Breeding Science 58: 347–354 (2008)

# Origins of traditional cultivars of *Primula sieboldii* revealed by nuclear microsatellite and chloroplast DNA variations

**Masanori Honjo[1,2], Takashi Handa[3], Yoshihiko Tsumura[4], Izumi Washitani[1] and Ryo Ohsawa*[3]**

[1] *Graduate School of Agricultural and Life Science, The University of Tokyo*, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
[2] *National Agriculture Research Center for Tohoku Region*, 92 Shimokuriyagawa-Nabeyashiki, Morioka, Iwate 020-0123, Japan
[3] *Graduate School of Life and Environmental Sciences, University of Tsukuba*, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8572, Japan
[4] *Department of Forest Genetics, Forestry and Forest Products Research Institute*, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan

We examined the origins of 120 cultivars of *Primula sieboldii*, a popular Japanese pot plant with a cultivation history of more than 300 years. In an assignment test based on the microsatellite allelic composition of representative wild populations of *P. sieboldii* from the Hokkaido to Kyushu regions of Japan, most cultivars showed the highest likelihood of derivation from wild populations in the Arakawa River floodplain. Chloroplast DNA haplotypes of cultivars also suggested that most cultivars have come from genets originating in wild populations from the same area, but, in addition, that several are descended from genets originating in other regions. The existence of three haplotypes that have not been found in current wild populations suggests that traditional cultivars may retain genetic diversity lost from wild populations.

**Key Words:** assignment test, gene bank, genetic resource, haplotype, horticulture.

## Introduction

Knowledge of the origins of cultivated plant species can be useful in understanding the evolution of crop species (Matsuoka 2005). Recent developments in molecular markers and population genetics statistics, such as assignment tests that assign each individual to a reference population in which its multilocus genotype is most likely to occur (Manel *et al.* 2005), enable us not only to find ancestral species (Fofana *et al.* 1999, Huang and Sun 2000, Matsuoka *et al.* 2002, Molina-Cano *et al.* 2005), but also to determine the geographic origins of cultivated species (Friesen *et al.* 1999, Sefc *et al.* 2000, Olsen and Schaal 2001, Matsuoka *et al.* 2002, Deguilloux *et al.* 2003, Harter *et al.* 2004).

Primroses (*Primula* spp.) are among the most popular garden plants in the world (Richards 2003). In Japan, more than 300 cultivars of *Primula sieboldii* E. Morren (Primulaceae) are known, and show diverse floral characteristics, including traits scarcely found in the wild (Torii 1985, Richards 2003, Yoshioka *et al.* 2005, Yoshida *et al.* 2008). This species is a heterostylous and perennial herb that occurs in moist habitats from Hokkaido to Kyushu in Japan, the Korean peninsula, northeastern China, and eastern Siberia (Yamazaki 1993), and has been bred as a traditional garden herb for some 300 years from the Edo period (1603–1867) in Japan (Torii 1985). The establishment periods of most cultivars have been estimated from horticultural literature (Torii 1985). These traditional cultivars of *P. sieboldii* are believed to have been established by finding genets with an uncommon appearance in wild populations, and by subsequent intraspecific crossing (Torii 1985). Each cultivar is clonally propagated by the separation of pips; therefore, there is basically no intra-cultivar genetic variation. Wild populations of *P. sieboldii* in the Arakawa River floodplain in a suburb of Edo (present-day Tokyo) are widely held to be the most likely geographic source of *P. sieboldii* cultivars (Torii 1985); however, to date, the origins of the cultivars of *P. sieboldii* have not been scientifically determined.

Although the ancestral species of the cultivars is considered to be exclusively *P. sieboldii*, it is suspected that congeners may have been used, considering the diverse floral variation; congeners have often been used in the breeding of other garden *Primula* species (Richards 2003). Kato and Mii (2000) showed that *P. kisoana* Miq., a closely related species of *P. sieboldii* native to Japan, can be successfully hybridized with *P. sieboldii,* and the inter-specific hybrid can be used for breeding *P. sieboldii*. Determining the origins of traditional *P. sieboldii* cultivars will contribute to understanding the history of the horticultural development of this species.

In a previous study, we analyzed nuclear microsatellite variation in representative wild populations of *P. sieboldii* from the Hokkaido to Kyushu regions of Japan (Honjo *et al.* 2008a); that study revealed significant genetic differentiation among populations. We considered that this regional feature of genetic variation may allow the origins of cultivated stocks of *P. sieboldii* to be deduced by assignment tests, and we therefore performed a self-assignment test to

Communicated by N. Mori
Received March 26, 2008. Accepted August 8, 2008.
*Corresponding author (e-mail: osawaryo@sakura.cc.tsukuba.ac.jp)

confirm whether the genotypic information of wild populations was sufficient to estimate the origins of the stocks (Honjo *et al.* 2008b). The test assigned 99.6% of the genets to the population from which they had been sampled, implying that this high probability of correct assignment could therefore allow the origins of the cultivars of *P. sieboldii* to be inferred.

Chloroplast DNA (cpDNA) variation is also becoming a useful tool for tracing the origins of cultivars. We previously analyzed sequence variation in five noncoding regions of cpDNA in 304 genets from 60 wild populations and 31 *ex situ* stocks of *P. sieboldii* in Japan: we detected 32 cpDNA haplotypes with local specificity (Fig. 1; Honjo *et al.* 2004, Honjo *et al.* 2008b). This regional feature of cpDNA variation could therefore contribute to evaluation of the maternal origins of *P. sieboldii* cultivars.

In this study, we used both nuclear microsatellite and cpDNA variations to examine the origins of *P. sieboldii* cultivars. Firstly, to confirm the ancestral species of these cultivars, we compared genetic similarity among the cultivars, wild populations, and *P. kisoana*. Secondly, to reveal the geographic origins of the cultivars, we performed an assignment test based on the microsatellite variation of representative wild populations of *P. sieboldii*, including two extant populations in the Arakawa River floodplain. In addition, we examined the distribution of cpDNA haplotypes of cultivars in wild populations of *P. sieboldii*.

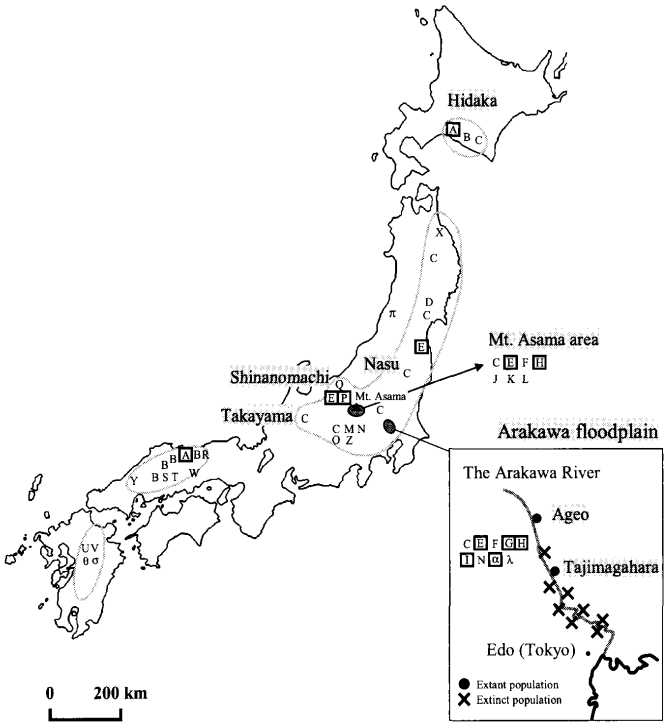


**Fig. 1.** Current distribution area of *Primula sieboldii* in Japan (southern Hokkaido, northeastern Honshu, western Honshu, and central Kyushu) and geographic distribution of cpDNA haplotypes (alphabetical and Greek letters) found in wild populations and *ex situ* stocks of the species (Honjo *et al.* 2004, Honjo *et al.* 2008b). Haplotypes found from cultivars of *P. sieboldii* are indicated by squares.

## Materials and Methods

*Plant materials*

At the time of flowering (April–May) in 2002 and 2003, we collected fresh leaves from 120 cultivars of *P. sieboldii* grown at the Agricultural and Forestry Research Center of the University of Tsukuba, and the Horticultural Laboratory of Saitama Prefectural Agriculture and Forestry Research Center (Table 1). These cultivars were supposed to have been developed between the early 1700s and the late 1900s (Table 1). We also collected leaves from two genets of *P. kisoana* clonally cultivated at the University of Tsukuba.

*Genotyping by nuclear microsatellites*

Total DNA was extracted from leaves using a modified CTAB method (Murray and Thompson 1980). The samples were genotyped at the following eight microsatellite loci, which were used in a previous analysis of wild populations (Honjo *et al.* 2008a): *PS2* (Isagi *et al.* 2001), *ga0235*, *ga0381*, *ga0668*, *ga01277* (Ueno *et al.* 2003), *ga0653*, *ga0666* (Ueno *et al.* 2005), and *Pri0146* (Kitamoto *et al.* 2005b). PCR and electrophoresis using a 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA) were performed as described by Honjo *et al.* (2008a).

*Data analysis for microsatellite variation*

To deduce the origins of the cultivars, we performed an assignment test based on the microsatellite allelic composition of 24 populations of *P. sieboldii* from the Hokkaido to Kyushu regions, as described in a previous study (Honjo *et al.* 2008b). We calculated the likelihood of a cultivar's multilocus genotype occurring in each reference population using Rannala and Mountain's (1997) criterion with the program GeneClass2 (Piry *et al.* 2004). The probability that the genotype belonged to the population of highest likelihood was then evaluated using the Monte Carlo resampling method of Paetkau *et al.* (2004); if the probability value $\alpha$ was below 0.01, the origin of the cultivar was rejected from the population. Such a combination of an assignment test and exclusion methodology has been used in previous studies (Manel *et al.* 2005, Frantz *et al.* 2006, Hare *et al.* 2006, Honjo *et al.* 2008b).

Further, to deduce the relatedness between cultivars, we calculated the number of alleles in common (NAC) (Surles *et al.* 1990) between all pairs of cultivars, which represent the degree of allele sharing. In the case of the eight microsatellite loci, this value ranged from 1 to 16, with higher values indicating more sharing. We calculated an average of pairwise NACs between a cultivar and each of all other cultivars.

*Sequencing of cpDNA*

In a previous study (Honjo *et al.* 2004, Honjo *et al.* 2008b), we determined cpDNA haplotypes of *P. sieboldii* based on the sequences of five noncoding regions of cpDNA. Since it is possible to distinguish all but three haplotypes

**Table 1.** The period of establishment, assigned population based on the assignment test using eight microsatellite loci, and the cpDNA haplotype for each *Primula sieboldii* cultivar

| Cultivar name | Period of establishment[a] | Assigned population[b] | cpDNA haplotype | Cultivar name | Period of establishment[a] | Assigned population[b] | cpDNA haplotype |
|---|---|---|---|---|---|---|---|
| Adesugata | Early 1900s | Karuizawa** | β | Kuninohikari | Early 1900s | Karuizawa* | G |
| Akatonbo | Late 1800s | Tajimagahara* | β | Kurama | unknown | Ageo** | β |
| Akinoyoso-oi | Early 1800s | Karuizawa** | δ | Kyoho | Early 1900s | Ageo** | P |
| Amegashita | Late 1800s | Nasu | γ | Kyoganoko | unknown | Hidaka | γ |
| Aobanofue | Late 1700s | Ageo** | E | Madono-ume | Late 1900s | Ageo** | P |
| Aounabara | Early 1800s | Ageo* | γ | Maiogi | Early 1800s | Ageo** | P |
| Asagiri | Early 1900s | Ageo** | E | Makino-o | Early 1800s | Nasu | γ |
| Asahi | Late 1800s | Ageo* | H | Manzairaku | unknown | Hidaka* | H |
| Asahinotamoto | Late 1700s | Karuizawa | P | Matsunoyuki | Early 1800s | Tajimagahara** | H |
| Asahizuru | Late 1800s | Ageo** | γ | Mihonokoji | Early 1800s | Karuizawa | γ |
| Ayanami | Late 1800s | Ageo** | γ | Mitajiman | Early 1900s | Ageo** | γ |
| Benijo-ou | Late 1800s | Ageo** | H | Momozono | Late 1800s | Nasu | γ |
| Bijonomai | Early 1900s | Ageo | G | Mutamagawa | Early 1800s | Karuizawa* | G |
| Botanjishi | Early 1800s | Ageo** | γ | Myochiriki | Early 1800s | Karuizawa | H |
| Bureiko | Late 1900s | Ageo** | β | Nankinkozakura | Early 1700s | Ageo** | α |
| Chidorigai | Early 1800s | Ageo** | H | Nio-ume | Late 1700s | Ageo** | E |
| Edasango | Early 1800s | Nasu* | γ | Osuma | Early 1800s | Tajimagahara* | A |
| Fujigoshi | Late 1800s | Ageo** | γ | Origamitsuki | Early 1900s | Ageo** | β |
| Fujinosato | Late 1900s | Ageo** | γ | Ozasanoyuki | Early 1900s | Ageo** | γ |
| Fukiagezakura | Early 1900s | Karuizawa** | E | Rashomon | Early 1800s | Ageo** | H |
| Fuyo | Early 1800s | Ageo** | γ | Ruriden | Early 1800s | Ageo** | β |
| Garyobai | Late 1700s | Ageo** | E | Sangokuko | Early 1800s | Ageo** | γ |
| Ginkujaku | Late 1800s | Ageo** | γ | Sankahaku-u | Late 1900s | Ageo** | γ |
| Ginsekai | Late 1700s | Tajimagahara** | γ | Seiobo | Early 1800s | Ageo** | γ |
| Godaishu | Late 1800s | Ageo* | G | Sensho | Early 1900s | Ageo** | P |
| Gyokukobai | Early 1800s | Ageo | β | Setsugetsuka | Early 1800s | Tajimagahara | I |
| Hagino-uwakaze | unknown | Karuizawa* | γ | Shiboritatsuta | Early 1800s | Shinanomachi* | α |
| Hahanoai | Early 1900s | Ageo** | H | Shikinomine | Early 1900s | Ageo** | γ |
| Hahanomegumi | Early 1900s | Ageo** | H | Shinkiro | unknown | Hidaka | H |
| Hakutaka | Late 1900s | Karuizawa* | γ | Shiokemuri | unknown | Hidaka* | γ |
| Hanakujaku | Early 1900s | Shinanomachi | β | Shiratama | Late 1900s | Karuizawa* | γ |
| Hanataisho | Early 1800s | Ageo** | H | Shiro-usagi | Late 1900s | Ageo** | E |
| Harutsugedori | Late 1900s | Gunma | γ | Shishifunjin | Early 1800s | Nasu | γ |
| Hatsusugata | Late 1900s | Gunma** | γ | Shishinden | Late 1900s | Ageo** | γ |
| Hatsuzakura | Late 1900s | Hidaka | γ | Shiunryu | Early 1800s | Ageo* | γ |
| Hien | Late 1800s | Ageo** | G | Soto-orihime | Late 1800s | Ageo** | γ |
| Hinokasane | Late 1900s | Ageo** | H | Sumidanohanabi | Early 1900s | Ageo** | H |
| Hiryu | Early 1800s | Ageo* | H | Shunko | Early 1900s | Tajimagahara | P |
| Hitomaru | Early 1900s | Ageo** | γ | Shunsho | Late 1900s | Ageo* | γ |
| Hokutosei | Early 1900s | Ageo** | β | Tagasode | Late 1800s | Ageo** | γ |
| Ichinenriki | Early 1800s | Takayama* | γ | Takanenoyuki | Late 1900s | Ageo** | γ |
| Iwatokagura | Early 1800s | Ageo** | G | Takasagozome | Late 1700s | Ageo** | γ |
| Janomegasa | Late 1700s | Nasu | H | Tamasango | Late 1800s | Ageo** | H |
| Jinpu | Late 1800s | Ageo** | γ | Tebyoshi | Early 1800s | Ageo** | γ |
| Joshibori | Early 1900s | Ageo** | γ | Tokinohina | Early 1800s | Ageo** | γ |
| Junihitoe | Early 1800s | Hidaka | γ | Tochiirimen | Early 1800s | Nasu | γ |
| Kagero | Late 1800s | Ageo** | γ | Tsukumojishi | Early 1800s | Ageo** | γ |
| Kansenden | Early 1800s | Karuizawa* | γ | Tsurezuru | Late 1800s | Ageo** | α |
| Kaoruhanakaze | Early 1800s | Ageo** | γ | Uchunosakura | Late 1800s | Ageo** | γ |
| Kasuminokoromo | Early 1800s | Ageo** | β | Umegae | Early 1800s | Ageo* | P |
| Kayoikomachi | Early 1800s | Ageo** | γ | Usujanome | Early 1800s | Tajimagahara** | E |
| Kazaguruma | Early 1900s | Gunma | β | Wakafuji | Late 1800s | Ageo** | γ |
| Keshonomai | Early 1800s | Ageo* | γ | Yodainoyume | Early 1900s | Ageo** | γ |
| Kinyoshu | Early 1800s | Ageo* | H | Yubae | Early 1800s | Tajimagahara** | H |
| Koshijinoyuki | Early 1800s | Karuizawa* | γ | Yuhibeni | Early 1900s | Ageo** | γ |
| Kotobuki | unknown | Shinanomachi* | P | Yukarinosode | Early 1800s | Karuizawa | γ |
| Koanoharu | Early 1900s | Shinanomachi* | E | Yukarinotamoto | Late 1800s | Hidaka | γ |
| Koroho | Early 1800s | Karuizawa* | γ | Yukinohada | Late 1800s | Tajimagahara** | γ |
| Kozakuragenji | Late 1700s | Ageo** | P | Zansetsu | Late 1800s | Ageo** | E |
| Kuisakigami | Early 1800s | Hidaka | G | Zendaimimon | Early 1800s | Ageo** | H |

[a] The establishment period of cultivars was according to Torii (1985).

[b] Asterisks show the probability of membership of the genotype to the population; where *: with $0.01 \leq \alpha < 0.05$, and **: $\alpha \geq 0.05$. The larger value of $\alpha$ indicates a higher probability that the cultivar originated in the population.

A, R and θ, by sequencing only three noncoding regions, we determined the haplotypes of *P. sieboldii* cultivars based on these three regions: (1) the spacer between *trnT* (UGU) and *trnL* (UAA) 5′ exon (Taberlet *et al.* 1991), (2) *trnL* (UAA) intron (Taberlet *et al.* 1991), and (3) the spacer between *trnD* (GUC) and *trnT* (GGU; Honjo *et al.* 2004). When a haplotype was assumed to be A, R, or θ, or when a haplotype showed sequences distinct from known haplotypes, we sequenced two additional regions: the spacers between *trnL* (UAA) 3′ exon and *trnF* (GAA; Taberlet *et al.* 1991), and that between *trnH* (GUG) and *psbA* (Demesure *et al.* 1995, Hamilton 1999). PCR and sequencing using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) were performed as described by Honjo *et al.* (2004).

*Data analysis of cpDNA variation*

The geographic distributions of the cultivars' cpDNA haplotypes were determined by reference to Honjo *et al.* (2004) and Honjo *et al.* (2008b), which examined cpDNA variation in wild populations from Hokkaido to Kyushu. To show the relatedness of haplotypes, we constructed a median-joining network (Bandelt *et al.*, 1999) with the epsilon value set to zero using the software Network 4.5.0.0. (http://www.fluxus-engineering.com). For a hypervariable site, i.e., a tandem repeat of mononucleotides in the intergenic spacer between *trnT* and *trnL*, we down-weighted the character to half of other loci as recommended by the protocol. As an outgroup, we used a closely related species, *P. kisoana*.

## Results

*Assignment test based on microsatellite variation*

All eight microsatellite loci were successfully amplified in all 120 cultivars of *P. sieboldii* and showed polymorphism, with the number of alleles per locus ranging from 3 (*PS2*) to 18 (*ga0666*). In contrast, only two loci (*pri0146* and *ga0381*) were successfully amplified in *P. kisoana* under the same PCR amplification conditions. In the assignment test, 97 cultivars were assigned to a wild population with a probability of membership $\alpha \geq 0.01$ (Table 2). Among these 97 cultivars, 78 showed the highest likelihood of occurrence of their genotypes in populations in the Arakawa River floodplain (Ageo and Tajimagahara populations), 12 in populations around Mt. Asama (Gunma and Karuizawa populations), and five in other populations in central Honshu (Nasu, Shinanomachi, and Takayama). The other two cultivars were assigned to Hidaka in Hokkaido, which is located far from central Honshu. Populations to which cultivars were assigned with a highest probability of membership ($\alpha \geq 0.05$) were those in the Arakawa River floodplain or the area around Mt. Asama (Table 2). Twenty-three cultivars were not assigned to any population with $\alpha \geq 0.01$, although they showed the highest likelihood of occurrence in the above-mentioned populations in central Honshu or Hokkaido (Table 2).

**Table 2.** The number of *Primula sieboldii* cultivars assigned to each wild population

| Assigned population | Locality | Number of cultivars | | |
|---|---|---|---|---|
| | | Probability of membership[a] | | Total |
| | | $\alpha \geq 0.01$[b] | $\alpha < 0.01$ | |
| Hidaka | Hokkaido | 2 (0) | 6 | 8 |
| Nasu | Central Honshu | 1 (0) | 6 | 7 |
| Ageo | Central Honshu (Arakawa floodplain) | 71 (62) | 2 | 73 |
| Tajimagahara | Central Honshu (Arakawa floodplain) | 7 (5) | 2 | 9 |
| Gunma | Central Honshu (Mt. Asama area) | 1 (1) | 2 | 3 |
| Karuizawa | Central Honshu (Mt. Asama area) | 11 (3) | 4 | 15 |
| Shinanomachi | Central Honshu | 3 (0) | 1 | 4 |
| Takayama | Central Honshu | 1 (0) | 0 | 1 |
| | | 97 | 23 | 120 |

[a] The probability of membership of the genotype to the population was calculated using the Monte Carlo resampling method (Paetkau *et al.* 2004). If $\alpha \geq 0.01$, we considered that the cultivar originated in that population.

[b] Values in parentheses indicate the number of cultivars assigned to the population with $\alpha \geq 0.05$.

*Relatedness between cultivars*

The average of pairwise NACs between a cultivar and each of all other cultivars ranged from 3.277, 4.563, and 5.311 in 'Setsugetsuka', 'Akatonbo', and 'Osuma' to 9.370, 9.429, and 9.790 in 'Hagino-uwakaze', 'Tamasango', and 'Fuyo', respectively.

*Chloroplast DNA haplotypes detected in the cultivars*

From the cultivars of *P. sieboldii*, 10 cpDNA haplotypes (A, E, G, H, I, P, α, β, γ, and δ; Table 1 and Fig. 1) were identified. These 10 haplotypes were already present in cultivars established by the early 1800s (Table 3). Among these haplotypes, 7 have been found in wild populations or *ex situ* stocks (Honjo *et al.* 2004, 2008b); haplotypes E, H, and I were found in wild populations in the Arakawa River floodplain, around Mt. Asama, or both, haplotype G and α in *ex situ* stocks originating from the Arakawa River floodplain, haplotype P in a population near Mt. Asama, and haplotype A was only found in populations in western Honshu and Hokkaido. Haplotypes β, γ, and δ were newly detected and were not found in wild populations. The nucleotide sequences of the new haplotypes will appear in the DDBJ database under accession numbers AB381924–AB381926. Among the 120 cultivars, almost half (59 cultivars) showed haplotype γ. Each of haplotypes A, I, and δ was detected in only one cultivar, 'Osuma', 'Setsugetsuka', and 'Akinoyoso-oi', respectively.

The median-joining network analysis of cpDNA haplotypes showed that three major groups exist in Japanese *P. sieboldii* populations (Fig. 2). Haplotypes E, G, H, I, and

**Table 3.** The appearance of cpDNA haplotypes in *Primula sieboldii* cultivars during each cultivar establishment period

| cpDNA haplotype | Establishment period of cultivars[a] | | | | | | | Total number and percent |
|---|---|---|---|---|---|---|---|---|
| | 1700s | | 1800s | | 1900s | | Unknown | |
| | Early | Late | Early | Late | Early | Late | | |
| A | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 (0.8%) |
| E | 0 | 3 | 1 | 1 | 3 | 1 | 0 | 9 (7.5%) |
| G | 0 | 0 | 3 | 2 | 2 | 0 | 0 | 7 (5.8%) |
| H | 0 | 1 | 9 | 3 | 3 | 1 | 2 | 19 (15.8%) |
| I | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 (0.8%) |
| P | 0 | 2 | 2 | 0 | 3 | 1 | 1 | 9 (7.5%) |
| α | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 3 (2.5%) |
| β | 0 | 0 | 3 | 1 | 5 | 1 | 1 | 11 (9.2%) |
| γ | 0 | 2 | 23 | 14 | 7 | 10 | 3 | 59 (49.2%) |
| δ | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 (0.8%) |
| Total | 1 | 8 | 45 | 22 | 23 | 14 | 7 | 120 |

[a] The establishment period of cultivars was according to Torii (1985).

P belong to group I; haplotypes A and δ to group II; and haplotypes α, β, and γ to group III. *Primula kisoana* showed a distinct haplotype differing from *P. sieboldii* in 34 mutations.

## Discussion

*Ancestral species of cultivars*

Cultivars of ornamental plants, including several primroses, have often been produced by interspecific hybridization (Iwata *et al.* 2000, Richards 2003, Tanaka *et al.* 2005, Ohta *et al.* 2006); however, it was considered that all cultivars analyzed in this study were derived from *P. sieboldii* only, as judged from cpDNA variation and successful PCR amplification of microsatellite loci. The cpDNA haplotypes detected in the cultivars were the same as, or closely related to, those of wild *P. sieboldii*, and were clearly different from those of its closely related species, *P. kisoana*. In accordance with this study, more extensive screening of microsatellite markers (Ohtani 2007) also suggested that only five markers were useable for *P. kisoana* among 250 microsatellites originally developed for *P. sieboldii*. These results suggest that cultivars of *P. sieboldii*, have been produced exclusively from intrinsic variation within the species.

*Geographic origin of cultivars*

Populations in the Arakawa River floodplain and in areas around the Mt. Asama volcano are believed to have constituted a historical metapopulation, the Asama-Arakawa metapopulation. We coined "the Asama-Arakawa metapopulation" herein. The individual populations within this metapopulation were considered to be connected by gene flow, which was the result of dispersal caused by occasional floodwaters. Several types of evidence suggest that populations of the Arakawa River floodplain originated from long-distance dispersal of seeds and clonal propagules carried by water flow from the riparian zones in the Mt. Asama area: the ancient Arakawa River flowed from the areas around Mt. Asama to the Tokyo area until about 3000 years ago (Hirai 1983), and the species' capability for dispersal by water flow has been reported (Kitamoto *et al.* 2005a, Nishihiro and Washitani 2006). Furthermore, historical connectivity between these populations has been supported by genetic clustering based on nuclear microsatellites (Honjo *et al.* 2008a) and the sharing of cpDNA haplotypes (Honjo *et al.* 2004). In the assignment test of this study, most cultivars were assigned to the Asama-Arakawa metapopulation as the origin (Table 2). Also, among 10 cpDNA haplotypes found in the cultivars, five (α, E, G, H, and I) have been detected in extant wild populations or *ex situ* stocks from the Asama-Arakawa metapopulation (Fig. 1). Haplotype P was also distributed in a population near Mt. Asama. These results suggest that most cultivars were produced from plants originating in the Asama-Arakawa metapopulation. Since the populations of *P. sieboldii* in the Arakawa River floodplain were closest to Edo, the region would have been very accessible to many people, including primrose enthusiasts.

Evidence obtained from cpDNA variation suggested that the breeding of some cultivars may have been unique. Haplotypes A and δ of group II were only detected in the cultivars 'Osuma' and 'Akinoyoso-oi', respectively (Table 1). Haplotypes of group II have only been detected in western Honshu and Hokkaido, and have not been found in extant populations of central Honshu (Honjo *et al.* 2004, Honjo *et al.* 2008b). In contrast, the assignment test based on nuclear microsatellites showed the highest likelihood of occurrence of these cultivar's genotypes in central Honshu. This result suggests two possibilities: that these cultivars were produced (1) by crossing between genets originating in central Honshu and western Honshu or Hokkaido, or (2) from plants belonging to group II that had been distributed in central Honshu. Since 'Osuma' exhibited the third lowest NAC value, this cultivar is likely to have gone through a different breeding process than the others. Likewise, 'Setsugetsuka' only

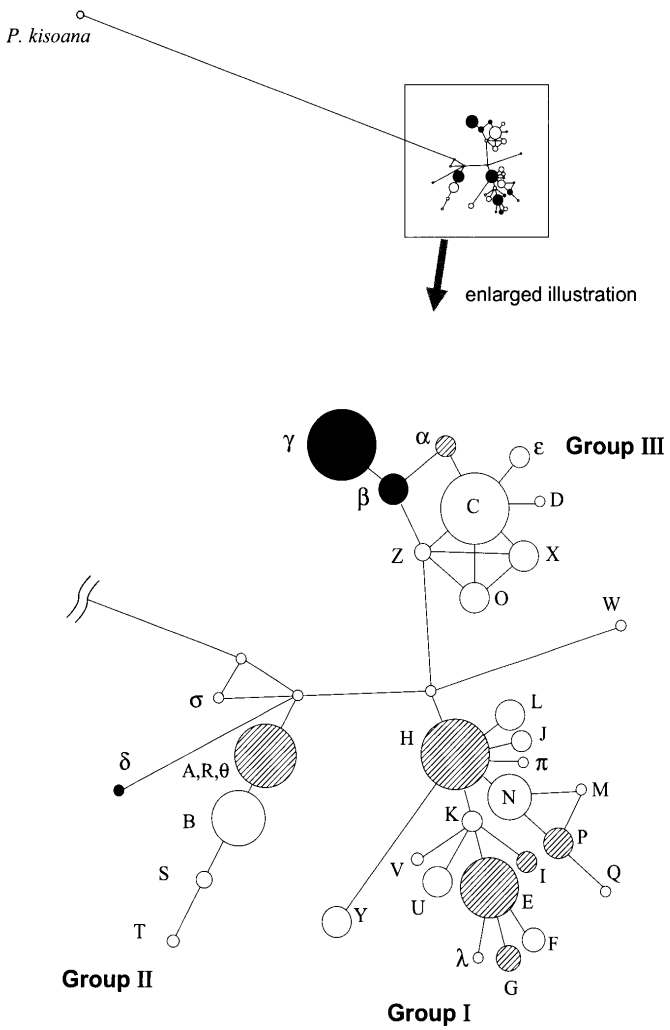


**Fig. 2.** Median-joining network of cpDNA haplotypes of *Primula sieboldii* based on the sequences of three non-coding regions. Each circle with an alphabetical or Greek letter corresponds to a haplotype, and the size of a circle is proportional to the haplotype's frequency in wild populations, ex situ stocks and cultivars of *P. sieboldii*. Haplotypes only found in the cultivars are black, and those detected in both the cultivars and wild populations or *ex situ* stocks are shaded. Nodes (small circles without letters) represent hypothetical ancestral haplotypes linking the presently extant haplotypes, or may represent haplotypes that, though presently extant, were not sampled.

showed haplotype I, and had the lowest NAC value. In addition, several cultivars that were assigned to Hidaka in Hokkaido as a possible origin may have also resulted from unique breeding. However, because the probability of membership of their genotypes to Hidaka was relatively low ($\alpha \leq 0.05$), and these cultivars did not show the cpDNA haplotypes detected in Hokkaido, further investigation will be needed to determine whether these cultivars descended from Hokkaido.

The assignment of almost half of the cultivars to haplotype γ suggests that individuals belonging to this haplotype were frequently used for breeding of *P. sieboldii* cultivars, although haplotypes β and γ have not been found in wild populations. However, these haplotypes might have been distributed in central Honshu because haplotypes C and α, closely related to haplotypes β and γ (Fig. 2), have been found in extant wild populations and *ex situ* stocks from the Arakawa River floodplain (Fig. 1; Honjo *et al.* 2004, Honjo *et al.* 2008b). As discussed below, because both the number and size of wild populations of *P. sieboldii* have decreased, a certain extent of genetic diversity might have been lost from wild populations.

In conclusion, most cultivars appear to have been improved by intraspecific crossing among *P. sieboldii* originating from the Asama-Arakawa metapopulation, but some are descended from *P. sieboldii* originating in other areas. The occurrence of natural populations near Edo would have significantly contributed to the horticultural development of *P. sieboldii*.

*Traditional cultivars may retain genetic diversity lost in the wild*

Although we examined representative extant wild populations in Japan, three haplotypes (β, γ, and δ) observed in cultivars have not been detected in the wild. Three factors should be considered as possible causes of this discrepancy. Firstly, these haplotypes may be specific to the cultivars. Secondly, these haplotypes may be extant but not detected in the wild populations. Thirdly, these haplotypes may have been lost from the wild populations. The likelihood of haplotypes being specific to the cultivars, however, is low considering the relatively short cultivation history of *P. sieboldii* compared to the low mutation rate of cpDNA. Although it is extremely difficult to sample all genetic variations remaining in extant wild populations, the possibility remains that genetic diversity has already been lost in them. The appearance of all 10 haplotypes in cultivars established in the early 1800s (Table 3) suggests that the horticultural base of *P. sieboldii* had been established by that time. Since then, the number and size of wild populations of *P. sieboldii* in Japan have decreased strikingly due to loss or fragmentation of habitats, the abandonment of traditional management of woodlands and grasslands, and excessive commercial collection (Environment Agency of Japan 2000). Although many populations grew along stretches of the Arakawa River, almost all have been lost because of the rapid growth of Tokyo (Torii 1985), and only two populations now remain (Ageo and Tajimagahara; Fig. 1). Such decline of wild populations might have led to a loss of genetic diversity. The conservation of both wild populations and traditional cultivars of *P. sieboldii*, which may serve as genebanks (Yoshida *et al.* 2008), is essential to maintain biodiversity and invaluable genetic resources.

## Acknowledgments

We sincerely thank Mr. Tatsuo Matsumoto of the Horticultural Laboratory of Saitama Prefectural Agriculture and Forestry Research Center, Dr. Yosuke Yoshioka, and the members of the Agricultural and Forestry Research Center of the University of Tsukuba for collecting plant materials,

and to anonymous reviewers for their valuable comments on this paper. This work was partly supported by the Fundamental Research Fund for the Future Environment from the Japan Ministry of the Environment, and by a grant for a Research Project for Utilizing Advanced Technology in Agriculture, Forestry and Fisheries from the Japan Ministry of Agriculture, Forestry and Fisheries.

## Literature Cited

Bandelt, H.J., P. Forster and A. Rohl (1999) Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16: 37–48.

Deguilloux, M.F., M.H. Pemonge, L. Bertel, A. Kremer and J. Petit (2003) Checking the geographical origin of oak wood: molecular and statistical tools. Mol. Ecol. 12: 1629–1636.

Demesure, B., N. Sodzi and R.J. Petit (1995) A set of universal primers for amplification of polymorphic noncoding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. 4: 129–131.

Environment Agency of Japan (2000) Threatened Wildlife of Japan, Red Data Book, Vascular Plants. Environment Agency of Japan, Tokyo, p. 660.

Fofana, B., J.P. Baudoin, X. Vekemans, D.G. Debouck and P. du Jardin (1999) Molecular evidence for an Andean origin and a secondary gene pool for the Lima bean (*Phaseolus lunatus* L.) using chloroplast DNA. Theor. Appl. Genet. 98: 202–212.

Frantz, A.C., T. Pourtois, M. Heuertz, L. Schley, M.C. Flamand, A. Krier, S. Bertouille, F. Chaumont and T. Burke (2006) Genetic structure and assignment tests demonstrate illegal translocation of red deer (*Cervus elaphus*) into a continuous population. Mol. Ecol. 15: 3191–3203.

Friesen, N., S. Pollner, K. Bachmann and F.R. Blattner (1999) RAPDs and noncoding chloroplast DNA reveal a single origin of the cultivated *Allium fistulosum* from *A. altaicum* (Alliaceae). Am. J. Bot. 86: 554–562.

Hamilton, M.B. (1999) Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol. Ecol. 8: 513–525.

Hare, M.P., S.K. Allen Jr., P. Bloomer, M.D. Camara, R.B. Carnegie, J. Murfree, M. Luckenbach, D. Meritt, C. Morrison, K. Paynter, K.S. Reece and C.G. Rose (2006) A genetic test for recruitment enhancement in Chesapeake Bay oysters, *Crassostrea virginica*, after population supplementation with a disease tolerant strain. Conserv. Genet. 7: 717–734.

Harter, A.V., K.A. Gardner, D. Falush, D.L. Lentz, R.A. Bye and L.H. Rieseberg (2004) Origin of extant domesticated sunflowers in eastern North America. Nature 430: 201–205.

Hirai, Y. (1983) Geomorphic development of the alluvial lowlands in the central part of the Kanto plain, Japan. Geographical Review of Japan 56: 679–694.

Honjo, M., S. Ueno, Y. Tsumura, I. Washitani and R. Ohsawa (2004) Phylogeographic study based on intraspecific sequence variation of chloroplast DNA for the conservation of genetic diversity in the Japanese endangered species *Primula sieboldii*. Biol. Conserv. 120: 211–220.

Honjo, M., N. Kitamoto, S. Ueno, Y. Tsumura, I. Washitani and R. Ohsawa (2008a) Management units of the endangered herb *Primula sieboldii* based on microsatellite variation among and within populations throughout Japan. Conserv. Genet. DOI 10.1007/s10592-007-9292-4.

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

Huang, J.C. and M. Sun (2000) Genetic diversity and relationships of sweet potato and its wild relatives in *Ipomoea* series *Batatas* (Convolvulaceae) as revealed by inter-simple sequence repeat (ISSR) and restriction analysis of chloroplast DNA. Theor. Appl. Genet. 100: 1050–1060.

Isagi, Y., M. Honjo and I. Washitani (2001) Development of microsatellite markers for *Primula sieboldii* using degenerate oligonucleotides-primed PCR-amplified DNA. Mol. Ecol. Notes 1: 22–24.

Iwata, H., T. Kato and S. Ohno (2000) Triparental origin of Damask roses. Gene 259: 53–59.

Kato, J. and M. Mii (2000) Differences in ploidy levels of inter-specific hybrids obtained by reciprocal crosses between *Primula sieboldii* and *P. kisoana*. Theor. Appl. Genet. 101: 690–696.

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

Kitamoto, N., S. Ueno, Y. Tsumura, I. Washitani and R. Ohsawa (2005b) Development of microsatellite markers in *Primula sieboldii* E. Morren, a threatened herb. Jpn. J. Conserv. Ecol. 10: 47–51.

Manel, S., O.E. Gaggiotti and R.S. Waples (2005) Assignment methods: matching biological questions with appropriate techniques. Trends. Ecol. Evol. 20: 136–142.

Matsuoka, Y., Y. Vigouroux, M.M. Goodman, G.J. Sanchez, E. Buckler and J. Doebley (2002) A single domestication for maize shown by multilocus microsatellite genotyping. Proc. Natl. Acad. Sci. USA 99: 6080–6084.

Matsuoka, Y. (2005) Origin matters: Lessons from the search for the wild ancestor of maize. Breed. Sci. 55: 383–390.

Molina-Cano, J.L., J.R. Russell, M.A. Moralejo, J.L. Escacena, G. Arias and W. Powell (2005) Chloroplast DNA microsatellite analysis supports a polyphyletic origin for barley. Theor. Appl. Genet. 110: 613–619.

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

Nishihiro, M.A. and I. Washitani (2006) The spatial and temporal dispersal of seeds and the establishment of seedlings of *Primula sieboldii*. *In*: Washitani, I. (ed.) Conservation molecular genetic ecology on *Primula sieboldii*, University of Tokyo Press, Tokyo, pp. 97–114.

Ohta, S., S. Osumi, T. Katsuki, I. Nakamura, T. Yamamoto and Y. Sato (2006) Genetic characterization of flowering cherries (*Prunus* subgenus *Cerasus*) using *rpl16–rpl14* spacer sequences of chloroplast DNA. J. Jpn. Soc. Hort. Sci. 75: 72–78.

Ohtani, M. (2007) Conservation genetics of an endangered plant species, *Primula kisoana* Miquel var. *kisoana* (Primulaceae). Doctoral dissertation, The University of Tokyo.

Olsen, K.M. and B.A. Schaal (2001) Microsatellite variation in Cassava (*Manihot esculenta*, Euphorbiaceae) and its wild relatives: Further evidence for a southern Amazonian origin of domestication. Am. J. Bot. 88: 131–142.

Paetkau, D., R. Slade, M. Burden and A. Estoup (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation based exploration of accuracy and power. Mol. Ecol. 13: 55–65.

Piry, S., A. Alapetite, J.M. Cornuet, D. Paetkau, L. Baudouin and A. Estoup (2004) GENECLASS2: A software for genetic

assignment and first-generation migrant detection. J. Hered. 95: 536–539.

Rannala, B. and J.L. Mountain (1997) Detecting immigration by using multilocus genotypes. Proc. Natl. Acad. Sci. USA 94: 9197–9221.

Richards, A.C. (2003) PRIMULA 2nd edn. Timber Press, Portland, Oregon, p. 346.

Sefc, K.M., M.S. Lopes, F. Lefort, R. Botta, K.A. Roubelakis-Angelakis, J. Ibáñes, I. Pejić, H.W. Wagner, J. Glössl and H. Steinkellner (2000) Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars. Theor. Appl. Genet. 100: 498–505.

Surles, S.E., J. Arnold, A. Schnabel, J.L. Hamrick and B.C. Bongarten (1990) Genetic relatedness in open-pollinated families of two leguminous tree species, *Robinia pseudoacacia* L. and *Gleditsia triacanthos* L. Theor. Appl. Genet. 80: 49–56.

Taberlet, P., L. Gielly, G. Pautou and J. Bouvet (1991) Universal primers for amplification of three noncoding regions of chloroplast DNA. Plant Mol. Biol. 17: 1105–1109.

Tanaka, T., T. Mizutani, M. Shibata, N. Tanikawa and C.R. Parks (2005) Cytogenetic studies on the origin of *Camellia* × *vernalis*. V. Estimation of the seed parent of *C.* × *vernalis* that evolved about 400 years ago by cpDNA analysis. J. Jpn. Soc. Hort. Sci. 74: 464–468.

Torii, T. (1985) Sakurasou. Nihon-TV, Tokyo, p. 151.

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

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

Yamazaki, T. (1993) Primula. *In*: Iwatsuki, K., T. Yamazaki, D.E. Boufford and H. Ohba (eds.) Flora of Japan 3a, Kodansha, Tokyo, pp. 87–94.

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

Yoshioka, Y., H. Iwata, R. Ohsawa and S. Ninomiya (2005) Quantitative evaluation of the petal shape variation in *Primula sieboldii* caused by breeding process in the last 300 years. Heredity 94: 657–663.