Conserv Genet (2009) 10:257–267
DOI 10.1007/s10592-007-9292-4

ORIGINAL RESEARCH ARTICLE

Management units of the endangered herb *Primula sieboldii* based on microsatellite variation among and within populations throughout Japan

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Received: 27 July 2006 / Accepted: 12 January 2007 / Published online: 10 February 2007

Abstract To promote programs for the conservation and restoration of the endangered species *Primula sieboldii*, we examined genetic variation at eight microsatellite loci among and within 32 remnant wild populations throughout Japan. Total allelic diversity within a population was higher in larger populations, but not so after rarefaction adjustment. The positive relationship between population size and the inbreeding coefficient may suggest that more heterozygous genets tend to survive the habitat contraction possibly because of the higher fitness associated with heterozygosity. By principal coordinate analysis and Bayesian analysis, we detected four genetic groups (Hokkaido, northern Honshu, central Honshu, and western Japan), which could be recognized as management units of *P. sieboldii*. If supplementation with plants from other populations were planned, it should be conducted among populations which belong to the same management unit and which are likely to represent the same adaptive variation.

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Keywords Chloroplast DNA (cpDNA) · Conservation unit · Genetic differentiation · Genetic rescue · Restoration

Introduction

Declining plant populations often face an increased short-term risk of extinction because of reduced reproduction due to pollen limitation and inbreeding depression, and an increased long-term risk due to decreased ability to respond to environmental changes (Kery et al. 2000; Frankham et al. 2002). Restoration activities, including introduction of individuals (Demauro 1993; Robichaux et al. 1997; Hogbin and Peakall 1999), can result in recovery of fitness or genetic rescue, but risk jeopardizing evolutionary distinctiveness and the ecological viability of populations owing to genetic swamping by introduced genotypes and outbreeding depression (Hufford and Mazer 2003; Hedrick 2005). Therefore, if introduction is planned, it is desirable to do that among populations representing similar ecotypic forms (Crandall et al. 2000) and belonging to the same lineage or management unit (Moritz 1994, 2002), which is a group of populations having a statistically significant differences of allelic composition at nuclear or organelle loci from others.

Several reviews of the relationship between molecular and quantitative variation suggest that neutral loci often do not match patterns in ecologically important traits (Reed and Frankham 2001; Mckay and Latta 2002). Hence, we should evaluate historical distinctiveness of populations and adaptive divergence by appropriate genetic markers (Fraser

and Bernatchez 2001). Molecular marker assay enables us to estimate the degree of genetic connectivity among populations via gene flow and to detect endemic lineages that are the component of biodiversity (Newton et al. 1999; Soltis and Gitzendanner 1999; Gompert et al. 2006). Saltonstall (2002) revealed the extinction of local lineages of common reed (*Phragmites australis*) in North America by the cryptic invasion of lineages from Eurasia by using chloroplast DNA (cpDNA) variation. The investigation of heritable phenotypic variation enables the estimation of local adaptation and outbreeding depression (Montalvo and Ellstrand 2001; Hufford and Mazer 2003). Further, comparison of the degree of genetic differentiation among populations in quantitative traits (Q_{ST}) to that in neutral markers (F_{ST}) allows the evaluation of local adaptation caused by natural selection (Spitze 1993).

Primula sieboldii E. Morren (Primulaceae) is a perennial heterostylous herb that is distributed throughout Japan. In recent years, natural *P. sieboldii* populations have declined owing to loss or fragmentation of habitats, abandonment of traditional management of woodlands and grasslands, and commercial collection (Environment Agency of Japan 2000). In this context, restoration activities led by private citizens have been growing in a number of regions. Because of the failure of seed production due to the loss of either of the two heterostylous morphs (Matsumura and Washitani 2000) and to strong inbreeding depression (Kitamoto 2005; Nagai et al. 2006), the introduction of individuals may ensure population viability, as well as the abovementioned risk. Hence, it is urgent to characterize the genetic variation in *P. sieboldii* to ensure effective genetic conservation.

Honjo et al. (2004) examined the phylogeography of the species in Japan on the basis of the sequence variation in five noncoding regions of cpDNA. Twenty-two haplotypes belonging to three distinct clades were recognized, with some geographic structure. Furthermore, the use of nuclear genetic markers gives insight into genetic structure reflecting gene flow by pollen and seed among populations.

We consider that conservation units of a species should be determined to conserve both evolutionary distinctiveness and ecological viability of populations. The purpose of this study is to delineate the management units of *P. sieboldii* in Japan as the first step in determining conservation units. We examined microsatellite variation among and within 32 remnant wild populations throughout Japan. The geographical patterns are interpreted jointly with previously reported cpDNA data.

Materials and methods

Study species and sampling procedure

Primula sieboldii is a heterostylous clonal plant that occurs in moist habitats in northeastern Asia (Yamazaki 1993). Each genet is composed of physiologically independent ramets originating from short rhizomes. A main pollinator is the queen bumblebee, *Bombus diversus* (Washitani et al. 1995), and seed dispersal occurs by gravity. Secondary dispersal of seed and clonal propagules by water sometimes occur (Kitamoto et al. 2005a).

In Japan, *P. sieboldii* is distributed in five regions: southern Hokkaido, northern, central, and western Honshu, and central Kyushu. At flowering time (April–June) in 2000–2003, we collected fresh leaves from 32 populations (Table 1; Fig. 1), representative of the remnant populations in each region. In the Hidaka region of southern Hokkaido and the Karuizawa region of central Honshu, leaves were collected from eight and seven populations, respectively, within a radius of 10 km. For samples of Tajimagahara (population 16), we used plants grown from seedlings obtained in a previous study of *P. sieboldii* (Washitani and Kabaya 1988). To understand genetic diversity retained in remnant small populations precisely, we collected leaves from as many genets as possible in smaller populations consisting of less than 50 genets, while from randomly chosen genets in larger populations. The sample size was 920 genets in total, and ranged from 10 to 49 per population. Although we collected leaves by distinguishing genets in the field by visual observation of interclonal variations in floral morphology (i.e., size, color, shape of petal, and heterostyly; Washitani et al. 1991) and the spatial arrangement of ramets, we found several replicate multilocus genotypes within samples from the same population in the following genotyping experiment. Because those pairs of individuals were grown adjacently in each habitat and considered to belong to the same genet, we excluded the overlapped samples from the data analysis. The above-mentioned number of samples, 920, corresponds to the number of a distinct multilocus genotype.

DNA extraction and PCR amplification

Genomic DNA was extracted from leaves by a modified CTAB method (Murray and Thompson 1980). Genotypes of the samples were amplified by PCR with eight pairs of microsatellite primers (Table 2). PCR amplifications were performed in 10 μl reaction mix-

Table 1 Locality, size, and genetic diversity measurements of each sampled population of *Primula sieboldii* in Japan

Population	Population name	Locality	Population size	No. of samples examined	H_e	n_a	n_{10a}	n_{ra}	n_{10ra}	F_{IS}	Clade (haplotype)
1	Mukawa4	Southern Hokkaido	–	21	0.655	4.63	4.08	1	0.48	–0.047	II (B)
2	Monbetsu1	Southern Hokkaido	150	29	0.597	4.13	3.68	0	0.00	–0.054	II (A)
3	Monbetsu3	Southern Hokkaido	870	33	0.625	4.38	3.79	0	0.00	0.025	II (A)
4	Monbetsu5	Southern Hokkaido	140	27	0.611	4.88	3.96	4	1.48	–0.085	II (A)
5	Monbetsu7	Southern Hokkaido	130	32	0.637	4.38	3.83	0	0.00	0.038	II (A)
6	Monbetsu8	Southern Hokkaido	300	34	0.650	5.63	4.43	2	0.80	–0.071	II (A)
7	Monbetsu9	Southern Hokkaido	104	29	0.684	5.50	4.53	3	1.85	–0.001	II (A, B)
8	Shizunai	Southern Hokkaido	25	25	0.476	3.25	2.95	0	0.00	0.014	III (C)
9	Hachinohe	Northern Honshu, Aomori	31	28	0.628	4.25	3.82	4	2.93	0.033	III (X[a])
10	Morioka	Northern Honshu, Iwate	150	44	0.554	4.88	3.85	3	1.25	–0.061	III (C)
11	Sendai	Northern Honshu, Miyagi	18	18	0.420	2.88	2.73	0	0.00	–0.110	III (C)
12	Souma	Northern Honshu, Fukushima	130	49	0.646	4.25	3.74	2	1.19	–0.019	I (E)
13	Nasu	Northern Honshu, Tochigi	150	30	0.620	5.75	4.73	8	5.36	0.129	III (C)
14	Akagi	Central Honshu, Gunma	–	27	0.531	3.75	3.45	2	1.84	–0.136	III (C)
15	Ageo	Central Honshu, Saitama	10	10	0.716	6.13	6.13	3	3.00	–0.013	I (F, H, N, λ[a]), III (C)
16	Tajimagahara	Central Honshu, Saitama	–	34	0.656	7.13	5.15	9	4.82	–0.037	I (E, H, I), III (C)
17	Gunma4	Central Honshu, Gunma	125	30	0.718	6.63	5.13	7	4.78	–0.064	I (H)
18	Karuizawa2	Central Honshu, Nagano	16	14	0.748	6.63	5.94	6	4.77	–0.114	I (J, K)
19	Karuizawa7	Central Honshu, Nagano	180	38	0.753	8.50	5.95	11	3.93	–0.071	I (E, F, K), III (C)
20	Karuizawa8	Central Honshu, Nagano	370	40	0.763	9.13	6.27	10	3.26	0.001	I (E, F)
21	Karuizawa9	Central Honshu, Nagano	28	27	0.750	7.13	5.82	4	2.44	–0.026	III (C)
22	Saku1	Central Honshu, Nagano	25	24	0.645	5.25	4.24	4	2.50	–0.051	I (L)
23	Saku2	Central Honshu, Nagano	100	30	0.636	5.75	4.54	7	3.53	0.051	I (L)
24	Yatsugatake5	Central Honshu, Nagano	77	28	0.595	5.75	4.51	6	3.24	–0.006	I (N), III (O)
25	Shinanomachi1	Central Honshu, Nagano	145	38	0.640	5.88	4.47	4	2.77	–0.013	I (E)
26	Takayama	Central Honshu, Gifu	42	38	0.587	4.75	4.02	1	0.46	–0.065	III (C)
27	Hiruzen	Western Honshu, Okayama	17	17	0.672	6.38	5.40	6	4.52	–0.006	II (A, B, R)
28	Kofu	Western Honshu, Tottori	14	14	0.613	4.25	4.03	3	2.43	–0.157	II (B)
29	Dougoyama1	Western Honshu, Hiroshima	36	28	0.714	5.75	4.63	3	1.46	0.038	II (B)
30	Mirasaka1	Western Honshu, Hiroshima	10	10	0.697	4.88	4.88	10	10.00	–0.042	II (B, S, T)
31	Geihoku	Western Honshu, Hiroshima	35	35	0.701	5.50	4.50	4	2.27	–0.081	I (Y[a])
32	Aso	Central Kyushu, Kumamoto	940	39	0.786	9.13	6.47	14	8.72	0.092	I (U, V)

Population names correspond to Honjo et al. (2004). Genetic diversity within a population was evaluated by unbiased estimates of gene diversity averaged over loci (H_e), average number of alleles per locus (n_a), the expected number of alleles observed in ten genets of each population (n_{10a}), summed number of rare alleles whose frequency was less than 1% among all populations (n_{ra}), the expected number of rare alleles in ten genets (n_{10ra}), and mean inbreeding coefficient (F_{IS}). Chloroplast DNA haplotypes and clades are based on Honjo et al. (2004)

–, indicates lack of data

[a] Haplotypes not reported in Honjo et al. (2004)

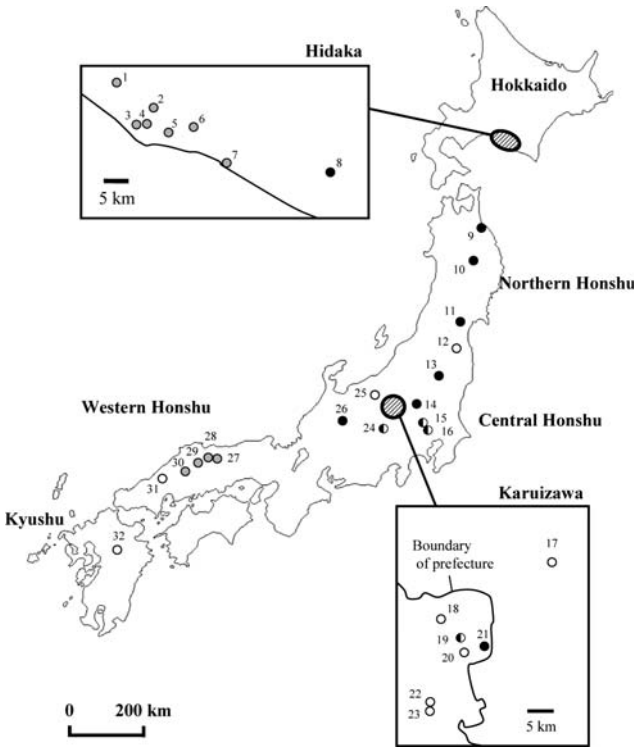

Fig. 1 Sampling locations. Numbers correspond to population numbers in Table 1. The colors of plots represent the clades to which the genets within the population belong, based on chloroplast DNA sequences (Honjo et al. 2004): white, clade I; gray, clade II; black, clade III

tures containing 10–15 ng template DNA, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 100 μM each dNTP, 0.02% Triton X-100, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.25 units *Taq* DNA polymerase (Promega, Madison, USA), and 0.2 μM each primer. Thermocycling conditions were as follows: 3 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at a primer-specific annealing temperature (Table 2), and 30 s at 72°C; with a final extension step at 72°C for 7 min. The PCR was carried out with a GeneAmp PCR System Model 9700 (Applied Biosystems, Foster City, USA). The PCR products were run on a 3100 Genetic Analyzer with GeneScan (Applied Biosystems).

Table 2 Microsatellite markers used in the analysis of *P. sieboldii* in Japan, annealing temperature, and the total number of alleles observed

Locus	Annealing temperature (°C)	No. of alleles	References
ga0235	60	19	Ueno et al. (2003)
ga0381	57	20	Ueno et al. (2003)
ga0653	60	12	Ueno et al. (2005)
ga0666	55	38	Ueno et al. (2005)
ga0668	52	10	Ueno et al. (2003)
ga1277	60	27	Ueno et al. (2003)
Pri0146	60	22	Kitamoto et al. (2005b)
PS2	55	12	Isagi et al. (2001)

Data analysis

To reveal the microsatellite diversity within each population, we obtained unbiased estimates of gene diversity averaged over loci (H_e; Nei and Roychoudhury 1974), average number of alleles per locus (n_a), and summed number of rare alleles over loci (n_{ra}), whose frequency was less than 1% among all populations. We also computed allelic richness (El Mousadik and Petit 1996), that is, the expected number of alleles (n_{10a}) and rare alleles (n_{10ra}) observed in 10 genets of each population (the smallest sample size). To infer the level of inbreeding in each population, we computed the mean inbreeding coefficient (F_{IS}). The significance of F_{IS} in terms of departure from random mating at the $P < 0.05$ level was tested by 5120 permutations of alleles among individuals within populations, with Bonferroni correction. This analysis also indicates the significance of the departure from Hardy–Weinberg equilibrium per locus and population. These calculations were performed with the aid of the software FSTAT 2.9.3 (Goudet 2001), except H_e, which was calculated by the program Arlequin 2.001 (Schneider et al. 2000). We examined the relationship between genetic diversity within populations (H_e, n_a, n_{ra}, n_{10a}, and n_{10ra}) and the logarithmic population size (total number of genets within a population) and latitude by multiple regression analyses. The latitude of each population was transformed into the plane orthogonal coordinate before analysis. The relationship between population size and latitude was examined by correlation analysis. We also examined the relationship between F_{IS} and population size or latitude by simple regression analysis. In all these analyses, we omitted Mukawa 4, Tajimagahara, and Akagi (populations 1, 14, 16), where we were unable to count the number of genets owing to the partial destruction of habitat by road construction, restricted access, and sampling before sufficient blooming.

Levels of microsatellite variation among and within the five geographical regions were estimated by hierarchical *F*-statistics (Yang 1998) using the software HIERFSTAT (Goudet 2005). Genetic divergence between each population pair was estimated by calculating population pairwise F_{ST} (Weir and Cockerham 1984) using FSTAT 2.9.3. To test the significance of correlation between geographic distance and pairwise F_{ST} values among all pairs of populations, a Mantel test (Mantel 1967) was conducted using FSTAT 2.9.3. The

genetic similarity ($1 - F_{ST}$) matrix was used in principal coordinate analysis (PCO) to extract the association of populations by using the software PCO 1.0 (Iwata 2004).

We also examined genetic clustering within species by using a Bayesian clustering method implemented in the program STRUCTURE 2.1 (Pritchard et al. 2000; Pritchard and Wen 2003), which estimates the number of clusters (K) present in a sample without the geographical locations of individuals and then assigns them to these clusters. We first ran the program for the total data set ($n = 920$) assuming correlated allele frequencies and admixture. We calculated ten independent runs of $K = 1$–20 with 10^5 MCMC cycles each for burn-in and data collection. Then, to detect the true number of clusters, we calculated ΔK (Evanno et al. 2005), which is based on the rate of change in posterior probability values for K [log probability of data; $L(K)$] between successive K values. Evanno et al. (2005) showed that the real number of clusters is best detected by the modal value of ΔK rather than $L(K)$ itself. They also showed that STRUCTURE tends to detect the uppermost level of a population hierarchy, and that within these clusters there can be sublevels of structuring. Therefore, we also explored whether substructure could be detected within each detected cluster in a similar manner to the first run (but $K = 1$–10).

Results

Genetic diversity within populations

All loci showed high polymorphism, with the number of alleles per locus ranging from 10 (*ga0668*) to 38 (*ga0666*; Table 2). In total, 160 alleles were detected in 920 distinct genets from 32 populations of *P. sieboldii* across eight loci. Genetic diversity measurements within each population, H_e, n_a, n_{ra}, n_{10a}, and n_{10ra} ranged from 0.420 to 0.786, 2.88 to 9.13, 0 to 14, 2.72 to 6.47, and 0 to 10, respectively (Table 1). Sendai (population 11) in northern Honshu showed the lowest value of every measurement. Four populations in Hokkaido (populations 2, 3, 5, 8) also do not have any rare alleles as well as Sendai. On the other hand, Aso (population 32) in southwestern Japan showed the highest or second highest values for all measurements. The mean inbreeding coefficient (F_{IS}) ranged from –0.157 of Kofu (population 28) to 0.129 of Nasu (population 13).

In the multiple regression analyses, five genetic diversity measurements (H_e, n_a, n_{ra}, n_{10a}, and n_{10ra}) were significantly correlated with latitude (Table 3). That is, all measurements, especially the number of rare alleles, significantly decreased with increasing latitude. Also, H_e, n_a, and n_{ra} were significantly correlated with population size. These measurements increased with increasing population size. On the other hand, n_{10a} and n_{10ra} were not correlated with population size (Table 3), indicating that allelic diversity in the same number of genets was not dependent on population size. Population size and latitude were not significantly correlated ($P = 0.11$). In the simple linear regression analysis, population size and F_{IS} were positively correlated ($P < 0.05$; Fig. 2), indicating an excess of heterozygotes in a smaller population. Latitude and F_{IS} was not related ($P = 0.91$). The tests for significance of F_{IS} showed that individual populations did not deviate significantly from Hardy–Weinberg equilibrium after Bonferroni correction.

Genetic differentiation among populations

According to hierarchical F-statistics, 10.0% of the total microsatellite variation occurred among regions, 12.1% among populations within regions, and 77.9% within populations. Population pairwise F_{ST} ranged from 0.002 to 0.430 (Table 4). The Mantel test revealed a significant correlation ($r = 0.691$, $P < 0.0001$) between pairwise F_{ST} values and the logarithm of geographic distances (Fig. 3).

PCO analyses of all 32 populations detected four genetic groups, corresponding to the geographic regions (Fig. 4). The first axis (PCO1) accounted for 22.0% of the variation and separated the Hokkaido populations from the more southern populations. Along the PCO2 axis, which accounted for 12.4% of the variation, the populations from western Japan (Kyushu and western Honshu), central Honshu, and northern Honshu were arranged in geographic order.

Table 3 Results of multiple regression analyses with genetic diversity measurements as the dependent variable and population size (pop. size) and latitude as the independent variables

Genetic diversity measurement	R^2	P	Independent variable	r	P
H_e	0.331	<0.01	Pop. size	0.349	<0.05
			Latitude	–0.575	<0.01
n_a	0.563	<0.001	Pop. size	0.561	<0.001
			Latitude	–0.697	<0.001
n_{ra}	0.563	<0.001	Pop. size	0.435	<0.01
			Latitude	–0.758	<0.001
n_{10a}	0.430	<0.001	Pop. size	0.299	0.066
			Latitude	–0.681	<0.001
n_{10ra}	0.427	<0.001	Pop. size	0.139	0.381
			Latitude	–0.682	<0.001

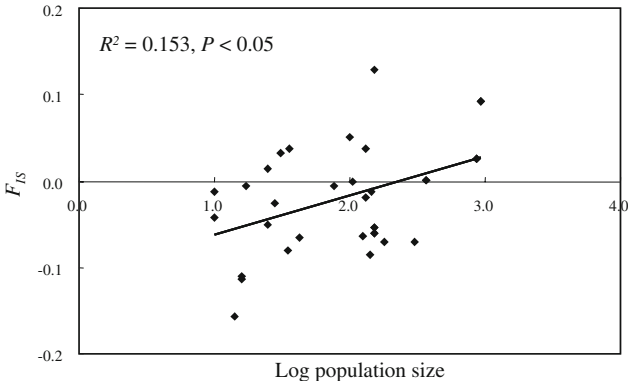

Fig. 2 Relationship between log population size and the inbreeding coefficient F_{IS} in populations of *P. sieboldii* in Japan

In the STRUCTURE analysis of the total dataset, the ΔK statistic clearly indicated that the sample included at least two distinct groups (Fig. 5A, B), though the log probability of data L (K) still increased after $K = 2$. Nearly all individuals from Honshu and Kyushu were assigned to cluster 1 and individuals from Hokkaido to cluster 2 (Fig. 6A). We also explored whether substructure could be detected within the clusters. Cluster 1 was split into three subclusters (1a, 1b, 1c; Figs. 5C, D, 6B), consisting of individuals mainly from western Japan, central Honshu, and northern Honshu, respectively, corresponding well to the geographic and PCO patterns (Fig. 4) except for Akagi (population 14) and Takayama (population 26). Most individuals of Akagi, which is located most proximal to northern Honshu among populations in central Honshu and was plotted in central Honshu group in PCO analysis, were assigned to cluster 1c. In Takayama, contrary to geographical location, a high rate of assignment to cluster 1c was detected, although the population was plotted in the PCO analysis by geographical location (namely, plotted between western Japan populations and central Honshu populations).

Discussion

Genetic diversity within populations

All microsatellite diversity measurements within a population were negatively correlated with latitude (Table 3). This result corresponds to the previous cpDNA analysis of *P. sieboldii* (Honjo et al. 2004), which revealed that only a few lineages might have expanded northward. Similar decrease of genetic diversity has been observed in some temperate plant species in Japan (Tomaru et al. 1997, 1998; Ohi et al. 2003). The Karuizawa region, where high haplotype diversity was observed (Honjo et al. 2004), also showed high microsatellite diversity (Table 1). These results suggest that Karuizawa was a glacial refugium or that it was a region of admixture of populations descended from distinct refugia (Walter and Epperson 2001).

Total allelic diversity within a population (n_a, n_{ra}) was higher in larger populations, but not so after rarefaction adjustment (n_{10a}, n_{10ra}; Table 3). Looking at the relationship between population size and F_{IS}, an excess of heterozygotes was observed in smaller populations (Fig. 2). Although this might be the result of demographic stochasticity because the relationship was relatively weak, it may suggest that more heterozygous genets tend to survive the habitat contraction possibly because of the higher fitness associated with heterozygosity as observed in several long-lived perennials (Schaal and Levin 1976; Rajimann et al. 1994; Oostermeijer et al. 1995; Luijten et al. 2000). The latter case may stem genetic decline of populations. Although further investigation is needed to determine whether more heterozygous genets have higher fitness or not, the purge of homozygosity of recessive detrimental genes may be likely, as strong inbreeding depression was observed in seed production, in germination, and in growth of seedlings of *P. sieboldii* (Kitamoto 2005; Nagai et al. 2006).

Genetic differentiation among populations

The data from different markers provide valuable information for understanding the evolutionary relationships of populations and identifying the appropriate units of biodiversity for conservation purposes. Previous cpDNA analysis (Honjo et al. 2004) did not detect obvious genetic differentiation between Hokkaido and Honshu populations of *P. sieboldii*. All three cpDNA haplotypes found from Hokkaido were also distributed in Honshu. However, PCO and STRUCTURE analysis based on nuclear microsatellite variation revealed clear genetic differentiation between those populations (Figs. 4, 6A). Ohshima (1990) suggested that Hokkaido and Honshu have been separated for about 100,000 years, even during the last glacial period. The genetic differentiation may result from long-term isolation, even though cpDNA sequences have not yet diverged. Along the PCO2 axis in Fig. 4, populations from western Japan, central Honshu, and northern Honshu paralleled their geographical location. This pattern supports the hypothesis based on cpDNA analysis (Honjo et al. 2004) that some lineages have migrated from western to northeastern Japan. Overall, the microsatellite allelic com-

Table 4 Population pairwise F_{ST} among 32 populations of *Primula Sieboldii*

Pop no.[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	0.000																															
2	0.076	0.000																														
3	0.057	0.031	0.000																													
4	0.042	0.041	0.002	0.000																												
5	0.054	0.064	0.023	0.014	0.000																											
6	0.065	0.052	0.030	0.038	0.030	0.000																										
7	0.104	0.099	0.092	0.101	0.092	0.064	0.000																									
8	0.197	0.195	0.171	0.160	0.177	0.135	0.191	0.000																								
9	0.209	0.227	0.181	0.189	0.162	0.173	0.154	0.286	0.000																							
10	0.258	0.285	0.229	0.232	0.197	0.198	0.191	0.293	0.099	0.000																						
11	0.305	0.344	0.275	0.292	0.265	0.271	0.251	0.430	0.169	0.177	0.000																					
12	0.242	0.274	0.242	0.251	0.224	0.235	0.172	0.332	0.140	0.196	0.209	0.000																				
13	0.221	0.260	0.208	0.222	0.197	0.208	0.184	0.308	0.136	0.178	0.186	0.140	0.000																			
14	0.314	0.353	0.308	0.314	0.290	0.274	0.288	0.323	0.244	0.291	0.376	0.243	0.240	0.000																		
15	0.187	0.227	0.188	0.201	0.168	0.150	0.151	0.243	0.156	0.203	0.286	0.152	0.115	0.148	0.000																	
16	0.178	0.209	0.176	0.187	0.149	0.141	0.176	0.262	0.168	0.222	0.229	0.177	0.160	0.214	0.068	0.000																
17	0.205	0.238	0.212	0.218	0.196	0.196	0.168	0.273	0.130	0.180	0.192	0.105	0.153	0.189	0.108	0.151	0.000															
18	0.191	0.199	0.178	0.191	0.168	0.176	0.150	0.283	0.123	0.203	0.228	0.076	0.122	0.214	0.085	0.112	0.052	0.000														
19	0.178	0.197	0.168	0.175	0.159	0.161	0.162	0.209	0.147	0.191	0.232	0.122	0.136	0.164	0.083	0.133	0.060	0.030	0.000													
20	0.167	0.203	0.171	0.176	0.153	0.150	0.154	0.205	0.142	0.183	0.233	0.127	0.149	0.135	0.079	0.124	0.058	0.046	0.012	0.000												
21	0.190	0.228	0.205	0.207	0.187	0.184	0.174	0.247	0.164	0.212	0.247	0.144	0.179	0.189	0.114	0.145	0.060	0.045	0.031	0.021	0.000											
22	0.263	0.268	0.249	0.263	0.248	0.256	0.229	0.348	0.221	0.310	0.334	0.171	0.217	0.279	0.168	0.199	0.126	0.047	0.088	0.097	0.108	0.000										
23	0.230	0.260	0.228	0.238	0.214	0.214	0.189	0.321	0.186	0.251	0.273	0.150	0.186	0.290	0.152	0.187	0.108	0.083	0.095	0.099	0.128	0.099	0.000									
24	0.247	0.287	0.244	0.245	0.219	0.227	0.230	0.312	0.207	0.251	0.300	0.187	0.209	0.261	0.188	0.203	0.141	0.144	0.117	0.107	0.161	0.205	0.135	0.000								
25	0.206	0.242	0.217	0.228	0.184	0.190	0.198	0.323	0.195	0.242	0.269	0.178	0.214	0.266	0.163	0.120	0.126	0.105	0.142	0.124	0.136	0.177	0.165	0.139	0.000							
26	0.222	0.274	0.229	0.233	0.198	0.205	0.229	0.287	0.213	0.263	0.348	0.198	0.175	0.203	0.107	0.142	0.214	0.167	0.155	0.137	0.199	0.231	0.215	0.204	0.203	0.000						
27	0.268	0.322	0.295	0.302	0.271	0.279	0.271	0.376	0.270	0.332	0.380	0.226	0.245	0.280	0.180	0.202	0.176	0.164	0.160	0.146	0.177	0.209	0.199	0.229	0.201	0.181	0.000					
28	0.255	0.327	0.299	0.306	0.256	0.271	0.243	0.381	0.238	0.296	0.371	0.223	0.238	0.319	0.182	0.206	0.186	0.178	0.194	0.165	0.198	0.244	0.207	0.248	0.193	0.196	0.133	0.000				
29	0.241	0.301	0.286	0.291	0.272	0.273	0.234	0.362	0.250	0.275	0.339	0.229	0.223	0.290	0.200	0.232	0.192	0.190	0.190	0.178	0.180	0.250	0.239	0.279	0.236	0.253	0.174	0.218	0.000			
30	0.203	0.277	0.250	0.258	0.247	0.241	0.231	0.390	0.275	0.333	0.393	0.267	0.263	0.339	0.194	0.231	0.224	0.209	0.206	0.195	0.211	0.261	0.229	0.289	0.253	0.278	0.238	0.238	0.178	0.000		
31	0.208	0.284	0.263	0.261	0.242	0.253	0.236	0.347	0.271	0.304	0.332	0.252	0.252	0.285	0.212	0.242	0.178	0.181	0.162	0.147	0.144	0.229	0.219	0.252	0.222	0.266	0.208	0.239	0.137	0.183	0.000	
32	0.183	0.238	0.214	0.218	0.187	0.201	0.175	0.307	0.205	0.237	0.299	0.192	0.200	0.277	0.154	0.192	0.195	0.162	0.164	0.149	0.155	0.230	0.217	0.242	0.216	0.186	0.182	0.186	0.147	0.163	0.178	0.000

[a] "Pop no." indicates population number in Table 1

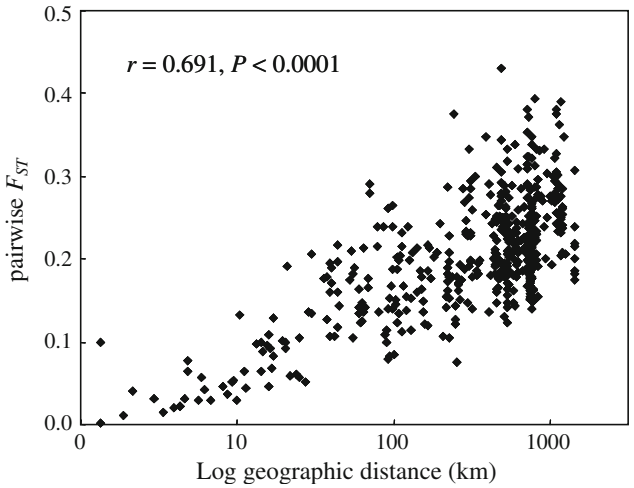

Fig. 3 Relationship between log geographic distance and pairwise F_{ST} between all pairs of populations of *P. sieboldii* in Japan

position was similar among geographically adjacent populations as shown in a significant isolation-by-distance pattern of Fig. 3, even when the cpDNA lineage of the populations differed. Hierarchical *F*-statistics revealed that 10.0% of the total microsatellite variation occurred among regions and 12.1% among populations within regions, while analysis of molecular variance (AMOVA; Excoffier et al. 1992) suggested that 59.9% of the total cpDNA variation existed among regions and 32.5% among populations within regions of *P. sieboldii* (Honjo et al. 2004). These relatively lower degrees of population differentiation estimated by nuclear microsatellites than cpDNA variation suggests that substantial pollen flow among populations might have historically occurred.

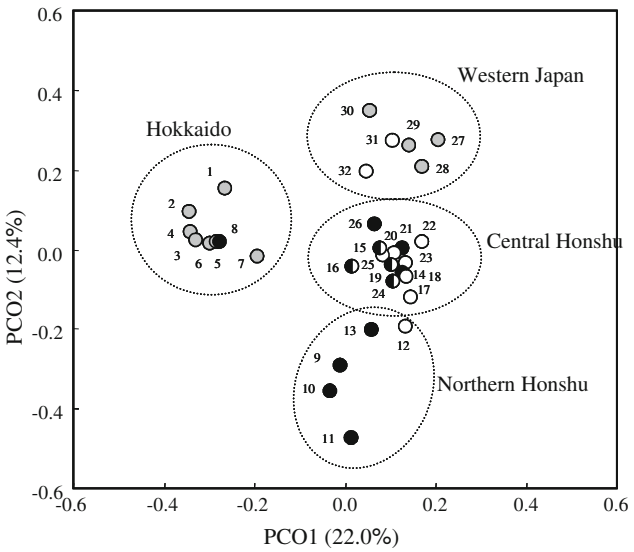

Fig. 4 Principal coordinate analysis (PCO) of 32 populations of *Primula sieboldii* analyzed at eight microsatellite markers. The first (PCO1) and second (PCO2) axes explain 22.0% and 12.4% of the total variation, respectively. Numbers refer to Table 1. Four genetic groups, corresponding to geographic regions, are encircled. The colors of plots indicate cpDNA clades according to Honjo et al. (2004): white, clade I; gray, clade II; black, clade III

Conservation units of *Primula sieboldii*

To recognize conservation units is important in the conservation and restoration of endangered species (Moritz 1994, 2002; Crandall et al. 2000; Fraser and Bernatchez 2001). We think that historical distinctiveness and adaptive divergence of populations should be considered in determining conservation units. From PCO and STRUCTURE analysis (Figs. 4, 6), we detected four genetic groups well corresponding to geographic regions (Hokkaido, northern Honshu, central Honshu, and western Japan), which could be recognized as management units (Moritz 1994, 2002) of *P. sieboldii*. Although it is not known whether these genetic groupings differentiate in adaptive traits, intraspecific endemic lineages merit conservation attention as a component of biodiversity. If supplementation with plants from other populations were planned, plant materials should be obtained from within the same management unit. Because the genetic composition of Takayama (population 26) may be somewhat different from geographically adjacent populations, careful management of this population may be needed. Admixture of individuals of northern Honshu descent and native one may be occurred. Also, since the assignment of Akagi (population 14) has varied between the PCO and STRUCTURE analysis, care may be needed for the handling of this population. This population might have experienced gene flow from both central and northern Honshu.

The patterns of neutral molecular and quantitative variation often do not correspond (Reed and Frankham 2001; Mckay and Latta 2002). For example, Cai et al. (2004) revealed that quantitative traits of common wild rice, *Oryza rufipogon*, were differentiated into perennial and annual types in response to different habitat conditions, whereas molecular markers showed a genetic isolation-by-distance pattern. A study of a vulnerable tree endemic to South America has shown that neutral DNA markers failed to detect genetic divergence in an ecologically important trait relating to drought tolerance (Bekessy et al. 2003). Steinger et al. (2002) found a strong correlation between molecular and quantitative genetic differentiation between pairs of populations, but the degree of differentiation in several quantitative traits was much larger than the

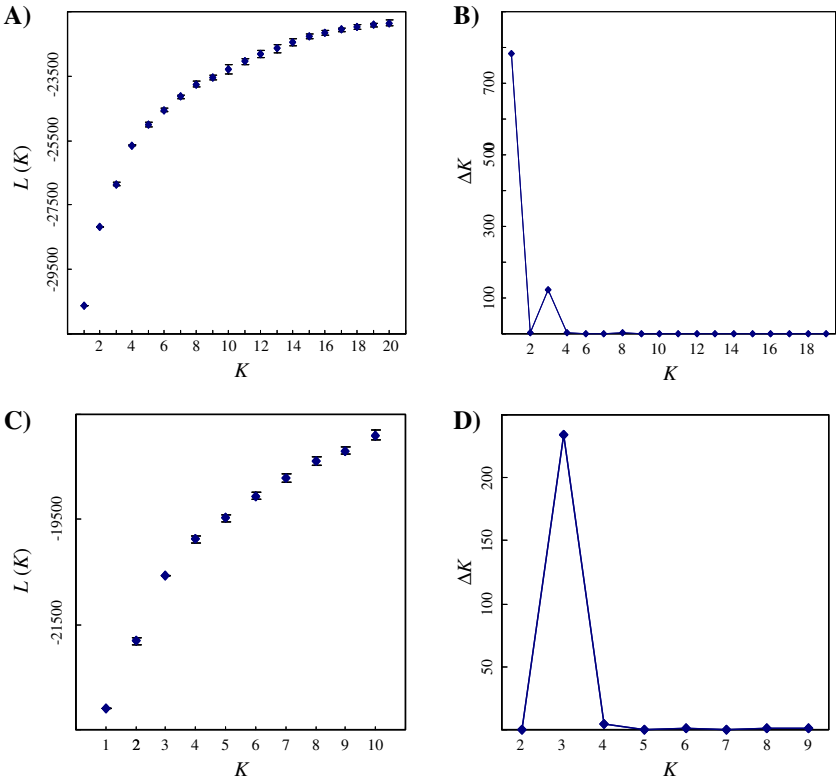

Fig. 5 Detection of the number of genetic clusters in the whole dataset (**A** and **B**) and in cluster 1, consisting of individuals of *P. sieboldii* mainly from Honshu and Kyushu in Japan (**C** and **D**; see text and Fig. 6). (**A**) and (**C**): Mean L (K) (±SD) over ten runs as a function of K. (**B**) and (**D**): ΔK (Evanno et al. 2005) as a function of K

neutral expectation. Therefore, we need to further evaluate population differentiation in adaptive traits to avoid outbreeding depression. A common garden experiment is effective to examine the genetic variation of phenotypic traits (Mckay and Latta 2002). Because each genet of *P. sieboldii* is composed of several clonally propagated ramets, we will be able to transplant some ramets to the common garden experiment while leaving other ramets in the wild habitat. By comparing relative levels of among-population divergence in quantitative traits (as measured by Q_{ST}) and neutral DNA markers (as measured by F_{ST}), we

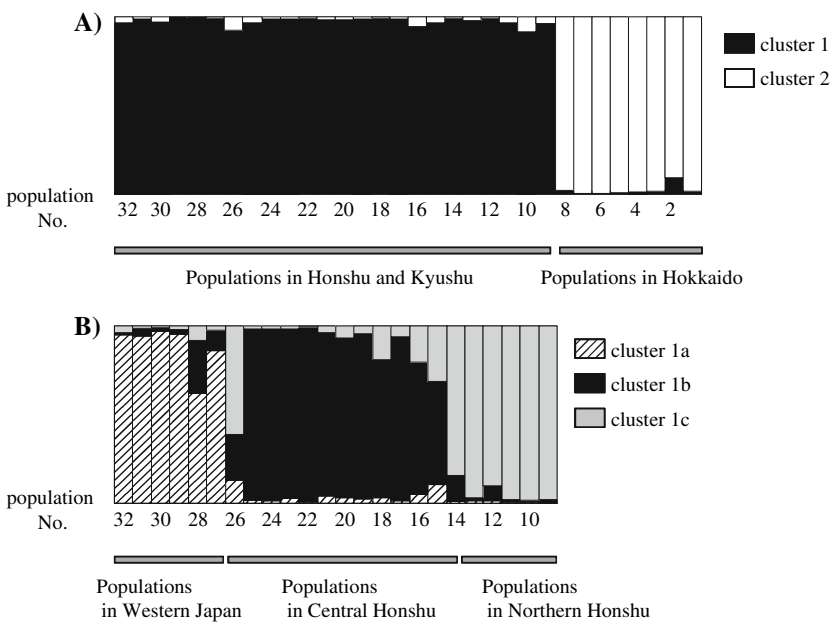

Fig. 6 Genetic clustering within *P. sieboldii* in Japan inferred by the STRUCTURE program. The relative lengths of each color in each bar represent average assignment probabilities of every population to each cluster. (**A**) In the analysis of the total dataset, two clusters were detected. Nearly all individuals from Honshu and Kyushu were assigned to cluster 1 and individuals from Hokkaido to cluster 2. (**B**) Cluster 1 was split into three clusters (1a, 1b, 1c), consisting of individuals mainly from western Japan, central Honshu, and northern Honshu, respectively

can examine the extent of local adaptation of phenotypic traits (Spitze 1993; Steinger et al. 2002).

We detected endemic cpDNA haplotypes and significant allelic variation at microsatellite loci among populations of *P. sieboldii*. These regional features of genetic variation may allow us to infer the origins of individuals of *P. sieboldii* with statistical methods such as an assignment test (Manel et al. 2005), as actually demonstrated in Honjo et al. (in press). Because commercial collection is posing an ever greater threat to the species, tracing the origin of each individual will contribute to managing species conservation. It will be also available to confirm the origins of *ex situ* stocks, which could be useful resources for genetic restoration.

Acknowledgments We express our sincere thanks to M. Ajima, S. Araki, M. Haga, H. Hata, M. Ide, Y. Kameyama, T. Kamijo, the Karuizawa Primrose Conservation Society, Y. Kato, K. Kida, T. Konishi, T. Kuwabara, K. Kuwata, M. Maeda, C. Matsumura, M. Mochida, H. Nakagaito, C. Nakano, M. Nakamura, J. Nishihiro, S. Ogawa, K. Oh, T. Ohmori, N. Ohtaki, T. Omozaki, A. Shinohara, K. Shirakawa, A. Suzuki, T. Suzuki, Y. Takahashi, Y. Takeda, T. Tsuchiya, S. Yamamoto, K. Yamanaka, J. Yokoyama, M. Yoshii, and Y. Yoshioka for collection of plant materials, and to anonymous reviewers for their comments for improving this paper. We also thank T. Kado and the members of the Laboratory of Plant Breeding at the University of Tsukuba for their assistance. This work was partly supported by the Fundamental Research Fund for Future Environment from the Japan Ministry of Environment.

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