Reconsideration for conservation units of wild *Primula sieboldii* in Japan based on adaptive diversity and molecular genetic diversity

YASUKO YOSHIDA[1], MASANORI HONJO[2], NAOKO KITAMOTO[1]
AND RYO OHSAWA[1]*
[1] *Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan*
[2] *National Agricultural Research Center for Tohoku Region, 4 Akahira, Shimo-kuriyagawa, Morioka, Iwate 020-0198, Japan*

Summary

Primula sieboldii E. Morren is a perennial clonal herb that is widely distributed in Japan, but in danger of extinction in the wild. In a previous study, we revealed the genetic diversity of the species using chloroplast and nuclear DNA and used this information to define conservation units. However, we lacked information on adaptive genetic diversity, which is important for long-term survival and, thus, for the definition of conservation units. In order to identify adaptive traits that showed adaptive differentiation among populations, we studied the genetic variation in six quantitative traits within and among populations for 3 years in a common garden using 110 genets from five natural populations from three regions of Japan. The number of days to bud initiation was adaptive quantitative trait for which the degree of genetic differentiation among populations (Q_{ST}) was considerably larger than that in eight microsatellite markers (F_{ST}). The relationship between this trait and environmental factors revealed that the number of days to bud initiation was negatively correlated, with the mean temperature during the growing period at each habitat. This suggests that adaptive differentiation in the delay before bud initiation was caused by selective pressure resulting from temperature differences among habitats. Our results suggest that based on adaptive diversity and neutral genetic diversity, the Saitama population represents a new conservation unit.

1. Introduction

The extent of genetic diversity that is maintained among and within remnant wild populations plays a key role in understanding the adaptive evolution of a species in response to fluctuating environments. Such knowledge can therefore support the conservation of endangered species (Frankham *et al.*, 2002). Similarly adaptive genetic diversity within and among local populations is particularly important when conservation procedures such as translocation of plant materials between sites or the restoration of local populations is promoted (McKay & Latta, 2002). The term 'Restoration' is commonly used for the maintenance of the genetic structure, i.e. the hierarchical structure of genetic variation, in a species and many researchers used different approaches for the implication of restoration efforts in endangered species (e.g. Gordon & Rice, 1998). In this context, a 'conservation unit' is a group of populations of a species whose genetic structure differs from that of other groups of populations (i.e. differs from other conservation units).

Genetic variation is mainly evaluated using quantitative traits and molecular markers. The evaluation of quantitative traits in common-garden studies began more than 60 years ago (Clausen *et al.*, 1940). The utilization of molecular markers in such studies has become increasingly common during the last several decades (Haig, 1998), because it is easier to evaluate genetic variation using molecular markers. The relationships between quantitative and molecular genetic variation within and among populations have

* Corresponding author: Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan. Tel.: +81-298-53-6674. Fax: +81-298-53-6674. e-mail: osawaryo@sakura.cc.tsukuba.ac.jp

been tested by many researchers (Karhu *et al.*, 1996; Merilä & Crnokrak, 2001; Reed & Frankham, 2001; Bekessy *et al.*, 2003; Carvajal-Rodriguez *et al.*, 2005), but no significant relationship was found between the two parameters in many cases. As a result, because molecular markers do not reflect the differentiation among populations in their adaptive traits, it has become necessary to directly evaluate the genetic variation of these populations based on quantitative traits in conservation efforts (Storfer, 1996; Mckay *et al.*, 2001; Holderegger *et al.*, 2006).

Population genetic structure is often quantified using Wright's F_{ST} parameter in combination with the analogous measure for quantitative traits, Q_{ST} (Spitze, 1993). When Q_{ST} is compared directly with F_{ST}, there are three possible patterns (Merilä & Crnokrak, 2001; McKay & Latta, 2002): (1) when $Q_{ST} > F_{ST}$, populations have become differentiated as a result of directional natural selection that acts on different phenotypes among the populations; (2) when $Q_{ST} = F_{ST}$, differentiation among populations has arisen mainly through genetic drift; or (3) when $Q_{ST} < F_{ST}$, stabilizing selection has favoured the maintenance of the same phenotype in different populations. Thus, by comparing Q_{ST} with F_{ST}, it becomes possible to identify adaptive traits that show adaptive differentiation among populations as a result of directional selection.

Understanding adaptive differentiation by comparisons of Q_{ST} with F_{ST} has become increasingly popular in the last 5 years (Leinonen *et al.*, 2008). Despite wide recognition of the importance of evaluating both adaptive and molecular genetic diversity, few conservation units have been defined for use in conservation efforts that address both adaptive and molecular genetic variation (de Guia & Saitoh, 2007). Many studies using molecular markers have assigned conservation priority to populations that are considered to represent conservation units (e.g. Moritz, 1994), but Crandall *et al.* (2000) and Fraser & Bernatchez (2001) emphasized that conservation units should be defined by considering both adaptive and molecular genetic variation. Although increasing numbers of studies have defined conservation units based on both parameters (e.g. Denoël, 2007; Cano *et al.*, 2008), few of these studies have focused on plants (see, de Guia & Saitoh, 2007).

Primula sieboldii E. Morren is a perennial clonal herb that occupies a range of moist habitats, from the understory of deciduous forests to well-managed grasslands. The species is distributed in Japan, on the Korean Peninsula, in northern China, and in eastern Siberia (Yamazaki, 1993). However, overexploitation and destruction of its habitats and horticultural collection are threatening wild populations with extinction, and the species has become endangered in Japan; originally classified in the lower rank of 'vulnerable',

the species was reclassified to 'near threatened' in 2007 (Iwatsuki, 2008).

The genetic diversity of *P. sieboldii* has been evaluated using cpDNA (Honjo *et al.*, 2004) and microsatellite markers (Honjo *et al.*, 2009) to define conservation units in Japan. These studies revealed that genetic differentiation among populations corresponded to the geographic distance between populations, and that genetic variation within populations correlated with population size. On this basis, we defined four conservation units.

In our previous research on quantitative traits, we investigated the genetic diversity in the petal shape and area of *P. sieboldii* in a common garden and revealed that genetic variation was mostly maintained within wild populations, i.e. low differentiation among populations (Yoshida *et al.*, 2008). These traits were not under directional selection and appeared to be basically selection-neutral. However, there is less information on adaptive traits, and understanding adaptive genetic diversity is required for more precise definition of conservation units for *P. sieboldii*.

In the present study, we evaluated genetic variation of six quantitative traits within and among wild populations of *P. sieboldii* to obtain new information that would help us understand the adaptive traits and genetic structure of this species. By comparing Q_{ST} with F_{ST}, calculated using eight microsatellite (simple sequence repeat (SSR)) markers, we estimated the cause of genetic differentiation among the populations and identified potentially adaptive traits of *P. sieboldii*. In addition, we investigated the relationships between quantitative genetic variation and five ecological factors in order to detect the potential causes of any differentiation among populations. Ultimately, we attempted to use this new knowledge to redefine the conservation units for *P. sieboldii* by considering both adaptive and molecular genetic variation.

2. Materials and methods

(i) *Plant species and sampling populations*

Each genet is composed of various numbers of physiologically independent ramets, which propagate clonally by means of short rhizomes. In early spring, these rhizomes bud and subsequently flower from April to June. After July, new rhizomes for next year are generated from a mother ramet below the ground.

In 2000, we collected 110 genets of *P. sieboldii* from five wild populations in three regions of Japan (Table 1): Hokkaido (a lowland area in northern Japan), Saitama (a lowland area in central Japan) and Nagano (a highland area in central Japan). In the Hokkaido and Nagano 1 populations, we randomly

Table 1. *Locations of the five Japanese populations of* P. sieboldii, *number of genets (total and number sampled) and average monthly temperatures at each site*

Population	Location	Latitude	Longitude	Altitude	Total no. of genets in population	No. of genets sampled	Average temperature (°C)				
							February	March	April	May	June
Hokkaido	Southern Hokkaido	42°31′N	142°01′E	30 m	140	26	−5·1	−0·7	4·9	9·9	13·9
Saitama	Central Honshu, Saitama	35°50′N	139°36′E	10 m	>300	18	4·2	7·5	13·3	17·9	21·3
Nagano 1	Central Honshu, Nagano	36°12′N	138°31′E	960 m	166	27	−1·3	2·6	9·2	14·4	18·4
Nagano 2	Central Honshu, Nagano	36°19′N	138°38′E	940 m	28	26	−3·4	0·0	6·6	11·6	15·4
Nagano 3	Central Honshu, Nagano	36°21′N	138°31′E	1120 m	16	13	−3·4	0·0	6·6	11·6	15·4

Average temperatures represent means from 1979 to 2000 obtained from the Japan Meteorological Agency Web site (http://www.jma.go.jp/jma/indexe.html).

sampled 26 and 27 genets, respectively, from a population that contained more than 100 genets. In the Nagano 2 and Nagano 3 populations, we sampled nearly all genets (26 and 13, respectively). For the Saitama population, we used 18 genets that had been grown from seeds obtained from a natural population in Saitama region in a previous experiment (Washitani & Kabaya, 1988). These 18 genets were from several different mothers and originated from the region surrounding Saitama population from the results of both an assignment test based on nuclear DNA markers and cpDNA (Honjo et al., 2004, 2008; Honjo, 2005).

After these samples were obtained, the ramets were acclimatized in Tsukuba (a lowland area of central Japan) for 3 years. Each genet used in the present study had a unique SSR genotype (Honjo et al., 2009).

In this study, we selected three regions (Hokkaido, Saitama and Nagano) with sample sites distributed over a wide range of altitudes (from 10 to 1120 m asl) and latitudes (from 35°50′N to 42°31′N) (Table 1). We selected three Nagano populations to investigate whether adaptive differentiation has occurred on a small geographical scale.

Meteorological data for each habitat were obtained from the Japan Meteorological Agency Web site (http://www.jma.go.jp/jma/indexe.html) (Table 1). Mean temperatures from February to June (the growing period of *P. sieboldii*) were obtained using data from 1979 to 2000. Because the Nagano 2 and Nagano 3 sites are so close to each other, they had the same mean temperature.

(ii) *Common-garden experiment*

From 2003 to 2005, we grew 425 to 450 ramets from the 110 genets in a greenhouse at the University of Tsukuba (140°10′E, 36°11′N) to evaluate genetic variation in quantitative traits. Clonally propagated shoots were planted into 12-cm pots at a depth of 1 cm below the soil surface in Metro-Mix 350 (Hyponex Japan, Corp.) in January. The pots were assigned randomly to their positions in the greenhouse. Four ramets from each genet were used as replications, when available; otherwise, we used two or three ramets.

We measured six quantitative traits: budding date, first flowering date, number of flowers, number of leaves, and the lengths of the peduncle at the start and end of flowering. Because the heights of the plants surrounding *P. sieboldii* in the habitats increased greatly from early spring to early summer, and the environmental conditions of the habitats between the start and end of the flowering period also changed greatly, we measured the two lengths of the peduncle. We measured all traits for each ramet in each of the 3 years of the study period. We scored the date when

Table 2. *Details of the eight microsatellite markers used in this study, and the gene diversity averaged over loci* (H_e), *expected number of alleles observed in 13 genets* (n_{13a}) *detected for the eight loci in* P. sieboldii

Locus	Annealing temperature (°C)	No. of alleles	References	Hokkaido		Saitama		Nagano 1		Nagano 2		Nagano 3	
				H_e	n_{13a}	H_e	n_{13a}	H_e	n_{13a}	H_e	n_{13a}	H_e	n_{13a}
*ga*0235	60	12	Ueno *et al.* (2003)	0·727	4·83	0·690	5·58	0·770	5·59	0·828	7·00	0·788	8·00
*ga*0381	57	8	Ueno *et al.* (2003)	0·314	2·95	0·231	2·74	0·532	2·89	0·615	3·64	0·727	5·00
*ga*0653	60	8	Ueno *et al.* (2005)	0·587	2·99	0·721	5·44	0·724	6·24	0·802	6·54	0·655	5·00
*ga*0666	55	24	Ueno *et al.* (2005)	0·707	5·17	0·796	8·07	0·437	4·11	0·906	11·00	0·928	13·00
*ga*0668	52	7	Ueno *et al.* (2003)	0·572	3·00	0·700	4·50	0·486	2·00	0·558	3·89	0·489	3·00
*ga*1277	60	16	Ueno *et al.* (2003)	0·809	5·90	0·585	5·43	0·798	7·15	0·824	6·15	0·795	8·00
*Pri*0146	60	15	Kitamoto *et al.* (2005)	0·613	4·69	0·515	5·19	0·646	4·84	0·865	7·71	0·814	7·00
PS-2	55	6	Isagi *et al.* (2001)	0·543	2·74	0·590	2·99	0·754	4·91	0·608	3·00	0·735	4·00
Mean		12		0·609	4·03	0·604	4·99	0·643	4·71	0·751	6·12	0·741	6·63

a plant attained a height of 1 cm above the soil surface as the budding date. To ensure that we did not miss budding or first flowering, we surveyed the plants daily at the start of each growing season. Before analysis, we converted the budding date and the first flowering into the number of days to bud initiation and flowering from the first budding date observed for the entire population of ramets. We measured the lengths of the peduncle on the first flowering day and at the end of the flowering period, i.e. the date when all flowers had abscised. We recorded the number of flowers and leaves at the end of the flowering period.

(iii) *DNA extraction and genotyping of microsatellite markers*

We extracted the genomic DNA of each plant from frozen leaves using a modified Cetyl trimethyl ammonium bromide (CTAB) method (Murray & Thompson, 1980). We have already developed many microsatellite markers for this species (Ueno *et al.*, 2009), but we have no information on linkage between these markers and quantitative traits. We determined the genotypes of the 110 genets using the eight pairs of microsatellite PCR primers (Table 2) used by Honjo *et al.* (2009). The PCR conditions for each primer followed the protocol described by Honjo *et al.* (2009). The PCR products were analysed using a 3100 Genetic Analyzer and the GeneScan software (Applied Biosystems).

3. Data analysis

(i) *Evaluation of quantitative traits*

We used factorial analysis to examine the effects of population, year, the population × year interaction and genet on the investigated traits using the values for each ramet. We performed restricted maximum likelihood (REML) tests to estimate the contribution of variance among populations, among genets within a population and among ramets within a genet to the total phenotypic variance for each investigated trait. The variation among ramets was assumed to represent environmental variation, because each ramet of a given genet has an identical genotype. Thus, the variation among genets and populations was considered to represent genetic variation, and we calculated broad-sense heritability (h^2) by dividing the genotypic variance by the total variance (Falconer, 1981). We used the Tukey–Kramer multiple-comparison test (with $P < 0.05$ considered significant) to detect significant differences in the average values among pairs of populations for all traits. All statistical tests were performed using the JMP 6.0 statistical software (SAS Institute Inc.).

(ii) *Genetic variation within populations*

The genetic variation within populations in quantitative traits, i.e. the variation among genets V_g was calculated as

$$V_g = \frac{(\text{MS genets} - \text{MS ramets})}{r}, \tag{1}$$

where MS represents the mean square. To represent the number of ramets per genet, we used r, the effective number of replicates:

$$r = \left(\sum_{i=1}^{a} r_i - \sum_{i=1}^{a} r_i^2 \Big/ \sum_{i=1}^{a} r_i \right) \Big/ (a-1), \tag{2}$$

where a is the number of genets and r_i is the number of replicates of genet i.

To evaluate the genetic variation for the eight microsatellite markers, we used the FSTAT 2.9.3 software (Goudet, 2001) to calculate unbiased estimates of gene diversity averaged over all loci (H_e; Nei & Roychoudhury, 1974); the expected number of alleles observed in 13 genets from each population,

Table 3. *Number of genets and years analysed for each trait in this study*

Traits	Number of genets	Number of years
Days to bud initiation	104	3
Days to flowering	93	2
Length of peduncle at the start of flowering	91	2
Length of peduncle at the end of flowering	88	2
Number of flowers	90	2
Number of leaves	92	3

which represents the smallest sample size (n_{13a}, allelic richness).

(iii) *Genetic variation among populations*

We estimated the degree of population differentiation for each of the investigated traits using Q_{ST} (Spitze, 1993), which was calculated as

$$Q_{ST} = \sigma^2_{gb}/(\sigma^2_{gb} + 2\sigma^2_{gw}) \qquad (3)$$

where σ^2_{gb} is the between-population variance component and σ^2_{gw} is the within-population, i.e. among genets variance component taken from the nested ANOVA using the JMP software.

The degree of population differentiation in molecular markers (F_{ST}; Weir & Cockerham, 1984) was estimated using the genotype data for the 110 genets based on the eight pairs of microsatellite primers. Furthermore, we compared the pairwise Q_{ST} and F_{ST} values to provide insights into the potential mechanisms responsible for genetic differentiation among the populations. We tested for correlations between each pair of Q_{ST} and F_{ST} values using the Mantel test (Mantel, 1967). We calculated F_{ST} and the pairwise F_{ST} and carried out the Mantel test using the FSTAT 2.9.3 software.

(iv) *Relationships between pairwise* Q_{ST} *and ecological factors*

To explore the potential causes of differentiation among populations, we investigated the relationships between five ecological factors and the traits that revealed to have differentiated among the populations by directional selection from the relationship between Q_{ST} and F_{ST}. We investigated between four environmental factors (temperature, altitude, latitude and longitude) and the average values of these traits per population, respectively. We also investigated the relationship between geographical distance and the pairwise Q_{ST} for these traits. These relationships were quantified using Spearman's rank-correlation coefficient, calculated using the JMP software.

4. Results

Because few genets flowered in 2005, we have only analysed the number of days to flowering, the number of flowers, and the lengths of the peduncle at the start and end of flowering using the 2003 and 2004 data. Table 3 shows the number of genets and years that we actually analysed.

(i) *Evaluation of quantitative traits*

All traits differed significantly among genets ($P < 0.01$; Table 4). This suggests that all the quantitative traits were determined by genetic factors. All traits differed significantly among years, with large F-values (Table 4), indicating that quantitative traits were greatly influenced by variations in environmental effects among the years. In 2004, the number of days to bud initiation was longer, the length of the peduncle at the start of flowering was longer, the length of the peduncle at the end of flowering was shorter and the number of leaves was smaller than in 2003; this was also true in 2005.

The number of days to bud initiation, number of days to flowering, number of flowers and number of leaves differed significantly among populations, but the two lengths of the peduncle did not ($P < 0.01$; Table 4). The number of days to bud initiation showed the largest F-value of the six traits (Table 4), and the Saitama population produced buds consistently and significantly earlier than the other populations in all 3 years, whereas Nagano 2 tended to produce buds later than the other four populations, with the difference significant in 2003 (Table 5). The number of days to flowering tended to differ between years in each population (Table 5). For the number of flowers, the differences among populations were smaller in 2004 than in 2003, and there were fewer significant differences. The genets from the Saitama and the Nagano1 populations tended to have a large number of leaves in all 3 years (Table 5).

We found a significant population × year interaction for three traits (Table 4): the number of days to bud initiation and flowering (both $P < 0.01$) and the length of the peduncle at the end of flowering ($P < 0.05$).

Table 6 presents the broad-sense heritability values for the six traits. The heritabilities of all traits were largest in 2003, with one exception: the value for the number of leaves was largest in 2005. In each year, the number of days to bud initiation consistently showed the smallest variance among the ramets and the largest heritability (61·2–74·6), whereas the

Table 4. *Results of the factorial analysis for the six investigated traits*

Factors	Days to bud initiation			Days to flowering			Length of peduncle at the start of flowering		
	df	MS	F	df	MS	F	df	MS	F
Population	4	6851·6	43·89**	4	326·9	5·31**	4	31·2	1·79
Year	2	80512·7	515·72**	1	17298·1	280·96**	1	150·3	8·62**
Population × Year	8	780·2	5·00**	4	216·0	3·51*	4	8·3	0·48
Genet	99	156·1	4·09**	88	61·6	5·17**	86	17·4	5·13**

Factors	Length of peduncle at the end of flowering			Number of flowers			Number of leaves		
	df	MS	F	df	MS	F	df	MS	F
Population	4	19·3	1·02	4	90·1	6·34**	4	159·1	10·53**
Year	1	559·4	29·43**	1	1241·3	87·42**	2	1026·8	67·96**
Population × Year	4	49·8	2·62*	4	23·5	1·65	8	20·5	1·36
Genet	83	19·0	5·76**	85	14·2	2·97**	86	15·1	2·72**

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

Table 5. *Mean values and genetic variation (V_g) for each trait within the five populations from 2003 to 2005*

	Days to bud initiation						Days to flowering				Length of peduncle at the start of flowering			
	2003		2004		2005		2003		2004		2003		2004	
	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g
Hokkaido	13·1b	12·54	22·2a	25·34	44·7b	14·61	47·35a	10·48	53·30a	10·46	6·21a	1·68	7·71a	0·62
Saitama	9·3c	12·17	11·1b	9·39	27·3a	19·65	45·94ab	14·30	47·21b	22·80	5·44a	4·71	6·49a	7·00
Nagano 1	14·4b	9·67	21·4a	12·18	45·6b	23·48	44·04b	6·42	50·43ab	5·71	5·11a	2·03	6·34a	3·09
Nagano 2	19·8a	12·07	24·5a	16·72	49·1b	26·28	48·60a	9·20	51·35a	11·40	6·42a	3·75	6·81a	2·99
Nagano 3	12·1bc	7·55	24·5a	10·71	45·6b	23·07	43·16b	10·61	51·55ab	3·06	5·85a	1·03	7·31a	1·70

	Length of peduncle at the end of flowering				Number of flowers				Number of leaves					
	2003		2004		2003		2004		2003		2004		2005	
	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g	Mean	V_g
Hokkaido	10·40ab	2·28	8·54a	0·74	7·61c	3·04	5·01b	0·63	5·83b	2·46	5·11b	3·69	3·27b	0·80
Saitama	8·88b	5·24	8·56a	8·09	8·74bc	1·65	7·09a	3·02	9·72a	5·27	7·66a	4·91	4·91a	0·31
Nagano 1	10·18b	2·30	7·44a	4·13	10·73a	1·65	6·72a	0·27	8·17a	2·87	6·14ab	1·32	4·38a	1·05
Nagano 2	11·94a	4·66	7·60a	2·66	9·89ab	4·67	5·97ab	2·31	6·19b	0·00	5·84ab	0·26	3·44b	0·62
Nagano 3	9·46b	1·14	8·05a	3·05	8·33bc	1·96	5·45ab	2·00	7·24ab	6·97	5·35ab	1·72	3·15b	0·15

Means within a year followed by different letters differ significantly among populations ($P < 0.05$).

number of leaves showed the biggest variance among ramets and the smallest heritability (32·4–40·1).

(ii) *Genetic variation in quantitative traits within and among populations*

Of the total variance in the number of days to bud initiation, an average of about 45% was partitioned among the populations across the 3 years, and the variance among populations was larger than that among genets (Table 6). For the other five traits, the variance among genets was larger than that among populations. This reveals a high degree of genetic variation within the populations.

The degree of genetic differentiation among populations (Q_{ST}) ranged widely, from 0·004 to

Table 6. *Proportions (%) of the three variance components (among populations, genets, and ramets) calculated by means of REML, and the broad-sense heritability (h^2) values for the investigated traits from 2003 to 2005. Q_{ST} represents the degree of genetic differentiation among populations*

	Days to bud initiation			Days to flowering		Length of peduncle at the start of flowering		Length of peduncle at the end of flowering		Number of flowers		Number of leaves		
	2003	2004	2005	2003	2004	2003	2004	2003	2004	2003	2004	2003	2004	2005
Population	42·6	44·3	47·0	17·6	16·9	3·4	1·7	18·7	0·4	16·4	9·0	16·0	9·6	18·3
Genet	32·0	21·0	14·2	45·5	39·7	53·8	47·0	54·4	49·2	34·9	24·0	19·8	22·8	21·8
Ramet	25·3	34·6	38·8	36·9	43·4	42·9	51·2	26·9	50·3	48·7	66·9	64·2	67·6	59·8
h^2	74·6	65·3	61·2	63·1	56·6	57·2	48·7	73·1	49·6	51·3	33·0	35·8	32·4	40·1
Q_{ST}	0·400	0·513	0·623	0·162	0·175	0·031	0·018	0·147	0·004	0·190	0·158	0·288	0·174	0·296

Variance values may not add to 100% due to rounding errors.

0·623 (Table 6). The Q_{ST} values for the number of days to bud initiation were particularly large (0·400–0·623), indicating that this trait was considerably differentiated among the populations. Of the other five traits, Q_{ST} ranged from 0·004 for the length of the peduncle at the end of flowering in 2004 to 0·296 for the number of leaves in 2005, and these Q_{ST} values were clearly smaller than those for the number of days to bud initiation.

(iii) *Molecular versus quantitative genetic variation*

According to our SSR analysis, each locus was polymorphic, with 6–24 alleles per locus (Table 2), and we detected a total of 96 alleles over the eight loci. Mean H_e ranged from 0·604 to 0·751, mean n_{13a} on ranged from 4·03 to 6·63.

The F_{ST} value calculated using the data for the eight SSR markers was 0·172 (two-sided 95% confidence interval, 0·115–0·232). The average Q_{ST} values for the number of days to bud initiation and the number of leaves were considerably and slightly larger than F_{ST}, respectively (Fig. 1), whereas the average Q_{ST} values for the lengths of the peduncle at the start and end of flowering were smaller than F_{ST}. The average Q_{ST} for days to flowering and the numbers of flowers were approximately equal to F_{ST}.

Pairwise Q_{ST} and pairwise F_{ST} were not significantly correlated for any parameter. However, the pairwise Q_{ST} value for the number of days to bud initiation differed greatly between the Saitama population and the other four populations in 2004 and 2005 (Fig. 2). These results suggest that the Saitama population differed considerably from the other populations. While the pairwise Q_{ST} value for the number of leaves ranged widely from 0 to 1 and Saitama population differed from each population expect Nagano 1 population in 2005, this difference is not yet pronounced as the days to bud initiation (Fig. 2).
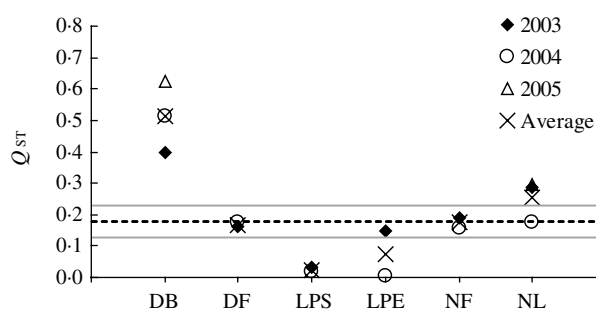

Fig. 1. Q_{ST} values for all traits in 2003, 2004 and 2005, and the F_{ST} value evaluated using the data for the eight microsatellite markers in Table 2. The grey lines represent the two-sided 95% confidence interval for F_{ST} (mean = 0·172, range = 0·115–0·232). Traits: DB, days to bud initiation; DF, days to flowering; LPS, length of the peduncle at the start of flowering; LPE, length of the peduncle at the end of flowering; NF, number of flowers; NL, number of leaves.

(iv) *Relationships between quantitative traits and ecological factors*

Because the average Q_{ST} values for the number of days to bud initiation and the number of leaves were larger than F_{ST} (Fig. 1), we examined the relationship between these parameters and the five ecological factors. The mean temperature of each month, from February to June, were significantly negatively correlated with the number of days to bud initiation in 2004 and 2005, and they were significantly positively correlated with the number of leaves in all 3 years (Fig. 3), suggesting that the differences in mean values of these parameters among populations correspond to differences in the mean temperature of each habitat. Latitude, longitude and altitude were not significantly correlated with the mean values of these two parameters, and geographical distance was not significantly correlated with the pairwise Q_{ST} for these two traits.
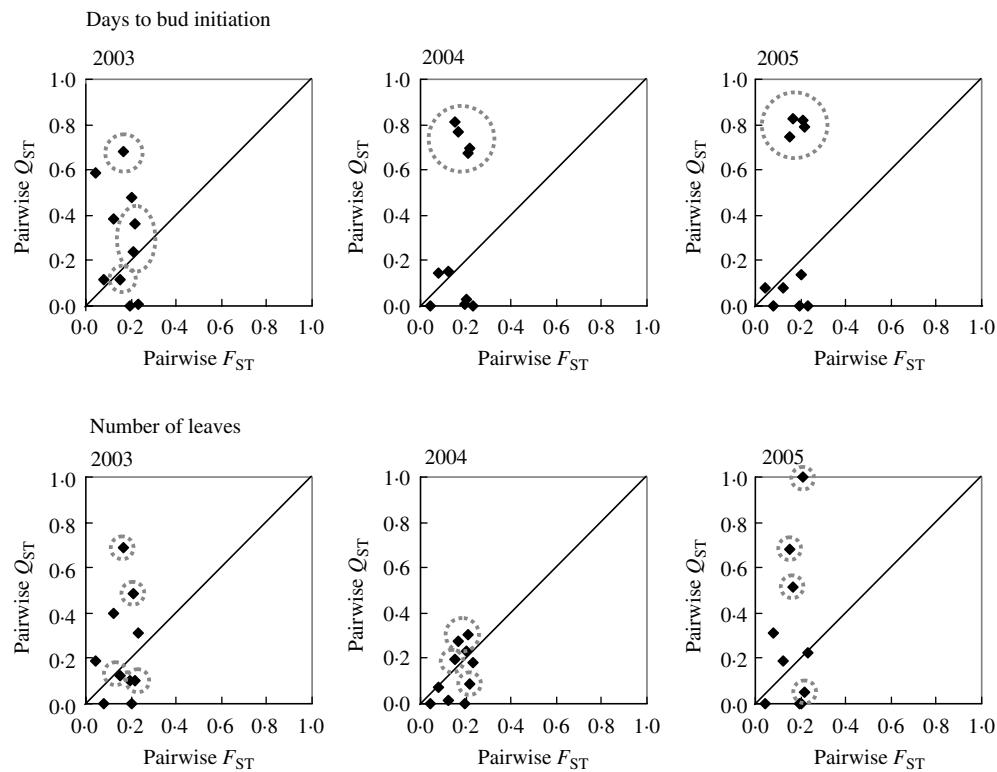
Fig. 2. Relationship between the pairwise Q_{ST} and pairwise F_{ST} values for days to bud initiation and the number of leaves. The data points surrounded by a dotted border represent the genetic distance between the Saitama population and the other four populations.
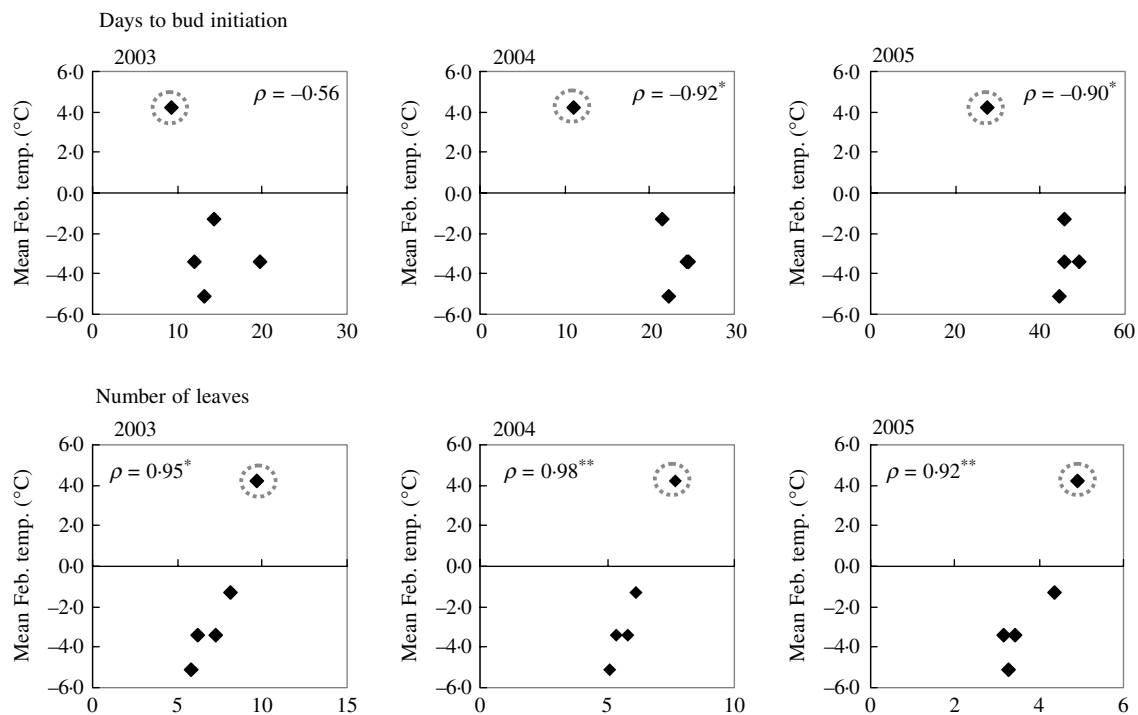

Fig. 3. The relationship between the mean temperature and the mean number of days to bud initiation and the mean number of leaves in 2003, 2004 and 2005. Because the relationships at each month were same patterns, only mean February temperature was shown. The data points surrounded by a dotted border represent the data of Saitama population. *$P<0.05$, **$P<0.01$.

5. Discussion

H_e and n_{13a} of five populations were compared to those of wild populations evaluated by Honjo *et al.* (2009), and genetic variation in the five populations is reflected in the mean variation that *P. sieboldii* have maintained in Japan.

(i) *What traits were adaptive in* P. sieboldii?

The lengths of the peduncle at the start and end of flowering did not differ significantly among populations (Table 4), although the length of the peduncle at the end of flowering varied among the years. The mean Q_{ST} was smaller than F_{ST} for both parameters (Fig. 1), suggesting that the length of the peduncle has not undergone directional selection and maintained mostly genetic variation within populations. However, the numbers of days to bud initiation, days to flowering, flowers, and leaves differed significantly among the populations (Table 4). The numbers of days to flowering and flowers appear to have become differentiated among populations mainly as a result of genetic drift because Q_{ST} was nearly equal to F_{ST} for these parameters (Fig. 1). Because high genetic diversity of these traits is maintained within the wild populations, Q_{ST} values were relatively low (Table 6). The mean Q_{ST} values for the number of days to bud initiation and the number of leaves were both larger than the corresponding F_{ST}. However, we do not necessarily conclude that the number of leaves is an adaptive trait from the result that this trait showed the smallest heritability in all years and is susceptible to environmental variation. Thus, the days to bud initiation is likely to be involved in adaptive differentiation among the populations as a result of directional selection.

The mean numbers of days to bud initiation in 2004 and 2005 were negatively correlated with the mean temperatures at each site (Fig. 3; $P < 0.05$), indicating that the mean temperature of the Saitama population, which appears to have differed from the other populations (Fig. 2), is considerably warmer (by more than 5 °C) than that of the other populations (Table 1). Thus, the winter temperature appears to be involved in determining the earliness of the budding date in *P. sieboldii*. In the Nagano region, which is located at the highest altitudes of the five study areas and showed correspondingly low temperatures, sudden late frost is often observed until the middle of May. Because *P. sieboldii* usually buds in early spring, genets with early budding are susceptible to frost injury and selection would favour genets with late budding in all except the Saitama population, where sudden late frosts are uncommon. In out-crossing forest trees such as Scots pine, *Populus* spp. and Douglas-fir, it has often been reported that traits related to budding, such as bud set and bud flush dates, are adaptive traits that are closely related to winter temperatures (Hurme *et al.*, 1997; Frewen *et al.*, 2000; Howe *et al.*, 2003).

Positive relationships between bud flush and cold hardiness have been reported, and their quantitative trait loci (QTLs) were detected at the same position as a result of pleiotropy or strong linkage (Howe *et al.*, 2003). Winter chilling, spring temperatures and frost are factors capable of controlling the timing of bud flush (Jermstad *et al.*, 2003). Thus, low temperatures in early spring appear to act as a selective pressure that selects genets with late budding in the three Nagano populations and the Hokkaido population. In contrast, genets with early budding appear to have been selected in the Saitama population. Frewen *et al.* (2000) noted that trees in which bud flush occurs too late in the spring have a shortened growing season that reduces their competitive ability and growth potential. Thus, temperature appears to be a selective factor involved in local adaptation, and phenotypic fixation of the budding date has occurred as a result of the selection of optimal genotypes in each habitat.

(ii) *Defining conservation units for* P. sieboldii *based on adaptive and neutral genetic markers*

Honjo *et al.* (2009) defined four *P. sieboldii* conservation units (Hokkaido, northern Honshu, central Honshu and western Japan) using eight microsatellite markers and cpDNA. Based on those results, the Saitama population and the three Nagano populations in the present study belonged to the same conservation unit, i.e. central Honshu, and only the Hokkaido population belonged to a different conservation unit. However, our budding date results suggest that the Saitama population differed considerably from the other populations. Therefore, the Saitama population should represent different conservation units. Among these Nagano populations, Nagano 2 differed significantly in the budding date from Nagano 1 and Nagano 3 in 2003 (Table 5). Therefore, further study is required to exploit the genetic basis of such differences that may be because of difference in the conservation unit.

As a result, the five populations in the present study could be divided into three conservation units previously proposed by Honjo *et al.* (2009) as well as at least one new conservation unit based on our analysis of both their adaptive traits and molecular markers. These results clearly demonstrate the importance of defining conservation units based on adaptive diversity, not just molecular genetic diversity.

Hypothetically speaking, if transplantation of genets is necessary to maintain remnant populations of *P. sieboldii*, it should be guided by a careful consideration of the genetic composition of the genets in

terms of the budding date, which represents an important adaptation to environmental conditions. The introduction of foreign genotypes that have adapted to a different environment can potentially disrupt the genetic architecture of native populations, which is optimal for long-term existence of these populations in their native habitat. This phenomenon has been called 'outbreeding depression' and has frequently been reported for some species (Keller *et al.*, 2000; Montalvo & Ellstrand, 2001). For *P. sieboldii*, the temperature during the budding stage appears to be a strong selection force for the budding date. Therefore, it should be possible to roughly estimate the timing of budding using the mean February temperature at a genet's native habitat. Wild genets or populations used for transplantation between regions should be selected based on the temperature during the budding stage, not based solely on geographical distance.

References

Bekessy, S. A., Ennos, R. A., Burgman, M. A., Newton, A. C. & Ades, P. K. (2003). Neutral DNA markers fail to detect genetic divergence in an ecologically important trait. *Biological Conservation* **110**, 267–275.

Cano, J. M., Mäkinen, H. S., Leinonen, T., Freyhof, J. & Merilä, J. (2008). Extreme neutral genetic and morphological divergence supports classification of Adriatic three-spined stickleback (*Gasterosteus aculeatus*) populations as distinct conservation units. *Biological Conservation* **141**, 1055–1066.

Carvajal-Rodriguez, A., Rolan-Alvarez, E. & Caballero, A. (2005). Quantitative variation as a tool for detecting human-induced impacts on genetic diversity. *Biological Conservation* **124**, 1–13.

Clausen, D. J., Keck, D. D. & Hiesey, W. M. (1940). Experimental studies on the nature of species. I. Effect of Varied Environments on Western North American Plants. Washington, DC: Carnegie Institute of Washington Publications. No. 520.

Crandall, K. A., Bininda-Emonds, O. R. P., Mace, G. M. & Wayne, R. K. (2000). Considering evolutionary processes in conservation biology. *Trends in Ecology and Evolution* **15**, 290–295.

de Guia, A. P. O. & Saitoh, T. (2007). The gap between the concept and definitions in the evolutionarily significant unit: the need to integrate neutral genetic variation and adaptive variation. *Ecological Research* **22**, 604–612.

Denoël, M. (2007). Priority areas of intraspecific diversity: Larzac, a global hotspot for facultative paedomorphosis in amphibians. *Animal Conservation* **10**, 110–116.

Falconer, D. S. (1981). *Introduction to Quantitative Genetics*, 2nd edn. London, UK: Longman.

Frankham, R., Ballou, J. D. & Briscoe, D. A. (2002). *Introduction to Conservation Genetics*. New York, NY: Cambridge University Press.

Fraser, R. & Bernatchez, L. (2001). Adaptive evolutionary conservation: towards a unified concept for defining conservation units. *Molecular Ecology* **10**, 2741–2752.

Frewen, B. E., Chen, T. H. H., Howe, G. T., Davis, J., Rohde, A., Boerjan, W. & Bradshaw, H. D. Jr (2000). Quantitative trait loci and candidate gene mapping of bud set and bud flush in *Populus*. *Genetics* **154**, 837–845.

Gordon, D. R. & Rice, K. J. (1998). Patterns of differentiation in Wiregrass (*Aristida beyrichiana*): Implications for restoration effects. *Restoration Ecology* **6**, 166–174.

Goudet, J. (2001) FSTAT, A Program to Estimate and Test Gene Diversities and Fixation Indices (version 2.9.3). Available from http://www2.unil.ch/popgen/softwares/fstat.htm

Haig, S. M. (1998). Molecular contributions to conservation. *Ecology* **79**, 413–425.

Holderegger, R., Kamm, U. & Gugerli, F. (2006). Adaptive vs. neutral genetic diversity: implications for landscape genetics. *Landscape Ecology* **21**, 797–807.

Honjo, M. (2005). Molecular ecological genetics for the conservation of genetic diversity in *Primula sieboldii*. Doctoral dissertation, University of Tsukuba (in Japanese).

Honjo, M., Kitamoto, N., Ueno, S., Tsumura, Y., Washitani, I. & Ohsawa, R. (2009). Management units of the endangered herb *Primula sieboldii* based on microsatellite variation among and within populations throughout Japan. *Conservation Genetics* **10**, 257–267.

Honjo, M., Ueno, S., Tsumura, Y., Handa, T., Washitani, I. & Ohsawa, R. (2008). Tracing the origins of stocks of the endangered species *Primula sieboldii* using nuclear microsatellites and chloroplast DNA. *Conservation Genetics* **9**, 1139–1147.

Honjo, M., Ueno, S., Tsumura, Y., Washitani, I. & Ohsawa, R. (2004). Phylogeographic study based on intraspecific sequence variation of chloroplast DNA for the conservation of genetic diversity in the Japanese endangered species *Primula sieboldii*. *Biological Conservation* **120**, 215–224.

Howe, G. T., Aitken, S. N., Neale, D. B., Jermstad, K. D., Wheeler, N. C. & Chen, T. H. H. (2003). From genotype to phenotype: unraveling the complexities of cold adaptation in forest trees. *Canadian Journal of Botany* **81**, 1247–1266.

Hurme, P., Repo, T., Savolainen, O. & Pääkkönen, T. (1997). Climatic adaptation of bud set and frost hardiness in Scots pine (*Pinus sylvestris*). *Canadian Journal of Forest Research* **27**, 716–723.

Isagi, Y., Honjo, M. & Washitani, I. (2001). Development of microsatellite markers for *Primula sieboldii* using degenerate oligonucleotide-primed PCR-amplified DNA. *Molecular Ecology Notes* **1**, 22–24.

Iwatsuki, K. (2008). Endangered vascular plants in Japan – present status and a proposal for conservation. *Proceedings of the Japan Academy, Series B, Physical and Biological Sciences* **84**, 275–286.

Jermstad, K. D., Bassoni, D. L., Jech, K. S., Ritchie, G. A., Wheeler, N. C. & Neale, D. B. (2003). Mapping of quantitative trait loci controlling adaptive traits in coastal Douglas Fir. III. Quantitative trait loci-by-environmental interactions. *Genetics* **165**, 1489–1506.

Karhu, A., Hurme, P., Karjalainen, M., Karvonen, P., Kärkkäinen, K., Neale, D. & Savolainen, O. (1996). Do molecular markers reflect patterns of differentiation in adaptive traits of conifers? *Theoretical and Applied Genetics* **96**, 215–221.

Keller, M., Kollmann, J. & Edwards, P. J. (2000). Genetic introgression from distant provenances reduces fitness in

local weed populations. *Journal of Applied Ecology* **37**, 647–659.

Kitamoto, N., Honjo, M., Ueno, S., Takenaka, A., Tsumura, Y., Washitani, I. & Ohsawa, R. (2005). Spatial genetic structure among and within populations of *Primula sieboldii* growing beside separate streams. *Molecular Ecology* **14**, 149–157.

Leinonen, T., O'Hara, R. B., Cano, J. M. & Merilä, J. (2008). Comparative studies of quantitative trait and neutral marker divergence: a meta-analysis. *Journal of Evolutionary Biology* **21**, 1–17.

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**, 209–220.

Mckay, J. K., Bishop, J. G., Lin, J.-Z., Richards, J. H., Sala, A. & Mitchell-Olds, T. (2001). Local adaptation across a climatic gradient despite small effective population size in the rare sapphire rockcress. *Proceedings of the Royal Society London B* **268**, 1715–1721.

McKay, J. K. & Latta, R. J. (2002). Adaptive population divergence: markers, QTL and traits. *Trends in Ecology and Evolution* **17**, 285–291.

Merilä, J. & Crnokrak, P. (2001). Comparison of genetic differentiation at marker loci and quantitative traits. *Journal of Evolutionary Biology* **14**, 892–903.

Montalvo, A. M. & Ellstrand, N. C. (2001). Nonlocal transplantation and outbreeding depression in the subshrub *Lotus scoparius* (Fabaceae). *American Journal of Botany* **88**, 258–269.

Moritz, C. S. (1994). Defining 'evolutionarily significant units' for conservation. *Trends in Ecology and Evolution* **9**, 373–375.

Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321–4325.

Nei, M. & Roychoudhury, A. K. (1974). Sampling variance of heterozygosity and genetic distance. *Genetics* **76**, 379–390.

Reed, D. H. & Frankham, R. (2001). How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. *Evolution* **55**, 1095–1103.

Spitze, K. (1993). Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. *Genetics* **135**, 367–374.

Storfer, A. (1996). Quantitative genetics: a promising approach for the assessment of genetic variation in endangered species. *Trends in Ecology and Evolution* **11**, 343–348.

Ueno, S., Tsumura, Y. & Washitani, I. (2003). Development of microsatellite markers in *Primula sieboldii* E. Morren, a threatened Japanese perennial herb. *Conservation Genetics* **4**, 809–811.

Ueno, S., Kitamoto, N., Ohsawa, R., Tsumura, Y. & Washitani, I. (2005). Nine additional microsatellite markers for *Primula sieboldii* E. Morren. *Conservation Genetics* **6**, 1063–1064.

Ueno, S., Yoshida, Y., Taguchi, Y., Honjo, M., Kitamoto, N., Washitani, I., Ohsawa, R. & Tsumura, Y. (2009). Development of 120 microsatellite markers for *Primula sieboldii* E. Morren for linkage mapping. *Conservation Genetics* DOI 10.1007/S10592-009-9861-9.

Washitani, I. & Kabaya, H. (1988). Germination responses to temperature responsible for the seedling emergence seasonality of *Primula sieboldii* E. Morren in its natural habitat. *Ecological Research* **3**, 9–20.

Weir, B. S. & Cockerham, C. C. (1984). Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370.

Yamazaki, T. (1993). *Primula*. In *Flora of Japan*, vol. IIIa (ed. I. Kunio, Y. Takashi, B. E. David & O. Hideaki), pp. 87–94. Tokyo: Kodansha. (in Japanese).

Yoshida, Y., Honjo, M., Kitamoto, N. & Ohsawa, R. (2008). Genetic variation and differentiation of floral morphology in wild *Primula sieboldii* evaluated by image analysis and SSR markers. *Breeding Science* **58**, 301–307.