

## Phylogeographic Study of *Phyllodoce aleutica* (Ericaceae) in the Japanese Archipelago

Hajime Ikeda<sup>1,\*</sup> and Hiroaki Setoguchi<sup>2</sup>

<sup>1</sup>Department of Botany, National Museum of Nature and Science,  
Amakubo 4-1-1, Tsukuba, Ibaraki 305-0005, Japan

Present address: Institute of Plant Science and Resources, Okayama University,  
Chuo 2-20-1, Kurashiki, Okayama 710-0046, Japan (ike@rib.okayama-u.ac.jp)

<sup>2</sup>Graduate School of Human and Environmental Studies, Kyoto University,  
Yoshida-nihonmatsu-cho, Sakyo-ku, Kyoto 606-8501, Japan

\*Hajime Ikeda (ike@kahaku.go.jp)

(Received 15 February 2013; accepted 25 March 2013)

**Abstract** Previous phylogeography on Japanese alpine plants exhibited intra-specific geographic differentiation between populations in central and northern Japan across various taxa. Accordingly, their biogeographic history in the Japanese archipelago was hypothesized that independent colonization into the archipelago occurred at least twice and that populations in central Japan have been persisted with isolation from the northern region. In addition, although northern populations harboured mostly homogenous genetic structure due to a single expansion history, unique genetic structure was sometimes detected in populations of Tohoku. Such unique genetic structure, but with less consistent tendency, implied that disjunct populations were also persisted in this region. Here, we attempted to examine these biogeographic hypotheses by conducting phylogeographic investigation of *Phyllodoce aleutica* (Ericaceae). Based on haplotype distribution of chloroplast DNA, populations in central Japan harboured a distinct genetic structure from those in northern Japan. This geographic pattern is consistent with the persistence of isolated populations in central Japan. In addition, populations in Tohoku harboured haplotypes exclusively found in this region, which emphasised the relict populations in this region distinct from other regions. Thus, our study confirmed the north-south differentiation of alpine plants in *P. aleutica* and suggests the importance of future study focusing on Tohoku region.

**Key words** : alpine plants, chloroplast DNA, *Phyllodoce aleutica*, phylogeography.

### Introduction

Pleistocene climatic oscillations caused repeated changes of species' range, resulting in their current genetic structure within species (Hewitt, 2004). Molecular phylogeographic studies elucidated genetic variation throughout species' range and inferred history of range shifts following Pleistocene climatic oscillations (Avice, 2000, 2009; Hewitt, 2000). Given that increasing the number of case studies enabled us to compare the genetic structure among various

species with similar distribution, phylogeographic studies have constructed the history of regional biota in terms of climatic changes. In particular, history of range expansion following retreats of glaciers was unambiguously elucidated in deciduous trees and alpine plants in Europe (Petit *et al.*, 2003; Schönswetter *et al.*, 2005). Since paleorecord regarding Pleistocene range shifts have been mostly confined to wind-pollinated species, the molecular phylogeographic approach could contribute much to infer the biogeographic history.

In contrast to the massive extent of continental glaciers in Europe and the North America during the glacial periods, occurrence of glacier in the Japanese archipelago was solely restricted in high mountains (Ono and Igarashi, 1992). Due to the ice-free areas in the Japanese archipelago, arctic-alpine plants had chance to expand their ranges into the Japanese archipelago. Boreal plants colonized into the archipelago during cold periods, whereas they experienced range contraction during warm periods including the current post-glacial period (Koizumi, 1919; Shimizu 1982). As a consequence, relict populations of alpine plants were found in high mountains in the Japanese archipelago. Following to such geological history, alpine plants in the Japanese archipelago was hypothesized to be relict plants that migrated into the archipelago during the last glacial period.

However, the previous phylogeographic studies mostly discarded this simple biogeographic history. Previous phylogeographic studies detected strong genetic differentiations between populations in central and northern Japan in most species investigated (Fujii *et al.*, 1997, 1999; Fujii and Senni, 2006). Because range expansion usually occurred by individuals with similar genetic variation (Hewitt, 1996), this geographic structure was unlikely to be explained by the single colonization history during the last glacial period. Thus, alpine plants in the Japanese archipelago were originated by at least two independent colonization events. In addition, populations in central Japan were persisted with isolation from northern populations (Ikeda *et al.*, 2009). Therefore, populations in central and northern Japan experienced vicariance history during the Pleistocene climatic oscillations.

Although genetic differentiation between central and northern Japan was unambiguous and consistent among various taxa, minor geographic structure was also found in regional scale, especially in the northern Japan. Unique haplotypes to populations in Tohoku region were found in *Potentilla matsumurae* (Ikeda *et al.*, 2006) and *Diapensia lapponica* (Ikeda *et al.*, 2008a) and

haplotypes in Tohoku was exclusively found in this region in *Cardamine nipponica* (Ikeda *et al.*, 2008b). In addition to the ancient vicariance as well as divergent history between central and northern Japan, relict history other than the post glacial range contraction may be plausible for the populations in Tohoku. Further investigation extending various taxa may make the relict hypothesis in Tohoku region more robust.

*Phyllodoce aleutica* is a perennial shrub growing around snowbed habitats in high mountains. Its range extends from high mountains in the Japanese archipelago to Aleutian and Alaska. Owing to the heather growth form, *P. aleutica* formed large populations and dominate the plant community around snowbed, especially in the Japanese archipelago. Therefore, this species is a good candidate to evaluate the phylogeographic history of alpine plants growing around snowbed.

In this study, we conducted phylogeographic structure of *Phyllodoce aleutica* (Spreng.) A. Heller (Ericaceae). Based on genetic structure of chloroplast DNA (cpDNA), we attempted to examine (i) the unique genetic structure in central Japan and (ii) genetic specificity in Tohoku region.

## Materials and Methods

### Sampling

Leaf material of *P. aleutica* was sampled from 10 populations in the Japanese archipelago (Table 1, Fig. 1) and 87 individuals were included in the analysis. All leaf samples were collected from individuals as distant from each other as possible, dried in silica gel, and stored at room temperature. Voucher specimens of all populations were deposited in the herbarium of Kyoto University (KYO).

### DNA extraction and sequencing

The dried leaf materials were freeze-dried using liquid nitrogen and then ground into fine powder. DNA was extracted by the CTAB method following the previous studies (Ikeda and Setoguchi, 2007). The extracted DNA was dis-

Table 1. Number of populations, location, coordination, numbers of analysed individuals (n), and haplotype composition of 10 populations of *Phyllodoce aleutica*

No.	Location	Coordination		Haplotype composition						
				n	A	B	C	D	E	F
Hokkaido										
1	Daisetsu-san	43.528	142.821	3		3				
Tohoku										
2	Choukai-san	39.097	140.047	10						
3	Gassan	38.544	140.027	10						3
4	Iide-san	37.851	139.679	7						7
Chubu										
5	Shirouma-dake	36.783	137.753	10	5		2		3	
6	Harinoki-dake	36.100	137.683	10	7		3			
7	Norikura-dake	36.114	137.557	10			1		9	
8	Ontake-san	35.887	137.485	7			1		6	
9	Kisokomagatake	35.786	137.807	10	4		4		2	
10	Hakusan	36.155	136.770	10					10	
Total				87						

solved in 100  $\mu$ L TE buffer and used as a template for the polymerase chain reaction (PCR). Two pairs of primers were used to amplify the *trnL* intron (Taberlet *et al.*, 1991) and an intergenic spacer (*rpl20–5'rps12*; Hamilton, 1999). PCR amplification was conducted in a total reaction volume of 25  $\mu$ L containing 14.7  $\mu$ L of autoclaved ion-exchanged water, 4  $\mu$ L of 2.5 mM dNTP mixture, 5  $\mu$ L of 10 $\times$  Ex Taq Buffer (Takara Ex Taq; Takara, Kyoto, Japan), 0.625 U of Ex Taq (Takara), 0.2  $\mu$ M of each primer and 1.25  $\mu$ L of DNA. Amplification was performed with an initial denaturation of 2 min at 94 $^{\circ}$ C followed by 30 cycles of denaturation of 1 min at 94 $^{\circ}$ C, annealing of 1 min at 50 $^{\circ}$ C and extension of 1 min at 72 $^{\circ}$ C. Following amplification, products were visualised on 0.5% TAE-agarose gels stained with ethidium bromide. PCR products were sequenced using the standard methods of the BigDye<sup>TM</sup> Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) using two primers [*trnL*(c), Taberlet, 1991; *rpl20*, Hamilton, 1999] on an ABI Genetic Analyzer 3100 (Applied Biosystems).

#### Data analysis

All sequence data were analysed and aligned using an Auto Assembler (Applied Biosystems). cpDNA haplotypes were determined based on

these aligned sequences, and a parsimony network was constructed using the programme TCS1.06 (Clement *et al.*, 2000). All indels were treated as substitutions. The geographic structure across populations in the Japanese archipelago was statistically evaluated by analyses of molecular variance (AMOVA) using Arlequin 3.1 (Excoffier *et al.*, 2005). The significance of differentiations were examined by 10 000 permutation tests.

## Results

#### *Phylogenetic relationships amongst haplotypes and geographic distribution of haplotypes*

The 512 or 513 bp and 715 or 716 bp were used for analysing genetic variations on *trnL* intron and *rpl20–5'rps12*, respectively. All sequences were deposited in DDBJ (Accession No. AB811032–AB811038). In total, four substitutions and one indels were found across samples, and seven haplotypes were identified (Table 2). The phylogenetic relationships among haplotypes were revealed in the parsimony network (Fig. 1). Their relationship was not unambiguously resolved perhaps due to recurrent mutations on the same site.

The distribution of haplotype exhibited geographic structure. Four haplotypes were found exclusively in central Japan (A, C, D, E; Fig. 1,

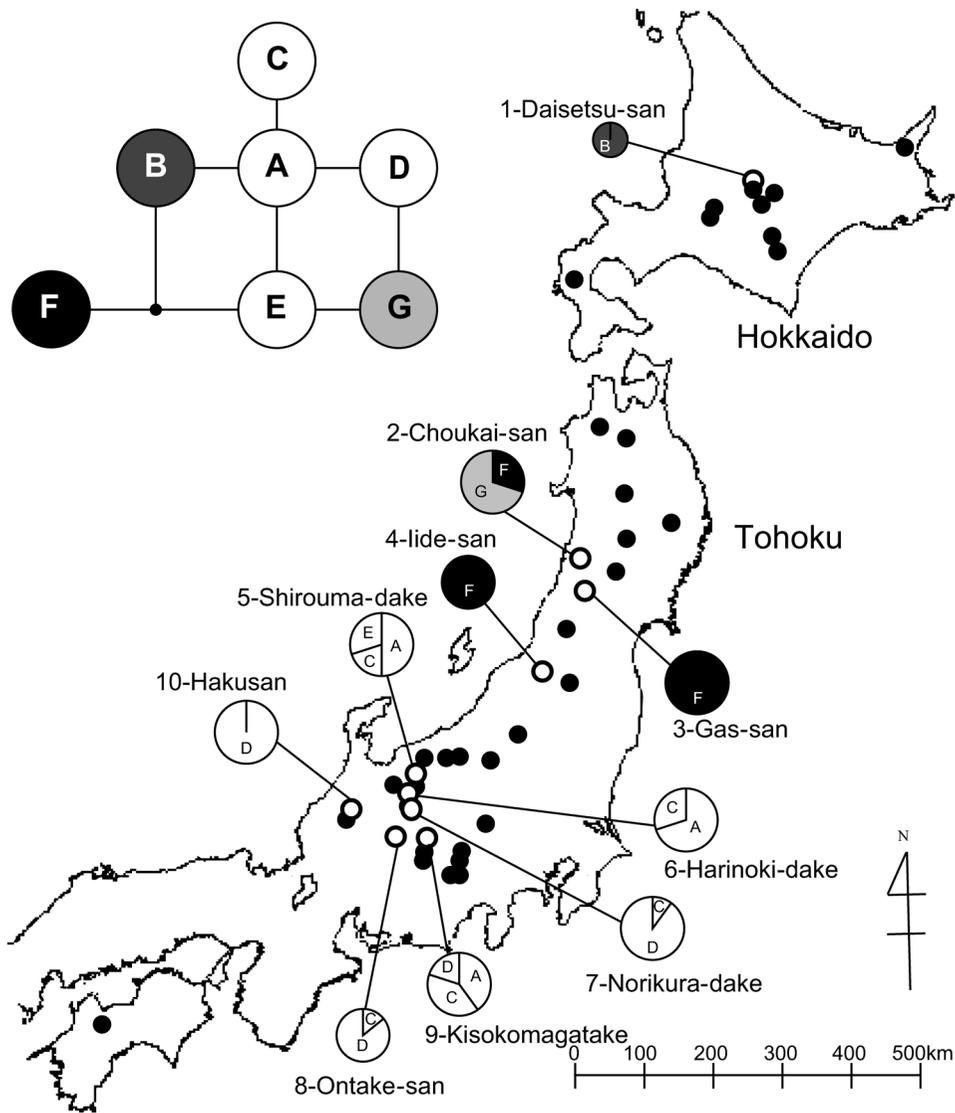


Fig. 1. Haplotype distribution of *Phyllodoce aleutica* in the Japanese archipelago and their relationship. The black and white circles on the map indicated the major populations of *P. aleutica* and analyzed ones in this study, respectively. Pie charts represent the haplotype frequency of each population (chart colours correspond to those in the network but those were not distinguished in central Japan). The details were shown in Table 1. Relationship among haplotypes was shown by parsimony network, where a black dot without alphabets represents a missing haplotype.

Table 1). While haplotype C was widespread and found in five of six populations, haplotypes A and D were dominated in most populations (Table 1). Two haplotypes were unique to Tohoku region (F, G), in which haplotype F was common among three populations (Fig. 1, Table 1). Daisetsu-san in Hokkaido was dominated by

haplotype B that occurred exclusively in this population among populations analysed in this study.

*Population differentiation and geographic structure*

According to AMOVA, a large portion of vari-

Table 2. Polymorphic sites and types of mutations in two regions of cpDNA. The number in parentheses represents the total number of analyzed sequences

Haplotype	<i>trnL</i> intron (512–513 bp)		<i>rpl20–5'rps12</i> (715–716 bp)		
	220	217	522	616	656
	G/T	A	G/T	G/A	G/A
A	G	–	G	G	G
B	G	+	G	G	G
C	G	–	G	G	A
D	G	–	G	A	G
E	T	–	G	G	G
F	T	+	T	G	G
G	T	–	G	A	G

Table 3. Results of analyses of molecular variance (AMOVA) of cpDNA sequence data from populations of *Phyllodoce aleutica* in the Japanese archipelago. Population numbers for each group are shown in parentheses

Source of variation	Percentage of variation (%)	Fixation Index	<i>P</i> value
Among populations (1–10)	64.99	0.64995	***
Within populations	35.01		
group 1: Hokkaido & Tohoku (1–4) vs. Chubu (5–10)			
Among groups	57.83	0.5783	**
Among populations within groups	24.54	0.8237	***
Within populations	17.63	0.5819	***
group 2: Tohoku (2–4) vs. Hokkaido & Chubu (1, 5–10)			
Among groups	60.32	0.6032	*
Among populations within groups	22.75	0.8307	***
Within populations	16.93	0.5733	***
group 3: Hokkaido (1) vs. Tohoku (2–4) vs. Chubu (5–10)			
Among groups	61.11	0.6111	**
Among populations within groups	21.30	0.8241	***
Within populations	17.59	0.5476	

\*\*\*\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

ation was explained by among populations across analyzed populations (65%,  $P < 0.001$ ; Table 3). By examining genetic differentiation among population groups based on three geographic regions (Hokkaido, Tohoku, and central Japan), the largest genetic variation was explained when each region was treated as distinct group (61%,  $P < 0.01$ ; Table 3). Combining Hokkaido with central Japan explained more variation among groups (60%,  $P < 0.01$ ; Table 3) than combining it with populations in Tohoku (58%,  $P < 0.01$ ; Table 3).

### Discussion

According to previous phylogeographic stud-

ies, alpine plants in the Japanese archipelago harboured unambiguous genetic differentiation between populations in central Japan and Tohoku and Hokkaido, northern Japan (Fujii and Senni, 2006; Senni *et al.*, 2005, but see Ikeda and Setoguchi, 2006, 2009). This genetic differentiation common to various taxa indicates that alpine plants experienced distinct biogeographic history between central and northern Japan; i.e., isolated populations have been persisted in high mountains in central Japan during Pleistocene climatic oscillations, while populations in northern Japan were originated from a single range expansion. Furthermore, this biogeographic history implies that alpine plants in the Japanese archipelago have colonized the archipelago at least twice

(Fujii *et al.*, 1997).

As consistent with the previous findings, the present investigation of cpDNA variation exhibited unique genetic structure of *P. aleutica* in central Japan (Tables 1, 3). Thus, the present genetic structure of *P. aleutica* would be explained by the previous biogeographic history of alpine plants. Populations of *P. aleutica* were persisted in high mountains in central Japan regardless of range shifts following Pleistocene climatic oscillations. In addition to the population persistence in central Japan, populations in northern Japan would be originated from another colonization process.

In contrast, no haplotypes were shared between populations in Tohoku and Hokkaido (Fig. 1, Table 1). This pattern of haplotype distribution in northern Japan was rare for other alpine plants. In addition, results of AMOVA showed that more variation was explained by among groups when assigning population in Hokkaido into a population group of central Japan (60%; Table 3, group 2) than into that of Tohoku (58%; Table 3, group 1). Furthermore, separating Hokkaido from each group of Tohoku and central Japan explained more variation among groups (61%; Table 3, group 3). These results indicate that northern Japan did not harbour homogenous genetic structure but did geographic differentiation between Hokkaido and Tohoku. The genetic affinity of Hokkaido with central Japan was attributed to their closer relationship of haplotypes than the relationship of haplotypes found in Tohoku and Hokkaido (Fig. 1). It was not implausible that this close relationship may represent a recent population divergence between these two disjunct regions. However, our study analysed solely a part of chloroplast regions, which may not resolve a robust phylogenetic relationship as shown in a network (Fig. 1). Accordingly, our study was not sufficient to infer the divergent process of populations as well as the regional biogeographic history in the Japanese archipelago. Nevertheless, the genetic uniqueness among regions suggests a distinct history not only in populations in central Japan

but also those in each of Tohoku and Hokkaido.

The important caveat of our result is the number of analysed populations and individuals. Because we analysed only three individuals from Daisetsu-san, the genetic structure of Hokkaido was not sufficiently elucidated. This implies that populations in Hokkaido may harbour haplotypes in Tohoku region. Nevertheless, regardless of our analysis including a number of samples from the major populations of *P. aleutica* in Tohoku, haplotypes in Hokkaido was not detected. This implies that unique haplotype in Hokkaido, haplotype B, may not distribute in Tohoku. Although further sampling may weaken the genetic differentiation between Tohoku and Hokkaido, a single colonization by restricted individuals would be unlikely for explaining the present genetic differentiation. Rather, more complicated history such that isolated populations persisted in both Tohoku and Hokkaido may be plausible for the origin of alpine plants in northern Japan.

Although a single colonization history is the major inference of origin of alpine plants in northern Japan, several studies found haplotypes that occurred exclusively in single populations of Tohoku or were unique to Tohoku region. For example, *Diapensia lapponica* and *Potentilla matsumurae* harboured unique haplotypes in some populations in Tohoku (Ikeda *et al.*, 2006, 2008a) and haplotypes in Tohoku were regional specific in *Cardamine nipponica* (Ikeda *et al.*, 2008b). These genetic structures in northern Japan were weaker than the genetic differentiation between central and northern Japan and any consistent tendency has not been detected across species. Therefore, although previous studies noted the persistence of populations in Tohoku following Pleistocene range shifts, such biogeographic history was not consistently supported.

Because phylogeographic studies of Japanese alpine plants mostly focused on the genetic differentiation as well as the vicariance history between northern and central Japan, less is inferred regarding to the detailed regional history. The present genetic structure of *P. aleutica* emphasized the persistent populations of alpine

plants in Tohoku as some studies noted. Future study focusing on the population history in Tohoku would be important to learn the more detailed population dynamics of alpine plants in the Japanese archipelago in terms of climatic oscillations.

### Acknowledgments

This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and The Japan Society for the Promotion of Science (13575011).

### References

- Avise, J. C. 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge.
- Avise, J. C. 2009. Phylogeography: retrospect and prospect. *Journal of Biogeography* 36: 3–15.
- Clement, M., Posada, D. and Crandall, K. A. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1659.
- Excoffier, L., Laval, G. and Schneider, S. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47–50.
- Fujii, N. and Senni, K. 2006. Phylogeography of Japanese alpine plants: biogeographic importance of alpine region of central Honshu in Japan. *Taxon* 55: 43–52.
- Fujii, N., Ueda, K., Watano, Y. and Shimizu, T. 1997. Intraspecific sequence variation of chloroplast DNA in *Pedicularis chamissonis* Steven (Scrophulariaceae) and geographic structuring of the Japanese “Alpine” plants. *Journal of Plant Research* 110: 195–207.
- Fujii, N., Ueda, K., Watano, Y. and Shimizu, T. 1999. Further analysis of intraspecific sequence variation of chloroplast DNA in *Primula cuneifolia* Ledeb. (Primulaceae): implications for biogeography of the Japanese alpine flora. *Journal of Plant Research* 112: 87–95.
- Hamilton, M. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* 8: 521–523.
- Hewitt, G. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* 58: 247–276.
- Hewitt, G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* 405: 907–913.
- Hewitt, G. M. 2004. Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences* 359: 183–195.
- Ikeda, H. and Setoguchi, H. 2006. Phylogeography of *Arctericia nana* (Ericaceae) suggests another range expansion history of Japanese alpine plants. *Journal of Plant Research* 119: 489–495.
- Ikeda, H. and Setoguchi, H. 2007. Phylogeography and refugia of the Japanese endemic alpine plant, *Phyllodoce nipponica* Makino (Ericaceae). *Journal of Biogeography* 34: 169–176.
- Ikeda, H. and Setoguchi, H. 2009. The homogenous genetic structure and inferred unique history of range shifts during the Pleistocene climatic oscillations of *Arctericia nana* (Maxim.) Makino (Ericaceae). *Journal of Plant Research* 122: 141–151.
- Ikeda, H., Fujii, N. and Setoguchi, H. 2009. Application of the isolation with migration model demonstrates the Pleistocene origin of geographic differentiation in *Cardamine nipponica* (Brassicaceae), an endemic Japanese alpine plant. *Molecular Biology and Evolution* 26: 2207–2216.
- Ikeda, H., Senni, K., Fujii, N. and Setoguchi, H. 2006. Refugia of *Potentilla matsumurae* (Rosaceae) located at high mountains in the Japanese archipelago. *Molecular Ecology* 15: 3731–3740.
- Ikeda, H., Senni, K., Fujii, N. and Setoguchi, H. 2008a. Survival and genetic divergence of an arctic-alpine plant, *Diapensia lapponica* subsp. *obovata* (Fr. Schm.) Hultén (Diapensiaceae), in the high mountains of central Japan during climatic oscillations. *Plant Systematics and Evolution* 272: 197–210.
- Ikeda, H., Senni, K., Fujii, N. and Setoguchi, H. 2008b. Consistent geographic structure among multiple nuclear sequences and cpDNA polymorphisms of *Cardamine nipponica* Franch. et Savat. (Brassicaceae). *Molecular Ecology* 17: 3178–3188.
- Koidzumi, G. 1919. Genetic and floristic phytogeography of the alpine flora of Japan. *Botanical Magazine, Tokyo* 33: 193–222.
- Ono, Y. and Igarashi, Y. 1991. Natural History of Hokkaido. Hokkaido University Press, Sapporo (in Japanese).
- Petit, R. J., Aguinagalde, I., De Beaulieu, J. L., Bittkau, C., Brewer, S., Cheddadi, R., Ennos, R., Fineschi, S., Grivet, D., Lascoux, M., Mohanty, A., Müller-Starck, G., Demesure-Musch, B., Palmé, A., Martín, J. P., Rendell, S., Vendramin, G. G. 2003. Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* 300: 1563–1565.
- Schönswetter, P., Stehlik, I., Holderegger, R., Tribsch, A. 2005. Molecular evidence for glacial refugia of mountain plants in the European Alps. *Molecular Ecology* 14: 3547–3555.
- Senni, K., Fujii, N., Takahashi, H., Sugawara, T. and Wakabayashi, M. 2005. Intraspecific chloroplast DNA variation of the alpine plants in Japan. *Acta Phytotaxo-*

- nomica et Geobotanica 56: 265–275.
- Shimizu, T. 1982. The New Alpine Flora of Japan in Color. Vol. 1. Hoikusha, Osaka (in Japanese).
- Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.