

where $\sigma^2_{g(b)}$ is the between-population variance component and $\sigma^2_{g(w)}$ is the within-population (i.e., among genets) variance component taken from the nested ANOVA.

To compare with Q_{st} and estimate the genetic differentiation in petal traits, F_{st} values at microsatellites was obtained. These parameters were calculated for the genotypic data of 108 genets using eight pairs of microsatellite primers using the program FSTAT 2.9.3 (Goudet 2001).

Results

Quantitative evaluation of floral traits

Only 94 out of 108 genets flowered and could be analyzed. These non-flowering genets were not from any particular population. The first three PCs for symmetrical variations identified by SHAPE is shown (Fig. 2). PC1 represented the relationship between the depth of the head notch and the maximum width of the petal and accounted for 58.4% of the total variance in petal shape. PC2 represented the aspect ratio, petal's width divided by petal's height, and accounted for 30.5% of the total variance. PC3 represented the position of the petal's center of gravity, the average location of the weight of a petal, and accounted for 3.8% of the total variance. Thus, these three PCs accounted for approximately 93% of the total variance.

The broad-sense heritabilities of petal shape PC1, PC2, and PC3 were 0.58, 0.68, and 0.53, respectively, whereas that of petal area was 0.69 (Table 2). Of these traits, the variances among ramets in PC1 and PC3 were larger than those in PC2 and petal area. That is, the relationship between the depth of the head notch and the maximum width and the petal's center of gravity were more likely to be affected by environmental factors than were the aspect ratio and petal area.

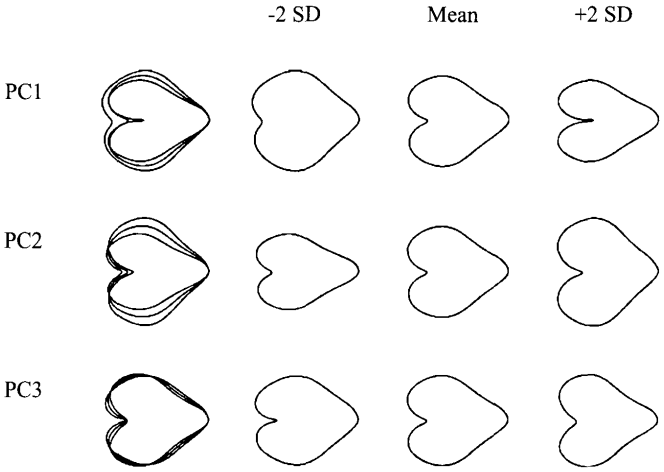

Fig. 2. The principal components that affect petal shape in *Primula sieboldii*. PC1, relationship between depth of head notch and maximum width; PC2, aspect ratio; PC3, position of the center of gravity. In the first column are superimposed images of the mean and range of shapes for each PC.

Table 2. Annealing temperature and number of alleles detected per locus of eight microsatellite markers used in this study

Locus	Annealing temperature (°C)	No. of alleles	References
ga0235	60	12	Ueno *et al.* (2003)
ga0381	57	9	Ueno *et al.* (2003)
ga0653	60	8	Ueno *et al.* (2005)
ga0666	55	24	Ueno *et al.* (2005)
ga0668	52	7	Ueno *et al.* (2003)
ga1277	60	16	Ueno *et al.* (2003)
Pri0146	60	15	Kitamoto *et al.* (2005)
PS-2	55	6	Isagi *et al.* (2001)

Genetic variation of floral traits within and among populations

The results of nested ANOVA demonstrated significant differences among the genets for all three PCs and petal area ($P < 0.01$), and there were significant differences among populations in PC1 ($P < 0.05$) and petal area ($P < 0.01$; Table 3). PC2 and PC3 showed no significant difference among populations ($P = 0.25$ and 0.17, respectively).

According to Bartlett's test, variation within populations did not differ significantly among populations in any of the four petal traits; that is, all the populations we studied had a similar degree of variation in all the traits (Table 4). Although the population size varied from 16 to more than 300 genets and sample size ranged from 13 to 28 genets per population, this result indicates that small populations maintain the same degree of variation in petal traits as that of large populations. However, the average values of PC1 and petal area differed significantly among populations (Fig. 3 and Table 4). In the Hokkaido population, PC1 was significantly smaller and petal area was significantly larger than in the other populations. Thus, plants in this population had a relatively distinct floral morphology.

The variance component among populations for petal area was larger than those of petal shape traits (PC1, PC2, PC3; Fig. 4). For all traits, the variance among populations was the smallest component and the variance among genets was the largest component of total variance. Q_{st} values for the relationship between the depth of the head notch and the maximum width of the petal (PC1), the aspect ratio (PC2), and the position of the petal's center of gravity (PC3) were 0.055, 0.006 and 0.000, respectively, and Q_{st} for petal area was 0.124.

Molecular versus Quantitative genetic variation

Each locus was polymorphic with 6-24 alleles per locus, and, in total, 97 alleles were detected over the eight loci (Table 2). F_{st} was 0.169 (two-sided 95% confidence interval, 0.118–0.222).

Comparing Q_{st} to F_{st}, Q_{st} was significantly smaller than F_{st} for the three petal shape PCs, but Q_{st} for petal area was similar to F_{st}.

Discussion

Washitani *et al.* (1991) reported that the genetic variation measured within *P. sieboldii* populations could be observed in the phenotypic variation of flowers. In this study, we also used floral morphology as a parameter to evaluate the genetic diversity in the field. The broad-sense heritability of floral morphological traits in *P. sieboldii* ranged from 0.53 to 0.69, and heritability of petal area tended to be greater than that of petal shape (Table 2). Thus, we conclude that the differences in petal traits among genets (accounting for more than 50% of total variance in all four petal traits) was largely determined by genetic factors rather than by environmental factors.

Many studies have evaluated broad-sense heritability of floral traits in wild plant species and reported a wide variability. For example, there is low heritability of flower size in *Eichhornia paniculata* ($h^2 = 0.19$–0.35; Worley and Barrett 2001), corolla size in *Campanula rapunculoides* ($h^2 = 0.25$; Vogler *et al.* 1999), and corolla size in *Echium vulgare* ($h^2 = 0.33$; Klinkhamer and van der Veen-van Wijk 1999), whereas high heritability has been reported for petal size in *Spergularia marina* ($h^2 = 0.67$–0.71; DeLesalle and Mazer 1995) and corolla length and corolla width in *Penstemon centranthifolius* ($h^2 = 0.86$ and 0.94, respectively; Randall

Table 3. Results of nested ANOVA and heritability of petal shape PC1, PC2, and PC3 and petal area

Factor	df	PC1			PC2			PC3			petal area		
		MS	F	h^2	MS	F	h^2	MS	F	h^2	MS	F	h^2
Population	4	0.0158	2.67*		0.0050	1.37		0.00051	1.56		19.093	8.06**	
Genet	94	0.0059	5.35**	0.58	0.0037	8.04**	0.68	0.00032	5.08**	0.53	2.370	6.85**	0.69
Ramet	249	0.0011			0.0005			0.00006			0.346		

*$P<0.05$, **$P<0.01$

Table 4. Average values and variance within and among populations in petal traits

		within-population					among-population
Petal traits		Hokkaido	Saitama	Nagano1	Nagano2	Nagano3	
PC1	Ave.	−0.0876	−0.0113	0.0077	0.0414	0.0404	−0.0045
	Var.	0.0036	0.0071	0.0043	0.0023	0.0032	0.0062
PC2	Ave.	0.0079	−0.0282	0.0458	−0.0273	−0.0085	0.0013
	Var.	0.0020	0.0017	0.0029	0.0026	0.0026	0.0032
PC3	Ave.	0.00137	0.00669	−0.00314	−0.00556	0.00634	0.00009
	Var.	0.00025	0.00031	0.00036	0.00040	0.00038	0.00035
Flower Area	Ave.	5.931	5.233	5.105	4.806	5.545	5.283
	Var.	0.785	0.774	0.677	0.659	0.868	0.864

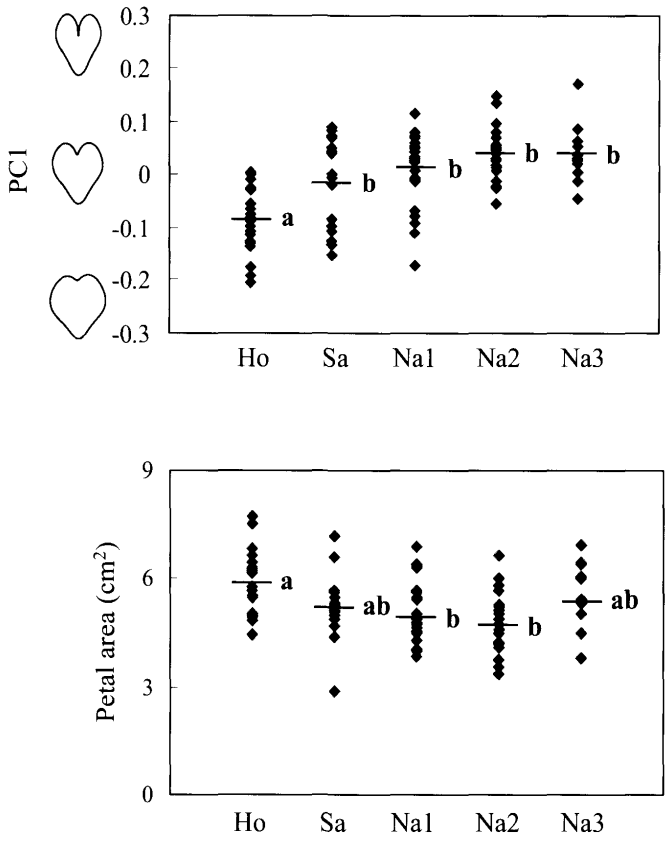

Fig. 3. Per-genet values for petal shape PC1 and petal area in *Primula sieboldii*. Horizontal lines indicate mean values of each trait; those followed by different letters are significantly different ($P<0.05$). Populations: Ho, Hokkaido; Sa, Saitama; Na1, Nagano 1; Na2, Nagano 2; Na3, Nagano 3.

and Shaw 1993). According to a review of 68 studies of flowering plants by Ashman and Majetic (2006), mean heritability of corolla size was 0.46, with a range from 0 to 1. Thus, the heritability of floral morphology in *P. sieboldii* appears to be relatively high.

Visual judgment of floral differences in the field is based not only on floral shape and area but also on color and its pattern. However, our results suggested that the evaluation of genetic diversity using floral morphology is not necessarily accurate, as genets may show only slight differences in certain characteristics. Conner *et al.* (2003) noted that the heritability under controlled conditions may be overestimated as compared to that under natural conditions. In wild *P. sieboldii*, however, we consider that genets can be efficiently and non-destructively identified in the field by the differences in the depth of the head notch (PC1), aspect ratio of the petal (PC2), and petal area obtained by visual observation. Because the center of gravity (PC3) made only a small contribution to the total variation (3.8%) and was more difficult to use this trait in order to distinguish among genets than the other traits, it is probably not an efficient parameter for field assessments. The environmental variation among years and among populations is expected to be considerably larger than that within populations, so visual assessment of genetic diversity would likely be effective only within populations.

Significant differences were found among genets in petal shape PC1, PC2, PC3, and petal area ($P<0.01$). In another study which used eight wild populations of *P. sieboldii*, Yoshioka *et al.* (2007b) reported that the petal's aspect ratio, the depth of the head notch, and the position of the center of gravity accounted for 49.2%, 45.0%, and 3.7% of total variation. Both that study and the present one found that the major PCs represented the same characteristics of petal shape in wild populations, and these three characteristics accounted for most of the total variation of petal shape. With regard to divergence of floral morphology, our previous study (Yoshioka *et al.* 2007b) noted the significant differences of major factors of petal shape (PC1–PC3) among populations, but in the field it was not possible to determine whether these differences were due to genetic or environmental factors. The present study, based on a common-garden experiment, showed that significant differences among populations in petal area and in the relationship between the head-notch depth and the maximum width (Fig. 4) are caused by genetic factors. On the other hand, the aspect ratio and the position of the petal's center of gravity are differentiated among populations due to local environmental factors.

The results of Bartlett's test showed that the same degree of genetic diversity in petal shape and area has been maintained in wild populations, despite consisting of different numbers of genets. This result represented that petal trait

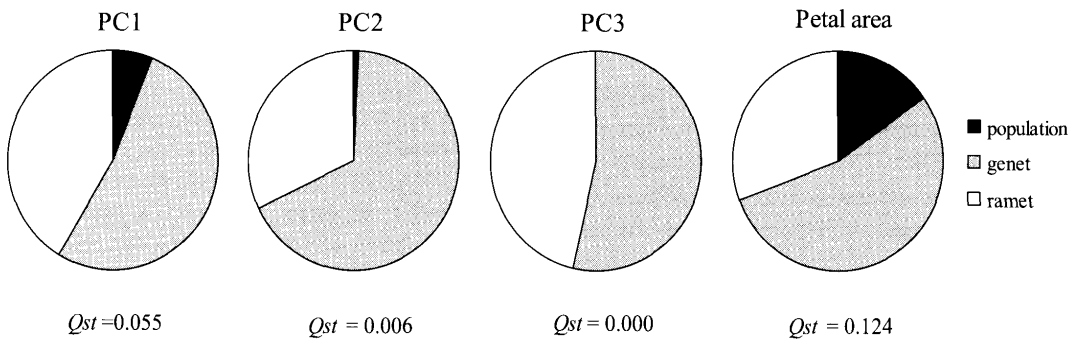

Fig. 4. Proportions of three variance components (among populations, genets, and ramets) in four petal traits (PC1, PC2, PC3, and petal area) revealed by nested ANOVA. Q_{st} represents the degree of genetic differentiation among populations.

variation within populations is not associated with population size. Thus, from a conservation perspective, it is important to maintain whole wild populations regardless of population size.

Q_{st} ranged from 0.000 in PC3 to 0.124 in petal area (Fig. 4), with the petal shape being especially low (ranging from 0.000 to 0.055). Because petal shape and area retained a high degree of variation among genets within populations, there appears to have been a low degree of differentiation among the populations. Q_{st} was smaller than F_{st} for all three petal shape PCs, indicating that the populations investigated have undergone selection favoring certain phenotypes. For petal area, Q_{st} was nearly equal to F_{st}, indicating that the genetic differentiation among populations has been caused mainly by random genetic drift. Because there was no directional selection on floral morphology, broad genetic diversity in floral morphology has been maintained within populations. Moreover, Yoshioka *et al.* (2007a) showed that there has been no directional selection on petal shape in *P. sieboldii*. Under current natural conditions, the innate or learned preference of bumblebees, the primary pollinator of this species, for certain flower characteristics may not be a strong selective force. Yoshioka *et al.* (2007a) mentioned that bumblebees made no distinction between petal shapes except for extremely narrow petals, which are not observed in wild populations. Thus, because these traits were not the targets of natural selection, the large genetic variation in petal shape observed was considered to be retained within wild populations naturally.

For all traits, genetic variation within populations accounted for a large proportion of the total variation, and the means of PC1 (head-notch depth) and area differed significantly among populations. Hokkaido plants had petals with large surface areas and the flowers of plants from Saitama and Nagano 2 had small petals. Hokkaido populations had shallow petal head notches and those from Nagano 2 and 3 populations had deep head notches. The genetic variation of PC2 and PC3 was mostly maintained within populations.

Based on molecular analysis, Honjo *et al.* (submitted) noted that various *P. sieboldii* garden cultivars originated mainly from a particular population along the Arakawa River in Saitama. Our results also suggested that other cultivars originated from the wide floral variation in the Saitama population. With regard to genetic variation in the depth of the head notch (PC1), Yoshioka *et al.* (2005) showed that wild *P. sieboldii* had a deeper head notch than cultivars; the shallower head notch may have resulted from previous breeding manipulations, such as crossing and selection. Currently, none of the more than 300 *P. sieboldii* cultivars have a deep head notch. Based on our assessment of wild populations, we propose the use of genets with deep notches from the Nagano 2 and Nagano 3 populations as breeding material. Thus, from the perspective of *Primula* breeding, the genetic diversity available within and among wild populations is a valuable resource.

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