Title	Ecotypic differentiation of northern plant species in mid-latitudes : the mechanisms of population maintenance in two ericaceous alpine species in northern Japan
Author(s)	和久井, 彬実; Wakui, Akimi
Degree Grantor	北海道大学
Degree Name	博士(環境科学)
Dissertation Number	甲第14332号
Issue Date	2021-03-25
DOI	https://doi.org/10.14943/doctoral.k14332
Doc URL	https://hdl.handle.net/2115/84540
Type	doctoral thesis
File Information	Akimi_Wakui.pdf

Ecotypic differentiation of northern plant species in mid-latitudes: the mechanisms of population maintenance in two ericaceous alpine species in northern Japan

Akimi Wakui

A Dissertation submitted to

Graduate School of Environmental Science, Hokkaido University

for the Degree of Doctor (Environmental Science)

2021

Contents

Summary

The pattern of geographic distribution and the genetic structure of marginal populations of Arctic-alpine plants often reflect the migratory and evolutionary histories of individual species. Many species of alpine plants in Japan are originated in Arctic tundra or boreal forests of high latitudes, and mountain regions in central Japan are located at the southern distributional edges for those species. Recent phylogeographic studies revealed that many alpine plants in Japan were classified into two phyletic groups, central Japan lineages and northern lineages. The lineages of central Japan commonly possess unique haplotypes different from the northern lineages, suggesting different origins.

In Hokkaido of northern Japan, several alpine plant species grow also in specific habitats at low elevations. However, the origin and maintenance mechanisms of the low-elevational populations are poorly understood. Given that Hokkaido was a gateway of migrations from northern regions to Japan for many Arctic-alpine plants during the glacial-interglacial cycles, phyletic lineages different from alpine populations may exist in the low-elevational populations. Because population size and environmental condition are very different between alpine and low-elevational habitats, furthermore, local adaptations to specific habitats might occur. In this study, I studied genetic and ecological characteristics of two ericaceous alpine species, *Vaccinium vitis-idaea* and *Rhododendron diversipilosum*, aiming to reveal how northern plants have migrated and maintained their populations under various environmental conditions in Japan.

In Chapter 1, I compared ploidy levels and genetic structures among *V. vitis-idaea* populations using microsatellite loci; for 30 populations in Japan and 11 populations

outside Japan. Although most alpine populations were diploid, low-elevational populations were mostly tetraploid. Diploid populations were distributed from Alaska to the Kuril Islands, whereas tetraploid populations were distributed from eastern Siberia to Sakhalin. There was a clear difference in genetic composition between diploid and tetraploid populations. In addition, the diploid populations in Japan were divided into two genetic groups: northern group and central group. In northern Japan, genetic differentiation among populations was greater in the tetraploids than in the diploids. The tetraploids showed lower clonal diversity within a population but higher genetic diversity among populations than the diploids. It is speculated that the diploids might migrate from Alaska via the Kuril Islands to Hokkaido, while the tetraploids might migrate from eastern Siberia via Sakhalin to Hokkaido. Large genetic differentiation among the tetraploid populations might be caused by the limitation of gene flow between populations and/or local adaptation to the specific habitats, because tetraploid populations are often isolated and exposed to different environments each other. High genetic diversity in the tetraploid populations despite low clonal diversity might be caused by the increasing number of alleles due to polyploidization.

In Chapter 2, I investigated reproductive performance and mating system of *V. vitis-idaea*, in order to reveal the mechanisms of population maintenance under different environmental conditions. In addition, morphological traits were compared among populations. Low-elevational populations of northern Japan (mostly tetraploids) and alpine populations of central Japan (diploids) showed moderate level of selfing rates under natural conditions, whereas alpine populations of northern Japan (diploids) showed negligibly small selfing rates. Pollination experiment conducted in northern Japan revealed that low-elevational populations showed moderate selfing ability, whereas alpine populations often showed obligate outcrossing as reported in the previous study. Even in the self-incompatible alpine populations, however, self-pollen germinated on stigmas and extended pollen tubes toward ovaries as outcross-pollen did. This suggests that the self-incompatibility in this species is regulated by early-acting inbreeding depression at the seed formation stage. The harmful effects of inbreeding depression might be mitigated by a polyploidization (for tetraploid populations) and/or past bottleneck event and subsequent purge effect of deleterious allele in the isolated small populations (for both of alpine populations in central Japan and low-elevational populations in northern Japan). Morphology of the tetraploid populations were generally characterized by larger sizes of plant organs, especially at the forest habitat. The increases in plant size and annual shoot growth in the low-elevational populations might be caused by a polyploidization and/or acclimation to local environments.

In Chapter 3, I investigated genetic structure, reproductive system, and morphological traits of *R. diversipilosum*, in the same way with *V. vitis-idaea* (Chapters 1 and 2). All *R. diversipilosum* populations in Japan and eastern Siberia were tetraploid. Although some genetic variations were detected among populations, there were no clear genetic differentiations across the habitat types. Genetic diversity and clonal diversity within a population were equally high across the habitat types. In addition, selfing rates under natural conditions were quite low in all populations, despite the selfing ability. Sizes of plant organs were significantly larger in the low-elevational populations, especially in the forest habitat. It is considered that all *R. diversipilosum* populations in Japan are originated in eastern Siberia, with lesser degrees of genetic differentiation and mating system variation compared to *V. vitis-idaea*. Large morphological plasticity might enable this species to acclimate to various environments after migration to Japan.

High outcrossing rate of *R. diversipilosum* seemed to reflect the high clonal diversity within a population and the generalist mating system characterized by frequent visitation of various pollinators.

Previous studies suggested that the separation of genetic groups in many Japanese alpine species might reflect the different migratory timing among lineages. However, little is known about the possibility that different lineages of single species might migrate to Japan separately through different pathways. For circumpolar Arctic-alpine species having wide distribution ranges across Eurasia to North America, Japanese populations might be composed of diverse lineages having different origins more than currently recognized lineages. Landscape from the westernmost Alaska to the Kuril Islands is dominated by tundra biome, whereas vegetation from eastern Siberia to Sakhalin is mostly occupied by boreal forests, i.e., taiga biome. Current habitats of diploid and tetraploid *V. vitis-idaea* populations seem to reflect their original environments. Therefore, habitat selection and population maintenance mechanisms of alpine plants in Japan may largely reflect their original environments, in addition to the species-specific ecological and genetic characteristics. Although *R. diversipilosum* showed less genetic and ecological differentiations in comparison with *V. vitis-idaea*, ecological characteristics of tetraploid populations were similar between the species, such as eastern Asia origin, growing at low-elevational habitats, selfing ability, and large plant size. These results indicate the contribution of polyploidization to the establishment and maintenance of alpine species in low-elevational environments.

General introduction

Geographic distributions of plant species, which are originated in northern regions, often reflect the migratory histories during the glacial–interglacial cycles in the Pleistocene (Hewitt 2004). This climatic oscillation created not only distributions of individual species but also distinct genetic structures among regional populations of single species. Recent phylogeographic analyses using molecular techniques have revealed the historical population dynamics of several Arctic-alpine species (Alsos *et al.* 2005; Winkler *et al.* 2012; Eidesen *et al.* 2013). These studies have provided remarkable insights into the histories of migration, adaptation, and species differentiation of Arctic-alpine plants at geographic scale.

These phylogenetic studies often emphasize the importance of marginal populations located at the distribution edges of individual species. Such marginal populations are usually isolated from one another because habitats that are suitable for a given species tend to exist quite locally and sparsely around the distribution edges (Vucetich and Waite 2003). Because of the geographic isolation and small population sizes, gene flow between local populations is often restricted by which the genetic structure of marginal populations may be maintained reflecting the migratory history of the species (Hampe and Petit 2005). For many plant species of Arctic elements, populations distributed in the mid-latitude mountains are recognized as marginal populations. Several previous studies reported that alpine populations isolated in the mid-latitude mountains often possess unique haplotypes, different from the high-latitude populations (e.g. DeChaine and Martin 2005; Schönswetter *et al.* 2005).

Marginal populations often have higher potential of adaptation to local environmental conditions (Abeli *et al.* 2014). From a view of population genetics, appearance of new alleles or fixation of advantageous mutations is especially beneficial in isolated populations (Kawecki 2008). Selfing ability and characteristics to combat inbreeding depression are thought to have evolved in isolated populations (Mee and Moore 2014) because genetic diversity often decreases in small populations, resulting in an increase in inbreeding or selfing (Arnaud-Haond *et al.* 2006; Mimura and Aitken 2007). Furthermore, several studies report the occurrence of polyploid populations at the margins of plant distribution ranges (Rivero-Guerra 2008; Karunarathne *et al.* 2018; Kudo and Hirao 2020). High ploidy levels may contribute to maintaining genetic variability and reducing negative effects of inbreeding depression, as well as improving the performance of vegetative properties (Soltis and Soltis 2000). Thus polyploidization may facilitate the maintenance of isolated populations and accelerate local adaptation (Abeli *et al.* 2014). Taken together, populations at the distributional margins are important from an evolutionary and conservation biology perspectives, as they greatly contribute to intraspecific variations of genetic and ecological traits, i.e., creation of genotypes, and sometimes resulting in local speciation (Kawecki 2008).

Many species of Japanese alpine plants are thought to have origins in northern regions, such as Arctic tundra and boreal forests, and populations in central Japan are located at the southern edges of the distribution ranges (Shimizu 1982). It is believed that the region from eastern Asia to western America, i.e., Beringia region, was not covered by ice sheets during the last glacial period (Frenzel 1968). Therefore, it is likely that Arctic and boreal plants migrated to Japan from both of eastern Siberia and North America via Sakhalin and/or the Kuril Islands during the last glacial period (Kawano 1971). In other words, Hokkaido was a contact zone of northern-element species migrated to Japan from different origins.

The distribution patterns of Japanese alpine plants are highly species-specific (Shimizu 1982). Some species grow only at the summits of high mountains, while others spread widely from alpine to subalpine zones. Additionally, southern limits of distributions are also different among species. Such species-specific distribution patterns of Japanese alpine plants might be caused by the migratory histories of individual species during the glacial periods, the differences in habitat characteristics in the original regions, such as Arctic tundra and boreal forests, and/or local adaptations in each habitat after migration. How alpine plants migrated to Japan and how they maintained local populations in the current habitat are important issues for understanding the determinants of plant distributions and the possibility of adaptation associated with climate change (Abeli *et al.* 2018).

Recent phylogeographic studies have revealed that populations of several alpine plant species are composed of different haplotypes between central Japan and northern Japan (Ikeda *et al.* 2008, 2009). This suggests that populations in central Japan may be old phyletic linages that migrated in former glacial periods and have survived repeated glacial and interglacial periods. On the other hand, northern phyletic linages are classified into the same haplotype with the Kuril Islands and Beringia regions, considered to have migrated during the last glacial period.

However, high mountains are not only habitat for northern species in Japan. Although most plant species originating in northern regions usually grow in alpine zone above the treeline in central Japan, some Japanese alpine species, such as *Vaccinium vitis-idaea* and *Rhododendron diversipilosum*, are distributed also at lower elevations in coniferous forests, coastal meadows, and high moors in northern Japan (Kawano 1971;

Sato 1995; Shimizu 2004). In these low-elevational habitats, adverse local conditions, such as cool temperature, oligotrophic soils, and strong winds, may suppress the growth of common lowland plants, resulting in a reduced competitive situation, and enable alpine plants to grow. Although species compositions and vegetation types of these specific habitats have been reported in several studies (Sato *et al.* 1993; Sato 1995; Wakui *et al.* 2017), phylogenetic or ecological studies of low-elevation populations have rarely been conducted in comparison with the typical alpine populations. Given that Hokkaido is the gateway of the migration of many alpine plants to Japan, specific phyletic lineages different from alpine populations may exist in the low-elevation populations. Because low-elevational populations are commonly small, isolated, and exposed to different environmental conditions from alpine populations, they may possess specific life-history strategies, genetic structure and/or morphological traits that are adapted to local habitat conditions at low elevations. Consequently, finer-scale phylogenetic analysis and ecological surveys in northern Japan may provide us new insights into the migratory and evolutionary histories of Japanese alpine plants.

In this study, I studied the formation of present distributions and the maintenance mechanisms of local populations of two ericaceous alpine plant species in Japan, on the basis of genetic and ecological perspectives. In Chapters 1 and 2, I focused on *Vaccinium vitis-idaea*, which is a typical Arctic-alpine plant distributed widely across northern hemisphere (Hulten 1968). In Chapter 1, I estimated the origins and genetic differentiations of Japanese *V. vitis-idaea*, by analyzing ploidy levels and microsatellite fragments of 30 populations in Japan and 11 populations in northern areas outside Japan. In Chapter 2, I studied how *V. vitis-idaea* have adapted to and maintained populations in various environments, from surveys of the reproductive system and morphological variations. In Chapter 3, I conducted genetic analysis and ecological surveys of *Rhododendron diversipilosum*, a species mainly distributed in boreal forests in eastern Asia, and compared its origins and the population maintenance system with *V. vitis-idaea*. Throughout the study, I aimed to reveal how northern plant species of various origins have migrated and maintained their populations under various environmental conditions in Japan. Finally, I discussed how the present studies contribute to the further understandings of distribution patterns of northern plants and adaptation to local habitats.

Chapter 1: Variations in genetic structure and ploidy levels of *Vaccinium vitis-idaea* populations in Japan

1.1 Introduction

About 25 % of Japanese alpine plant species are distributed in wide range across northern hemisphere, so called “Arctic-alpine plants” (Shimizu 1982). These species repeated range expansion and contraction associated with past glacial-interglacial cycles, resulting in the distinct genetic structure in the Arctic region (Hewitt 2000). Genetic structures of Arctic-alpine plants often reflect the geographical barriers, such as ice sheet or straits, and many of them possess different genetic lineages between Eurasia and North America (Abbott *et al.* 2000; Alsos *et al.* 2005). During the last glacial period, the regions from eastern Asia to Alaska were connected by a land bridge, which was called Beringia, and not covered with ice sheet (Frenzel 1968). Therefore, species compositions and genetic structures of plants in Beringia are historically related to both North America and Eurasia regions. However, the geographic pattern of intraspecific genetic variations in Arctic-alpine plants is variable among species. For instance, same lineage with North America is distributed in Beringia in *Rubus chamaemorus*, same lineage with Eurasia is distributed in Beringia in *Betula nana*, and both of North American and Eurasian lineages exist in Beringia in *Cassiope lycopodioides* (Eidesen *et al.* 2013). Because mountain regions in Japan are located in the southern edge of many Arctic-alpine plants, multiple lineages having different origins may exist in Japan.

During the past few decades, many phylogeographic and population genetic studies on Japanese alpine plants have been conducted. These studies revealed that populations distributed in the alpine areas of central Japan are composed of unique haplotypes, while populations in northern Japan showed similar haplotypes with populations at higher latitudes in Beringia (e.g. Ikeda *et al.* 2008, 2009). This indicates that the historical background of alpine plants distributed in central Japan is different from alpine plants in northern Japan, i.e., lineages in central Japan might immigrated to Japan during earlier glacial periods than lineages in northern Japan.

From the perspective of population genetics, alpine plants in central Japan tend to possess lower genetic diversity within a population and higher genetic differentiation among populations. This trend may be explained by small population size and strong isolation in southern populations than in northern populations (Hampe and Petit 2005). For instance, *Dryas octoptala*, a typical Arctic-alpine species, shows low genetic diversity but highly differentiated genetic structure in the populations of central Japan in comparison with northern populations (Hirao *et al.* 2017). Therefore, isolated populations at the marginal distribution area are exposed to high risk of regional extinction, while they are important driving force of diversification and evolution of Arctic-alpine plants. However, our ecological and evolutionary understanding of alpine plants at the marginal regions is limited.

Interestingly, some alpine species are distributed also at lower elevations in northern Japan. It is speculated that low-elevational populations of Arctic-alpine species may be different lineages from typical alpine populations, but such kind of aspects have been overlooked in the previous studies. *Vaccinium vitis-idaea*, which is distributed also at low elevations in northern Japan, is a typical Arctic-alpine species. Eidesen *et al.* (2013) conducted an AFLP analysis for *V. vitis-idaea* populations and reported the existence of different genetic lineages in eastern Asia and western part of North America. Ikeda *et al.* (2015) reported little genetic differentiations among populations along a latitudinal gradient in Japan when only alpine populations were compared. On the other hand, Shimokawabe *et al.* (2016) found that there are clear genetic difference between alpine and low-elevational populations in Hokkaido, suggesting the existence of different lineages between alpine and low-elevational habitats. Low-elevational populations of alpine plants may be regarded as the populations at a margin of the distribution range, judging from small population size, isolation from other populations, and large difference in the environmental condition from typical alpine habitat (Shimokawabe *et al.* 2015). Therefore, it is expected that low-elevational populations may have high evolutional potential for local adaptation and differentiation. Nevertheless, phylogeographic analysis and population genetics of low-elevational populations have rarely been studied for alpine plants.

In this chapter, I analyzed microsatellite fragments of *V. vitis-idaea* populations in Japan in order to evaluate the genetic traits of low-elevational populations compared to alpine populations. I also analyzed genetic relationships between Japanese populations and northern populations outside of Japan. In particular, I aim to clarify (1) the historical background of *V. vitis-idaea* populations in Japan, including low-elevational populations, and (2) the degree of genetic diversity and differentiation between low-elevational populations. Discussing the genetic characteristics of Japanese alpine plants, considering their origin, migration timing, and variation in the genetic structure, may contribute to deeper understanding of the migration history and evolutionary process of alpine plants in Japan.

1.2 Methods

Study species and study sites

Vaccinium vitis-idaea (Fig. 1-1) is an evergreen dwarf shrub (5–30 cm in height) with creeping stems (Ritchie 1955) that sometimes expands via stolon to form large clonal patches (> 30 m in diameter) (Persson and Gustavsson 2001). This species is broadly distributed in tundra wilderness, boreal forests and bogs across the northern hemisphere (Garkava-Gustavsson *et al.* 2005), and it mainly inhabits alpine regions in mid-latitudes. In Hokkaido, it grows mainly in alpine zone but also exists locally at lower elevations in unique environments, such as algific talus slopes and coastal grasslands. Flowering occurs in early summer, flowers are pollinated by bees, and fruits are dispersed by birds and mammals (Ritchie 1955). A partial self-incompatibility leads to reduced numbers of developed seeds due to early acting inbreeding depression (Guillaume and Jacquemart 1999). This species is commonly known to be diploid ($2n = 2x = 24$, Ritchie 1955), including Japanese alpine populations (Shimizu 1982). Based on the phylogeographic analyses by Ikeda *et al.* (2015), *V. vitis-idaea* populations in Japan are thought to have persisted since before the last glacial period.

During 2016–2019, I collected one leaf per shoot from 20–30 patches in each of 39 populations (22 in Hokkaido, 8 in Honshu, and 9 outside of Japan; Fig. 1-2, Table 1-1). Japanese populations were classified into four habitat types on the basis of elevation and growing environment as follows: alpine (typical alpine environment above the timberline: 1400–2800 m), open montane (open grassland in the montane zone: 200–900 m), montane forest (algific talus slopes surrounded by coniferous forests: 200–900 m), and coastal mire (coastal wet grassland or moors: 0–100 m). Generally, alpine populations are large and continuously distributed in each mountain area, while most low-elevational populations are small and isolated from one another. All sampling points in Japan were mapped using a GPS (GPSmap 64s, Garmin). In addition, leaves of specimens collected outside Japan were sampled from the herbarium of Hokkaido University Museum (SAPS); 11 samples from Sakhalin and 17 samples from Kuril Islands, respectively. In total, 991 samples from 41 populations were analyzed. Leaf samples were kept with silica gel and preserved at room temperature before analysis.

DNA extraction and fragment analysis

DNA extraction was conducted by the cetyltrimethylammonium bromide (CTAB) method. In brief, each sample was crushed in CTAB extraction buffer, chloroform-isoamyl alcohol was added, the aqueous layer containing the DNA was separated, the DNA was purified with isopropanol and ethanol, and the DNA was preserved in Tris–EDTA buffer. Eight microsatellite loci developed for a related species *Vaccinium microcarpon* (vm04249, vm09532, vm48827, vm51985, vm51409, vm10462, vm01649, vm89040; Zhu *et al.* 2012) were amplified using polymerase chain reaction (PCR). I used the following PCR program: 5 min at 95 ºC, followed by 30 cycles of 30 s at 95 ºC, 90 s at 60 ºC, and 30 s at 72 ºC with a final rest for 30 min at 60 ºC. Microsatellite fragments were analyzed using the Applied Biosystems 3730 Genetic Analyzer, and the genotypes were coded using Gene Mapper ver. 4.0 (Applied Biosystems). Gene Scan 500 LIZ Size Standard (Applied Biosystems) was used as the size standard. I calculated the number of genets of all populations, treating samples with the same genotypes in all loci as the same individual (genet).

Ploidy analysis and chromosome observation

The microsatellite analysis revealed that some populations showed more than two microsatellite peaks, which was unexpected for diploid species. Thus, I suspected the existence of polyploid populations. To test this possibility, I conducted a ploidy analysis based on the amount of DNA using a flow cytometry (Partec PA; Partec GmbH, Münster, Germany). From one to twelve flesh leaves from 38 populations (all sampling sites except for KAM) were used for ploidy analysis. Leaves were chopped in 0.2 mL of nuclei extraction buffer (CyStain UV precise P; Partec, Münster, Germany). After filtration through a 30-μm nylon mesh, crude nuclear samples were stained with 0.8 mL 4,6-diamidino-2-phenylindole (DAPI) solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1 % (w/v) PVP K-30, 0.1 % (v/v) Triton X-100, and 2 mg L^{-1} DAPI (pH 7.5) (Mishiba *et al.* 2000), incubated for 3 minutes at room temperature and relative DNA contents were measured.

In addition, direct observation of chromosome number was conducted. Seeds from three populations, one indicating increased number of microsatellite peaks (EN1) and two indicating one or two peaks per locus (KAU, UR), were sown on agar medium and germinated under the condition of 15 ºC dark and 25 ºC bright (12 h / 12 h). Root tips of seedlings were used for the chromosome observation. Root tips were immersed to 0.002 M 8-hidroxyquinoline for 24 hours and fixed in the compound liquid of acetic acid and ethanol (ratio of 1:3) for at least three hours. Then, cells were dissociated in 60 ºC hydrochloric acid for 10 minutes. Finally, samples were stained with acetic dahlia for 15 minutes and observed using a light microscope (BX43, Olympus).

Genetic structure

I assessed genetic similarity between the populations by discriminant analysis of principal components (DAPC) to reveal genetic structure of *V. vitis-idaea*. DAPC is a methodological approach to visualize genetical similarity among populations, that requires data transformation using a principal component analysis (PCA) as a prior step to a discriminant analysis (DA) (Jombart 2008). DA partitions genetic variation maximizing differences between clusters but minimizing within-cluster variation. I used Bruvo distances (Bruvo *et al.* 2004), which is particularly recommended for analyses with mixed-ploidy data (Dufresne *et al.* 2014). All genets were treated as unit of analysis. I implemented DAPC analysis in R 3.3.0 using the package poppr (Kamvar *et al.* 2014). DAPC analysis was conducted for all populations and for Japanese populations only. In addition, the analyses were conducted separately for diploid and tetraploid populations in northern Japan.

Gene flow among populations

To reveal the degree of gene flow among Japanese populations, the correlation between the *Rho* value and geographic distance (isolation by distance (IBD): Wright 1943) was examined based on the Mantel tests. I obtained *Rho* values (Ronfort *et al.* 1998) as the genetic differentiation indices for each pair of populations, because *Rho* statistics exhibit identical expectations for population differentiation in different ploidy levels under identical gene flow conditions (Hardy and Vekemans 2002). I calculated the geographic distance as three-dimensional straight-line distances considering elevational differences, calculated by QGIS 2.8.0. Isolation by distance was tested for all populations and for subsets of populations of different ploidy levels on varied spatial scales: all diploid populations, all tetraploid populations, diploid populations in Hokkaido, tetraploid populations in Hokkaido, and diploid populations in Honshu.

Genetic diversity within populations

To compare intra-population genetic diversity, I calculated clonal diversity, heterozygosity, and allelic richness of all Japanese populations. Clonal diversity was calculated based on Shannon-Wiener diversity (*H*) for 30 populations in Japan, considering abundance and evenness of genotypes (using R-package polysat: Clark and Jasieniuk 2011). Allelic richness (*Ar*), expected heterozygosity (*He*), and observed heterozygosity (*Ho*) were calculated as genetic diversity indices using SPAGeDi 1.5a (Hardy and Vekemans 2002). I conducted ANOVA for comparisons of clonal diversity, *Ar*, *He*, and *Ho*. Ploidy level (diploid, tetraploid), habitat type (alpine, open montane, montane forest, coastal mire), and geographic location (northern Japan, central Japan) were used as fixed effects.

1.3 Results

Ploidy levels of Vaccinium vitis-idaea in Japan and surrounding areas

Vaccinium vitis-idaea had been reported to be a diploid species with 24 chromosomes, as mentioned above (Ritchie 1955). Although one or two microsatellite peaks were expected for diploid species, genetic samples from 14 populations showed three or four peaks per locus. Flow-cytometry analysis revealed that all populations with more than two microsatellite peaks had twice as much DNA content as populations with one or two peaks (Fig. 1-3a). This indicates that there were two ploidy levels in the studied *V. vitis-idaea* populations. In direct chromosome observation, individuals in the KAU and

UR population, which showed only one or two microsatellite peaks and lower DNA content, had 24 chromosomes (Fig. 1-3bc), while EN1 population, which showed three or four microsatellite peaks and higher DNA content, had 48 chromosomes (Fig. 1-3d). Therefore, I concluded that populations with lower DNA content were diploid ($2n = 2x = 24$), and populations with double the amount of DNA content were tetraploid ($2n = 4x = 48$).

Distributions of diploid and tetraploid *V. vitis-idaea* in Japan were altitudinally separated (Fig. 1-2). Diploid populations were mainly distributed in alpine environment, higher than 1400 m above the sea level, except for three low-elevational populations along the Pacific coast in eastern Hokkaido (UR, OC, and ES). The tetraploid populations were mostly distributed at low elevations, except for one alpine population of the northern island in Hokkaido (RI). Outside Japan, tetraploids were only found at eastern Siberia and Sakhalin, while diploids occurred in Norway, Alaska, Kuril Islands, and eastern Siberia. Three populations in eastern Siberia were mixed-ploidy, where diploid and tetraploid individuals coexisted.

Genetic differentiation among populations

In the microsatellite analysis, I recorded a total of 66 alleles; 19 were found exclusively in the tetraploid populations, while the diploid populations had only four unique alleles. At the population level, one diploid population carried one unique allele each, and two tetraploid populations carried between one and two unique alleles each (Table 1-2). Among 991 leaf samples from all populations, 437 genets were recognized. The number of genets varied from 1 to 29 per population, indicating the existence of large clonal structures in some tetraploid populations and diploid populations in central

Japan, such as EN2, TEI, KAM, TES, HAM, KO, NAG, TA and HO (Table 1-2).

In the DAPC analysis, clear genetic difference was detected between the diploid and tetraploid populations (Fig. 1-4a). Japanese and Siberian tetraploids, including samples from Sakhalin, formed a single genetic group, whereas diploid populations in Japan were plotted near the populations in the Kuril Islands and Alaska. Three populations in Norway greatly diverged from the populations in other regions. Furthermore, Japanese diploids were divided into two groups, the group of Hokkaido and northern Honshu and the group of central Honshu (Fig. 1-4b). Taken together, there were three genetic groups in Japanese *V. vitis-idaea* populations; diploids in northern Japan, diploids in central Japan, and tetraploids at low elevations in northern Japan. DAPC analysis conducted for the populations with the same ploidy level in northern Japan showed that tetraploids had clearer genetic differentiation among populations than diploids (Fig. 1-4cd).

Mantel tests revealed that the effect of IBD was significant among all populations ($r = 0.23$, $P = 0.01$; Fig. 1-5a), all diploid populations ($r = 0.49$, $P = 0.01$; Fig. 1-5b), diploid populations in Hokkaido ($r = 0.41$, $P = 0.04$; Fig. 1-5c), and diploid populations in Honshu ($r = 0.62$, $P = 0.03$; Fig. 1-5d). However, there was no significant IBD among the tetraploid populations, both in full range of Japan ($r = 0.02$, $P = 0.47$; Fig. 1-5e) and Hokkaido ($r = 0.12$, $P = 0.16$; Fig. 1-5f).

Genetic diversity within populations

ANOVA comparison revealed that clonal diversity (*H*) was higher in the diploid populations than in the tetraploid populations ($P < 0.05$; Fig.1-6a), while the effect of habitat type or geographic location on genetic properties was not detected ($P > 0.1$). Indices of genetic diversity within a population (*Ar*, *He*, *Ho*) were greater in the tetraploid populations than in the diploid populations ($P < 0.001$; Fig. 1-6bcd). There were no significant differences in the genetic properties among habitat types and between geographic locations ($P > 0.1$).

1.4 Discussion

The genetic analysis revealed the existence of tetraploid *V. vitis-idaea* in most low-elevational populations. This is the first discovery of tetraploid populations in *V. vitis-idaea* ($2n = 4x = 48$) as this species had long been recognized as diploid ($2n = 2x = 24$; Ritchie 1955). The DAPC analysis revealed the clear genetic differentiation between diploids and tetraploids in Japanese populations. This means that tetraploid populations at low elevations have a genetically different origin from alpine diploid populations. This result is consistent with the report of Shimokawabe *et al.* (2016), and reinforces the generality of ecotypic differentiation between different elevations in this species.

At wider geographic scale, tetraploid *V. vitis-idaea* populations were only found in Siberia and Sakhalin. Japanese tetraploid populations formed a single genetic group, genetically similar to the tetraploid populations in Sakhalin and Siberia. This indicates that tetraploids in Japan may have a monophyletic origin, probably migrated to Japan from eastern Siberia via Sakhalin. On the other hand, the populations in Norway, Alaska, the Kuril Islands, and a part of eastern Siberia were diploid. The diploid populations in northern Japan were genetically similar to the populations in the Kuril Islands and Alaska, indicating that northern diploids might have migrated from North America via the Kuril Islands. In most previous studies, Japanese Arctic-alpine plants were considered to be originated from Beringia, and the populations in the Kuril Islands and Sakhalin were recognized to be similar genetic lineages with Japanese populations.

Although the Japanese archipelago is located immediately south of Beringia between Eurasia and North America, this is the first report that genetic lineages originating in different continents were found in Japanese alpine plants within a same species.

Two genetic groups were detected in the diploid populations in Japan; the group of northern Japan and the group of central Japan. Genetic divergence between the populations of northern and central Japan have been reported in several Arctic-alpine species (e.g. Ikeda *et al.* 2008, 2009), and mostly explained by differential migration timing. Unlike this study, no significant genetic divergence was detected in *V. vitis-idaea* populations between northern and central Japan by Ikeda *et al.* (2015). This inconsistency might be partly caused by the difference in genetic markers. The previous study was analyzed using markers of DNA regions with slow mutation rates, such as chloroplast DNA, while the microsatellite region used in this study is known to have high mutation rates (Varshney *et al.* 2005).

Genetic differentiation among populations was smaller in the diploid populations than in the tetraploid populations. IBD was significant in the pairs of all combination of diploid populations (Fig. 1-5). It is expected that gene flow occurs between diploid populations, which are geographically located close each other. Because alpine populations, especially in Hokkaido, are relatively large and exist continuously, gene flow via seed dispersal may be possible among populations in neighboring mountains, resulting in small genetic divergence among populations. In contrast, the lack of significant IBD in the low-elevational tetraploid populations might be because of isolated distributions of local populations accompanied with geographical barriers, and it may result in large genetic divergence among populations. On the other hand, local adaptation might accelerate the degree of inter-population genetic divergence (Kawecki

2008). Alpine populations are mostly distributed in similar environments with cold climate, strong wind, and little snow cover on ridges, whereas the low-elevational populations are isolated in local habitats of various environmental conditions in terms of local temperature, soil, and light conditions. Therefore, local adaptation is expected to occur in the tetraploid populations more frequently than in the alpine diploid populations. Furthermore, Increased genome size and high selfing rate of tetraploids may contribute to acceleration of local adaptation (Abeli *et al.* 2014).

Clonal diversity was lower but genetic diversity indices (*Ar*, *He*, *Ho*) were larger in the tetraploid populations in comparison with the diploid populations. Although low-elevational populations in northern Japan are not located at margins of the geographical distribution range, their population sizes are quite small and each population is strongly isolated from other populations. Therefore, low clonal diversity of the tetraploid population might have been affected by the past bottleneck events and/or limited gene flow among populations (Shimokawabe *et al.* 2016). The large allelic richness and heterozygosity may be certainly associated with ploidy duplication (Soltis *et al.* 2004). Genetic diversity in the tetraploid populations is usually high due to the increase in allele number that tetraploidy imparts. This means that heterozygosity is generally higher in the tetraploid populations than in the diploid populations, and it is maintained even in isolated populations (Rosche *et al.* 2016), resulting in lower risk of extinction in isolated populations than diploid species (Plue *et al.* 2018). Polyploids are known to have larger potential to adapt to new and isolated environments than diploids (Soltis and Soltis 2000; Otto 2007), and this advantage might be important for the tetraploid *V. vitis-idaea* to maintain the isolated populations in diverse environments at low elevations.

Genetic drift and repeated selfing or inbreeding under isolated conditions often result in a decrease in genetic diversity in marginal populations (Eckert *et al.* 2008). Reductions of allelic richness and heterozygosity were reported in many species, including Japanese alpine plants (e.g. Moracho *et al.* 2016; Hirao *et al.* 2017). However, in this study, clonal diversity, allelic richness, and heterozygosity were not decreased in the populations of central Japan compared to the populations of northern Japan. Because seeds of *V. vitis-idaea* are expected to be dispersed by animals to long distances, regeneration by long-dispersed seeds may be possible between populations, resulting in the maintenance of clonal diversity and allelic richness. Furthermore, genets of *V. vitis-idaea* can persist for long periods by clonal growth, rapid reduction in genetic diversity may be unlikely. Therefore, ecological situations affecting genetic properties of individual populations are highly varied between alpine environments and low-elevational environments. These differences may enable the existence and the maintenance of two ecotypes in northern Japan.

In this Chapter, I revealed that tetraploid populations exist in low-elevational habitats in northern Japan, having diverse genetic compositions different from diploid populations in alpine habitats. Existence of low-elevational populations may contribute to maintenance of intra-specific diversity and evolution of northern plants in Japan.

Fig. 1-1. *Vaccinium vitis-idaea*. (a) Inflorescence composed of bell-shaped flowers, (b) red-ripen fruits, and flowering plants in (c) diploid alpine population (KAU) and (d) tetraploid low-elevational population (EN1).

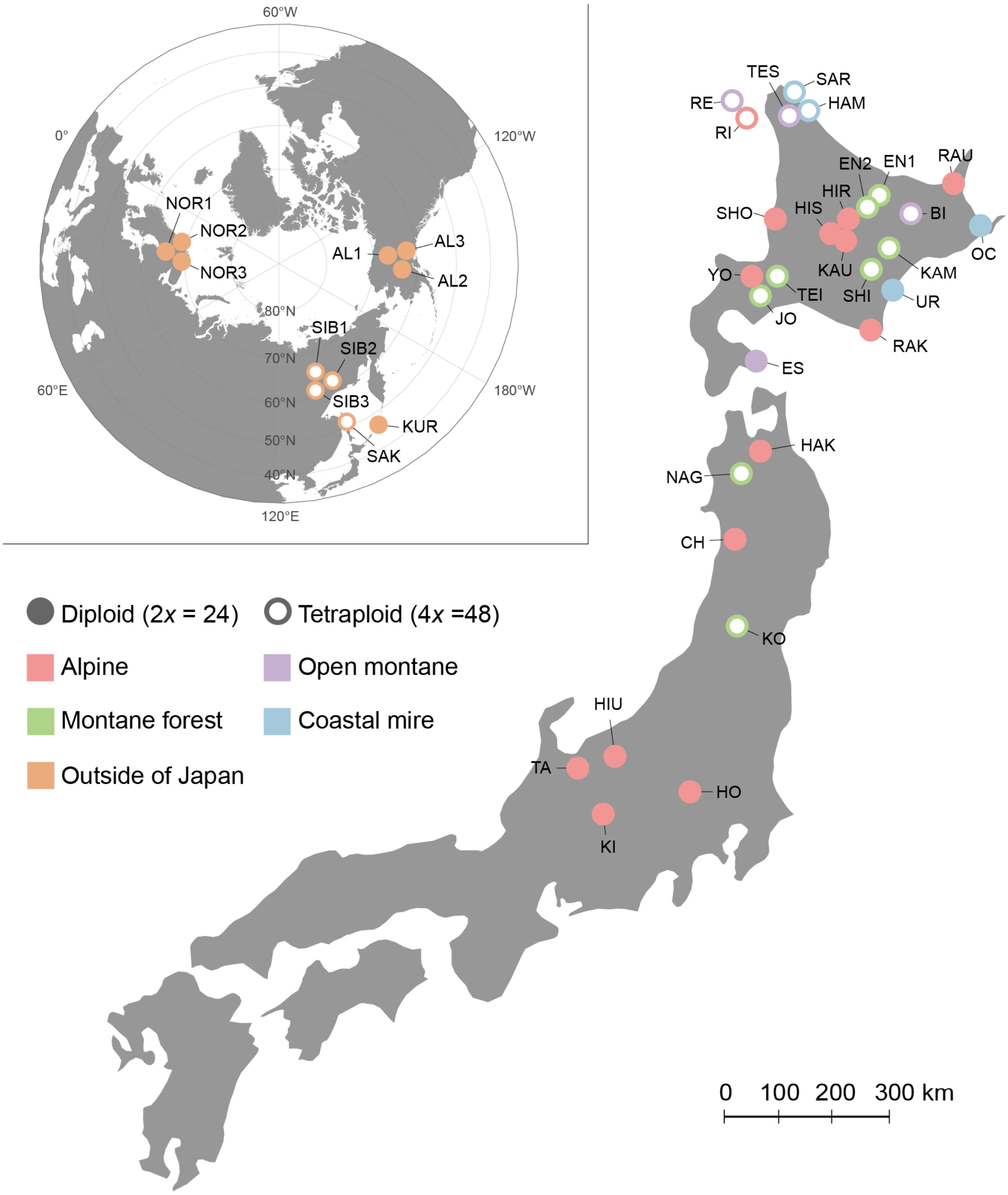


Fig. 1-2. Distribution, habitat type, and ploidy level of sampling sites. Red circles represent alpine habitat, purple circles represent open-montane habitat, green circles represent montane-forest habitat, blue circles represent coastal-mire habitat, and orange circles represent foreign sites. Diploid populations are represented as filled circles and tetraploid populations are represented as open circles. Population IDs correspond to those listed in Table 1-1.

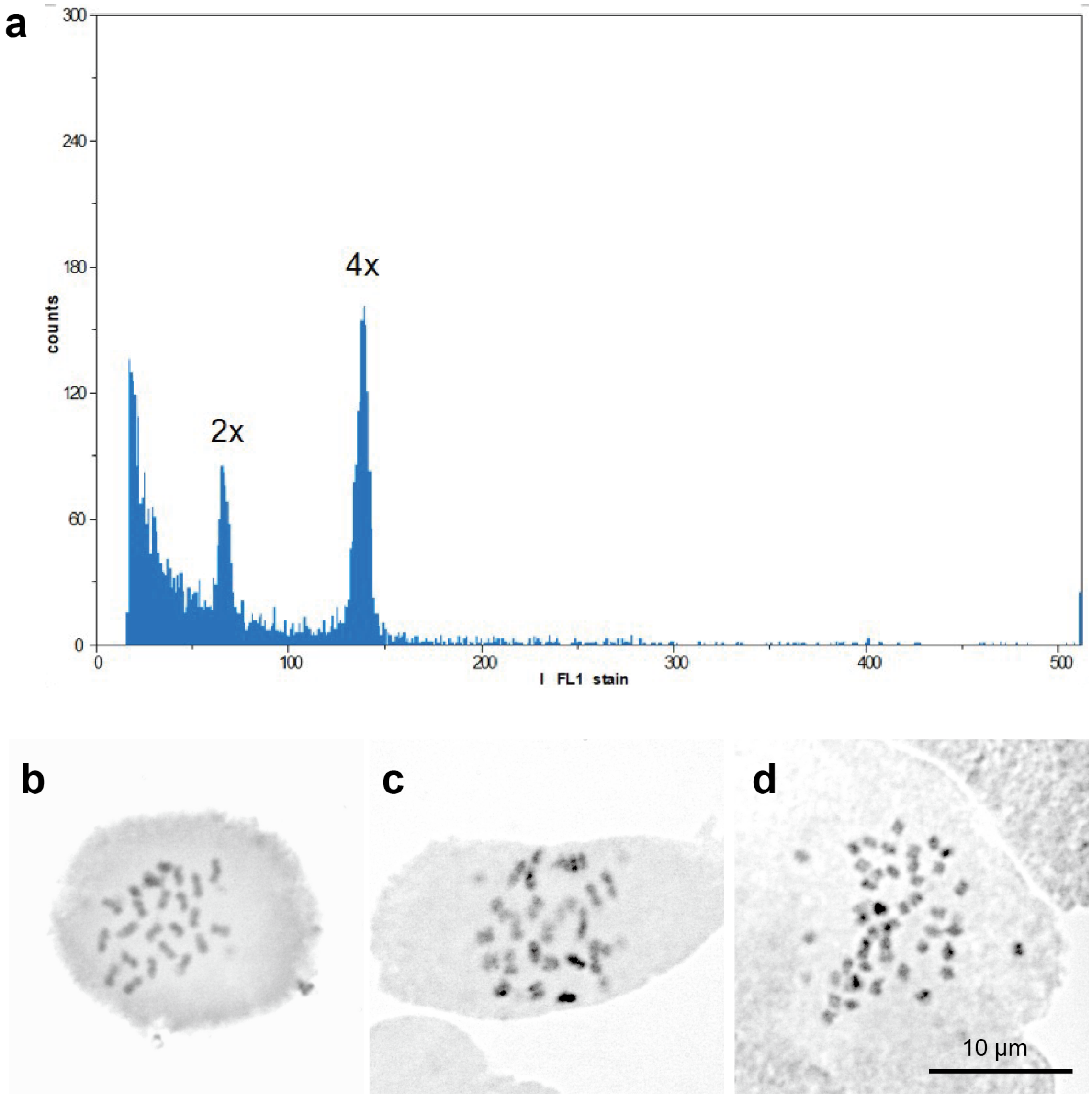


Fig. 1-3. (a) Result of flow-cytometry analysis of *V. virtis-idaea* from diploid and tetraploid populations (KAU and EN1, respectively) and image of chromosome of diploid (2*n* = 2*x* = 24) *V. vitis-idaea* ((b) KAU, (c) UR population) and tetraploid (2*n* = 4*x* =48) *V. vitis-idaea* ((d) EN1 population).

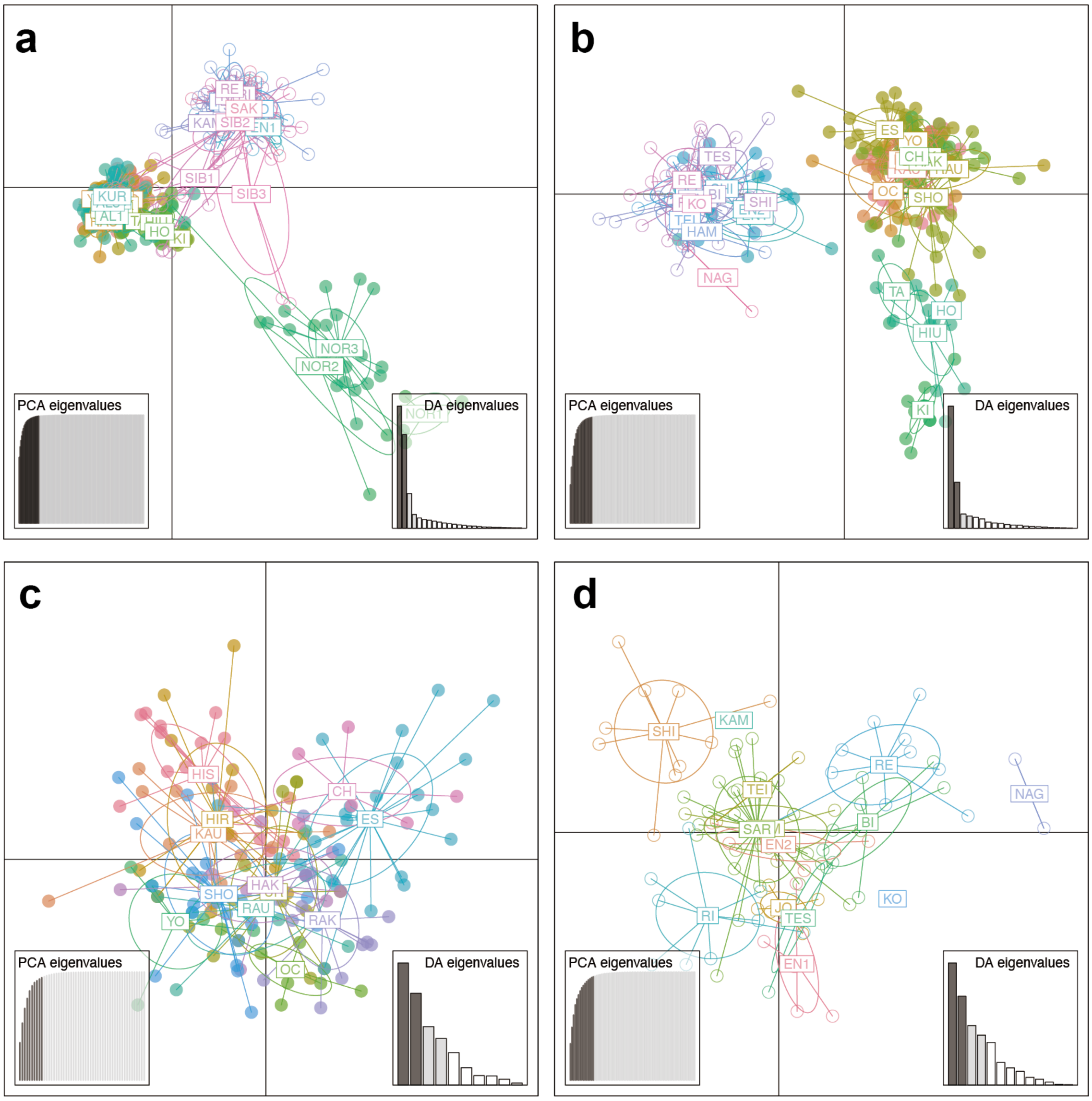


Fig. 1-4. Results of discriminant analysis of principal components (DAPC) of (a) all populations, (b) Japanese populations, (c) diploid populations in northern Japan, and (d) tetraploid populations in northern Japan. Each point represents one genotype. Diploids are represented as filled circles and tetraploids are represented as open circles. Population IDs correspond to those listed in Table 1-1.

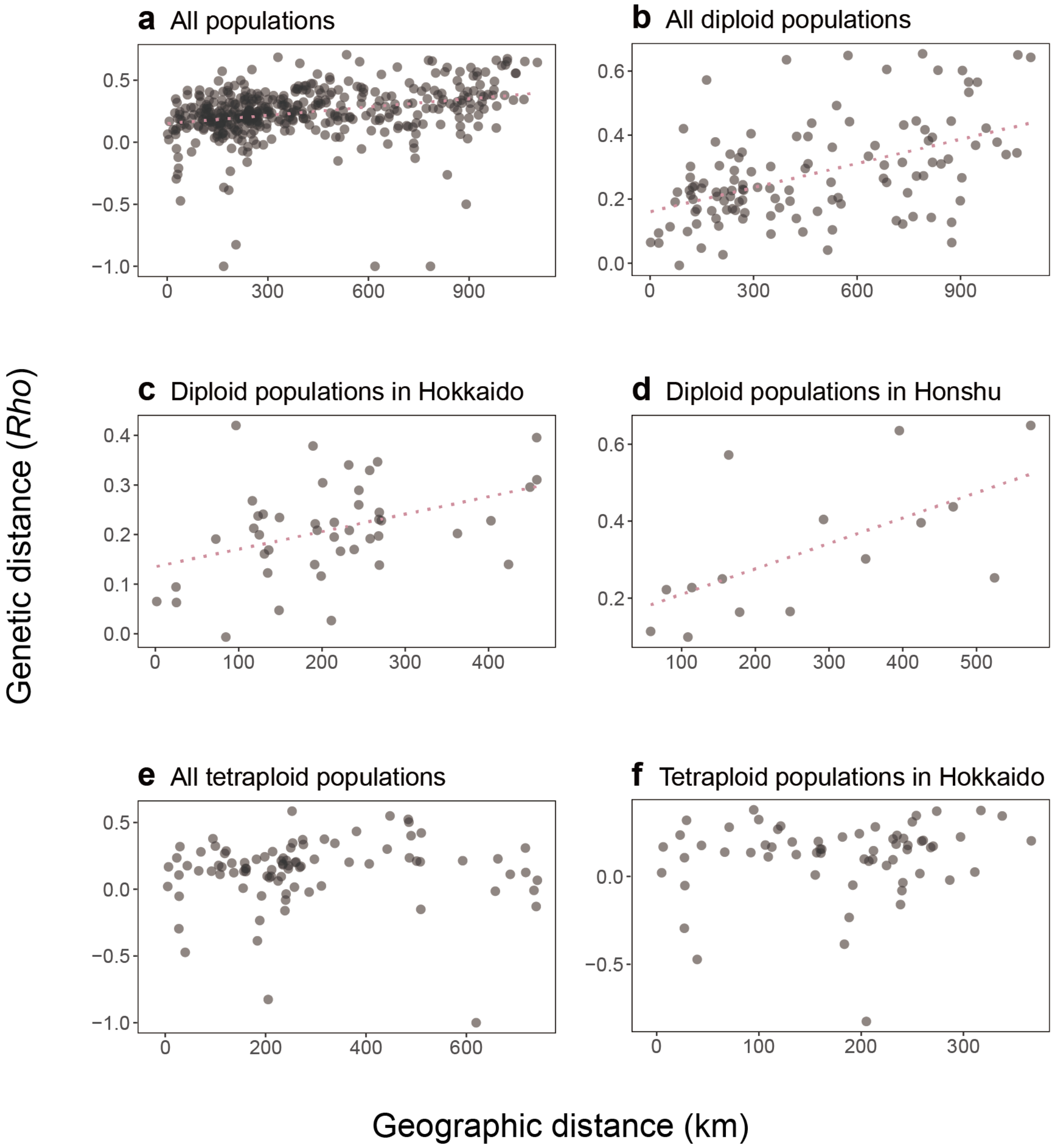


Fig. 1-5. Relationship between geographic distance (km) and genetic distance *Rho* in (a) all populations, (b) all diploid populations, (c) diploid populations in Hokkaido, (d) diploid populations in Honshu, (e) all tetraploid populations, and (f) tetraploid populations in Hokkaido. Geographic distance was calculated using QGIS, and *Rho* values (Ronfort *et al*. 1998) were calculated as genetic distance using SPAGeDi 1.5a.

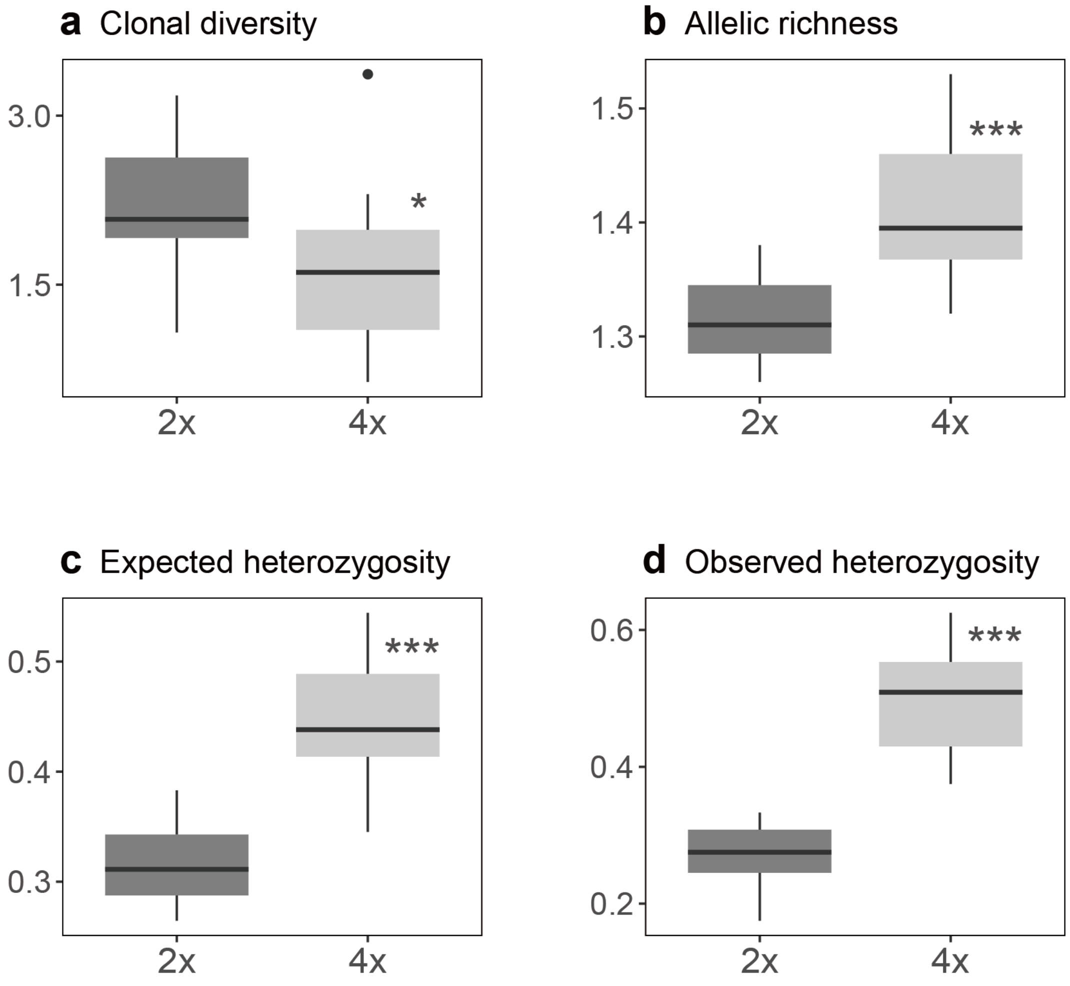


Fig. 1-6. Results of ANOVA comparison of (a) clonal diversity, (b) allelic richness, (c) expected heterozygosity, and (d) observed heterozygosity compared by ploidy levels. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$

Table 1-1. Geographic details of 41 sampling sites. See Fig. 1-2 for locations.

Population	ID	Habitat	Region	Latitude	Longitude	Elevation (m)
Hisago Lake	HIS	Alpine	Hokkaido	43° 32' 04"	142° 53' 20"	1671
Mt. Kaun	KAU	Alpine	Hokkaido	43° 34' 35"	142° 51' 06"	1907
Mt. Hira	HIR	Alpine	Hokkaido	43° 47' 10"	143° 00' 37"	1721
Mt. Yoichi	YO	Alpine	Hokkaido	43° 02' 32"	141° 01' 06"	1485
Mt. Rausu	RAU	Alpine	Hokkaido	43° 51' 45"	144° 52' 31"	1445
Mt. Shokanbetsu	SHO	Alpine	Hokkaido	43° 42' 36"	141° 32' 22"	1454
Mt. Rakko	RAK	Alpine	Hokkaido	42° 15' 17"	143° 07' 37"	1461
Mt. Esan	ES	Open montane	Hokkaido	41° 48' 12"	141° 09' 52"	352
Urahoro	UR	Coastal mire	Hokkaido	42° 42' 49"	143° 40' 30"	1
Ochiishi	OC	Coastal mire	Hokkaido	43° 12' 01"	145° 30' 37"	62
Mt. Hakkouda	HAK	Alpine	Tohoku	40° 40' 16"	140° 48' 25"	1538
Mt. Chokai	CH	Alpine	Tohoku	39° 06' 54"	140° 02' 21"	1725
Mt. Kisokoma	KI	Alpine	Central	35° 48' 01"	137° 48' 09"	2675
Mt. Tateyama	TA	Alpine	Central	36° 35' 58"	137° 37' 02"	2725
Mt. Hiuchi	HIU	Alpine	Central	36° 54' 08"	138° 07' 16"	2462
Mt. Hotaka	HO	Alpine	Central	36° 48' 30"	139° 08' 05"	2023
Mt. Rishiri	RI	Alpine	Hokkaido	45° 10' 35"	141° 13' 16"	1617
Engaru1	EN1	Montane forest	Hokkaido	43° 55' 48"	143° 20' 45"	371
Engaru2	EN2	Montane forest	Hokkaido	43° 55' 16"	143° 19' 03"	487
Shikaribetsu	SHI	Montane forest	Hokkaido	43° 11' 12"	142° 59' 18"	948

Continued

Jozankei	JO	Montane forest	Hokkaido	42° 51' 33"	141° 09' 23"	720
Teine	TEI	Montane forest	Hokkaido	43° 04' 30"	141° 11' 27"	725
Kamishihoro	KAM	Montane forest	Hokkaido	43° 22' 31"	143° 10' 52"	828
Bihoro	BI	Open montane	Hokkaido	43° 39' 07"	144° 14' 52"	525
Teshio	TES	Open montane	Hokkaido	44° 56' 56"	142° 01' 06"	434
Rebun	RE	Open montane	Hokkaido	45° 23' 07"	141° 01' 24"	422
Hamatonbetsu	HAM	Coastal mire	Hokkaido	45° 10' 00"	142° 20' 30"	9
Sarufutsu	SAR	Coastal mire	Hokkaido	45° 16' 10"	142° 13' 15"	8
Koyouzan	KO	Montane forest	Tohoku	37° 59' 17"	140° 11' 57"	661
Nagabashiri	NAG	Montane forest	Tohoku	40° 18' 05"	140° 30' 35"	202
Siberia 1	SIB1	Outside of Japan	Russia			
Siberia 2	SIB2	Outside of Japan	Russia			
Siberia 3	SIB3	Outside of Japan	Russia			
Alaska 1	AL1	Outside of Japan	Alaska			
Alaska 2	AL2	Outside of Japan	Alaska			
Alaska 3	AL3	Outside of Japan	Alaska			
Norway_Alta	NOR1	Outside of Japan	Europe			
Norway_Jotka 1	NOR2	Outside of Japan	Europe			
Norway_Jotka 2	NOR3	Outside of Japan	Europe			
Sakhalin	SAK	Outside of Japan	Russia			
Kuril	KUR	Outside of Japan	Russia			

Table 1-2. Genetic traits of populations at 30 study sites in Japan. Ploidy: ploidy level (2*x*: diploid, 4*x*: tetraploid), Location: geographic location (North: northern Japan, Central: central Japan), Genet: number of genets, P_A: number of private alleles, *H*: genotype diversity based on the Shannon–Wiener diversity index, *Ar*: allelic richness, *He*: expected heterozygosity, *Ho*: observed heterozygosity

Population	Habitat	Location	Ploidy	N	Genet	P_A	*H*	*Ar*	*He*	*Ho*
HIS	Alpine	North	2*x*	30	16	0	2.77	1.29	0.29	0.25
KAU	Alpine	North	2*x*	30	14	0	2.64	1.32	0.32	0.30
HIR	Alpine	North	2*x*	30	15	0	2.62	1.31	0.31	0.28
YO	Alpine	North	2*x*	25	5	0	1.61	1.27	0.27	0.18
RAU	Alpine	North	2*x*	25	8	0	2.08	1.27	0.27	0.20
SHO	Alpine	North	2*x*	30	22	0	3.00	1.35	0.35	0.26
RAK	Alpine	North	2*x*	30	14	0	2.48	1.38	0.38	0.33
ES	Open montane	North	2*x*	30	24	0	3.18	1.34	0.34	0.32
UR	Coastal mire	North	2*x*	30	13	0	2.56	1.29	0.29	0.24
OC	Coastal mire	North	2*x*	30	7	0	1.95	1.29	0.29	0.32
HAK	Alpine	North	2*x*	30	7	0	1.31	1.28	0.28	0.25
CH	Alpine	North	2*x*	30	9	1	1.88	1.38	0.38	0.29
KI	Alpine	Central	2*x*	30	11	0	2.00	1.26	0.26	0.28
TA	Alpine	Central	2*x*	30	3	0	1.08	1.35	0.35	0.33
HIU	Alpine	Central	2*x*	30	12	0	2.00	1.32	0.32	0.21
HO	Alpine	Central	2*x*	20	1	0	-	-	-	-

Continued

RI	Alpine	North	4*x*	30	9	0	1.92	1.46	0.49	0.44
EN1	Montane forest	North	4*x*	25	5	0	1.61	1.43	0.47	0.60
EN2	Montane forest	North	4*x*	25	4	0	1.39	1.53	0.54	0.63
SHI	Montane forest	North	4*x*	25	10	0	2.30	1.46	0.49	0.59
JO	Montane forest	North	4*x*	25	5	0	1.61	1.37	0.40	0.48
TEI	Montane forest	North	4*x*	25	2	0	0.69	1.32	0.35	0.38
KAM	Montane forest	North	4*x*	20	1	0	-	-	-	-
BI	Open montane	North	4*x*	25	8	0	2.08	1.33	0.38	0.48
TES	Open montane	North	4*x*	30	3	0	1.10	1.36	0.42	0.54
RE	Open montane	North	4*x*	30	10	2	1.96	1.39	0.43	0.39
HAM	Coastal mire	North	4*x*	25	3	1	1.10	1.46	0.51	0.54
SAR	Coastal mire	North	4*x*	30	29	0	3.37	1.40	0.45	0.53
KO	Montane forest	North	4*x*	30	1	0	-	-	-	-
NAG	Montane forest	North	4*x*	30	2	0	0.64	1.37	0.42	0.38

Chapter 2: Variations in reproductive properties and morphological characteristics of *Vaccinium vitis-idaea* populations across the diverse habitats and geographic locations

2.1 Introduction

Ecological situations vary greatly across the distributional range of individual plant species. In populations located at distributional margins, where environmental conditions are different from central distribution area, any ecological adaptation to local environments is expected to occur, as well as genetic differentiation (Hampe and Petit 2005). Abeli *et al.* (2014) noted that local adaptations of reproductive system, morphological traits, and ecological traits related to interspecific interactions tend to occur at marginal populations, because population size, intensity of isolation, and surrounding environments are obviously different between small populations under unfavorable environments at distribution limit and large populations at optimum environment. Such ecological variations at marginal environments are important to understand how organisms respond to climate changes and associated range shifts (Hampe and Jump 2011).

Generally, marginal populations are exposed to spatial isolation from other populations and low genetic diversity, which limits the opportunity of cross-pollination. Therefore, plants in isolated populations often express high selfing rate, low inbreeding depression, apomixes ability or vigorous clonal propagation (Mee and Moore 2014). Arctic-alpine species are known to have large variations in reproductive system along geographical gradient (Wada 2008; Popp *et al.* 2011) and/or local topographical difference (Kameyama and Kudo 2009). For instance, populations of a dioecious shrub

Salix herbasea are maintained by vegetative growth of several clones at the southern edge of the distribution range, where seed production is limited at very low level (Carbognani *et al.* 2019). These studies indicate that isolated conditions favor selfing ability or active clonal reproduction, as a reproductive assurance under restricted outcrossing. In addition, changes in morphological traits are expected at isolated populations because environmental conditions are different from the distributional center of the species (Mee and Moore 2014). Actually, leaf traits or plant size of Arctic-alpine plants often vary along climatic and environmental gradients (Herbert 2003; Choler 2005).

It is reported that *V. vitis-idaea* mainly produces outcrossing seeds because of a self-sterility system caused by early-acting inbreeding depression (Guillaume and Jacquemart 1999). Because alpine diploid populations in central Japan are generally small, fragmented, and isolated more than the alpine diploid populations in Hokkaido, the frequency of self-fertilization may be larger in central Japan. However, certain level of outcrossing may be maintained also in central Japan because the genetic diversity was not decreased in these populations (Chapter 1). On the other hand, low-elevational populations in northern Japan are extremely isolated and express low clonal diversity (Chapter 1), where seed production by selfing may be advantageous for the maintenance of populations. Therefore, evolution of selfing ability and reduced inbreeding depression are expected for the low-elevational populations. In another case, the low-elevational populations may be maintained by clonal growth. Additionally, environmental conditions, such as temperature, light condition, and length of growing season, are very different between alpine and low-elevational habitats. Thus, *V. vitis-idaea* populations at low elevations may have unique morphological traits reflecting the local environments different from alpine populations.

Furthermore, ploidy duplication is known to contribute to the rapid local adaptation, and related to collapse of the self-incompatibility system, moderation of inbreeding depression, and enlargement of morphological traits (Soltis and Soltis 1999; Otto 2007). Therefore, the difference in ploidy level between alpine and low-elevational populations (see Chapter 1) may be related to the mating system and morphological traits. In this chapter, I aim to confirm whether there is significant difference in ecological traits among different habitat types, and reveal how *V. vitis-idaea* maintain their populations under various environmental conditions in Japan.

2.2 Methods

Fruiting success, seed production, and selfing rate under natural conditions

For the comparisons of reproductive performance among habitat types and between ploidy levels, I conducted field survey for 15 *V. vitis-idaea* populations in Hokkaido (HIS, KAU, HIR, YO, RAU, UR, OC, EN1, EN2, SHI, JO, TEI, BI, HAM, SAR; Fig. 1-2) in 2016–2017, and in four populations in Honshu (HAK, TA, KO, NAG) in 2019. In early summer, one 50 m × 25 m plot was set in each population, where 50 shoots were marked and flower number per shoot, fruit-set success, and developed seed number per fruit were recorded for all marked shoots. Flower numbers were recorded in June and July, fruit numbers were recorded in August and September, and fruit-set rates (proportion of flowers setting fruits) were calculated for each population.

In 2016–2019, one fruit per shoot was sampled from 25–30 shoots in 26 population (HIS, KAU, HIR, YO, RAU, SHO, RAK, ES, UR, OC, HAK, CH, KI, TA, HIU, EN1,

EN2, SHI, JO, TEI, BI, RI, RE, HAM, SAR, NAG; Fig. 1-2) and the number of developed seeds per fruit was counted. To examine the mating system of *V. vitis-idaea* populations, I estimated selfing rates for each population based on multi-locus outcrossing rates (*tm*) from progeny arrays composed of genotype data of leaves and seeds from same shoots. One leaf and 1–3 seeds from each plant were used for genetic analysis for 25–30 samples in each population, using eight microsatellite markers in the same way with Chapter 1. In total, 746 leaves and 2318 seeds were analyzed. To calculate the selfing rates of each population, MLTR (Ritland 2002) was used for diploid populations and MLTET (Ritland 1990; Murawski *et al.* 1994) was used for tetraploid populations. Calculations were performed with 1,000 bootstraps with families as resampling units.

Pollination experiment and observation of pollen tube growth

I conducted additional pollination experiment across different habitat types and between ploidy levels in five populations (KAU, HIR, UR, EN1, JO; Fig. 1-2) in 2018, aiming to reveal the mating systems of each population. In early summer, 50 individuals with floral buds were randomly chosen and marked. Then, one inflorescence was selected and flower number was counted to measure fruit-set success under natural conditions. For 30 individuals, three more inflorescences were selected for a pollination experiment: one for a cross-pollination treatment, one for a self-pollination treatment, and one for an automatic-selfing treatment. These inflorescences were covered with fine-meshed nylon bags before flowering to prevent insect visits. After flowers opened, hand pollination was conducted for the inflorescences of the cross- and self-pollination groups. Inflorescences of the automatic-selfing group were left untreated to check for the seed-set rate via automatic self-pollination. The number of treated flowers was recorded, and fruit set was calculated in autumn. When fruits developed, a single fruit per inflorescence was sampled to count developed seeds. At the same time, I counted unfertilized ovules and aborted seeds, and calculated the seed-set rate per fruit. Relative seed-set rate was calculated as a product of fruit-set rate and seed-set rate. The mass of 30–50 developed seeds in each treatment group was measured after drying at room temperature.

In order to check the compatibility for pollen germination and pollen tube extension, furthermore, pistils after the pollination treatment were observed under a microscope. Stigmas were sampled from cross-pollinated and self-pollinated flowers in the five populations. Stigmas were left at least two hours after pollination treatment before sampling. They were fixed in FAA (formalin: acetic acid: alcohol (ethanol) = 5: 5: 90) for 24 hours and kept in 70 % ethanol until observation. Fixed stigmas were treated with 8 M NaOH for 15 minutes at 65 ºC, then washed with water, and stained with decolorized Aniline Blue. Stained stigmas were observed under a microscope (BX43, Olympus).

Measurements of floral morphology and vegetative morphology

To confirm if the difference in mating systems is related to floral morphology, the length and width of corolla and the length of pistil and stamen were measured in five populations which are identical to those for the pollination experiment. I randomly sampled 30 flowers from different individuals for this measurement. Corolla size was expressed using the volumetric formula of an ellipsoid. Distance between stigma and anther was estimated from the length of pistil and stamen.

Furthermore, vegetative traits were measured to compare plant performance among habitat types. Annual shoot growth and the number of current leaves were recorded for 30 shoots in 30 populations in Japan. One leaf per shoot was sampled from 30 shoots, and leaf area and dry mass (after drying using silica gel) were measured. The public-domain NIH ImageJ program (U.S. National Institutes of Health) was used to measure leaf area. Then, leaf mass per unit area (LMA) was calculated from leaf area and dry mass. Annual leaf production in each population was estimated from the annual leaf number and dry mass of a single leaf.

Statistical analysis

Reproductive success and morphological traits under natural conditions were compared among populations using generalized linear mixed models (GLMMs). GLMMs were conducted for flower number per shoot (with poisson error distribution), fruit-set rate (with negative binomial error distribution), seed number per fruit (with poisson error distribution), annual shoot growth (with gamma error distribution), annual leaf production (with gamma error distribution), leaf area (with gamma error distribution), and LMA (with gamma error distribution). Ploidy level (intercept = diploid), habitat type (intercept = alpine), and geographic location (intercept = northern Japan) were set as explanatory variables, since these ecological traits may be affected by both genetic background and environment. In addition, population ID and year of survey were set as random effects. Selfing rate ($s = 1 - tm$) was compared using ANOVA, including ploidy level, habitat type, and geographic location. For the analysis of the pollination experiment conducted in five populations, I used GLMs and Tukey's post-hoc test, in which fruit-set rate (with negative binomial error distribution), relative seed-set rate per fruit (with negative binomial error distribution), and seed weight (with gamma error distribution) were compared among pollination treatments in each population. Additionally, comparisons of flower size (with gamma error distribution), distance between stigma and anther (with gamma error distribution), ovule number (with poisson error distribution), and seed weight (with gamma error distribution) were conducted among populations.

2.3 Results

Reproductive performance and mating system

The GLMM results of reproductive traits revealed that flower production and fruit-set rate varied among 19 populations (Fig. 2-1), but clear trend was rarely detected between ploidy levels or among habitat types ($P > 0.1$; Table 2-1), except for a decrease in fruit-set rates in the populations of central Japan ($P < 0.05$). In contrast, seed number per fruit was significantly different between ploidy levels ($P < 0.001$); the diploid populations (11.1 ± 8.2) had more than twice as many seeds as the tetraploid populations (4.6 ± 3.5).

Average selfing rates were significantly higher in the tetraploid populations than in the diploid populations, and higher in the central populations than in the northern populations ($P < 0.001$; Table 2-2). Although the diploid populations in northern Japan often exhibited low selfing rates, there were some diploid populations having relatively high selfing rate in northern Japan (UR, YO). There were no significant differences in selfing rate among habitat types ($P > 0.1$).

Results of the pollination experiment in 2018 are summarized in Fig. 2-2. Fruit-set rate of the cross-pollinated inflorescences was greater than that of naturally pollinated inflorescences in every population (Fig. 2-2a), indicating that pollen limitation is common in this species. Fruit-set rates in the self-pollinated and automatic-selfing treatment groups varied among populations. Two diploid alpine populations, KAU and HIR, showed negligibly low fruit production in the self-pollinated inflorescences. In the coastal diploid population (UR), the fruit-set rates of the self-pollinated and bagged (automatic-selfing) inflorescences were comparable to the fruit-set rate of control, but lower than that of the cross-pollinated inflorescences. In the forest tetraploid populations, EN1 and JO, fruit-set rates of the self-pollinated inflorescences were comparable to that of the cross-pollinated inflorescences, while the fruit-set rate of the bagged inflorescences was lower than that of the cross-pollinated inflorescences.

In the alpine diploid populations, relative seed-set rates of the self-pollinated inflorescences were quite small (Fig. 2-2b). In the low-elevational populations, a certain level of seed-set rate was detected in the self-pollinated inflorescences, although relative seed-set rates by the self-pollination was lower than that of the cross-pollination in UR and JO. There were some variations in seed weight among the pollination treatments in two of five populations (Fig. 2-2c), i.e., the cross-pollinated and bagged inflorescences produced heavier seeds than the naturally pollinated inflorescences in UR, and the self-pollinated and bagged inflorescences produced heavier seeds than the cross-pollinated inflorescences in EN1, although these differences were all small. Unexpectedly, pollen germination and pollen tube growth were observed in all samples, regardless of cross- or self-pollination, and pollen tubes commonly reached the bottom of pistils (Fig. 2-3).

Variations in floral and vegetative morphologies

In the survey of floral morphology, every floral trait differed between alpine and low-elevational populations, regardless of ploidy levels ($P < 0.05$; Fig. 2-4). Smaller corollas and shorter distance between stigma and anther were common in the alpine populations. Ovule number was highest in the coastal-mire population (UR), and did not differ between the alpine (KAU, HIR) and forest (EN1, JO) populations. Seed weight was greater in the low-elevational populations than in the alpine populations, especially in the two tetraploid populations (EN1, JO).

Annual shoot length, leaf area, LMA, and annual leaf production all varied significantly among ploidy levels, habitat types and/or different geographic locations (Table 2-3). Annual shoot length differed significantly among habitat types and marginally among ploidy levels and geographic locations. The montane-forest populations (43.5 ± 13.7 mm) and the coastal-mire populations (34.3 ± 17.8 mm) showed larger annual shoot length than the alpine populations (16.9 ± 8.0 mm). Additionally, the tetraploid populations and the populations of central Japan were slightly larger in annual shoot length than the diploid populations and the populations of northern Japan, respectively ($P < 0.1$). Leaf area was significantly greater in the tetraploid populations (1.53 ± 0.66 cm^2) than in the diploid populations (0.58 ± 0.22 cm^2; $P < 0.001$), and the montane-forest populations had the largest leaf area (1.76 ± 0.72 cm^2; $P < 0.05$) among four habitat types, at almost three times that of the alpine populations (0.60 ± 0.30 cm^2). LMA was significantly lower in the populations of central Japan (133.2 ± 22.3 gm^{-2}) than that of northern Japan (146.3 ± 31.5 gm^{-2}; $P <$ 0.05). Among habitat types, the montane-forest populations showed lowest value, (134.6 ± 29.6 gm^{-2}), although the difference was not significant ($P > 0.1$). Annual leaf production was significantly different between ploidy level and among habitat types. The tetraploid populations (0.224 ± 0.103 g) had more than twice leaf production in the diploid populations (0.084 ± 0.049 g in northern Japan and 0.072 ± 0.039 g in central Japan) and the montane-forest population had largest leaf production in four habitat types (0.258 ± 0.106 g).

2.4 Discussion

In the survey of reproductive performance, there were no significant differences in flower production among genetic groups and habitat types. Fruit-set rate was lower in the populations of central Japan than the populations of northern Japan, while there was no significant difference between ploidy levels or among habitat types. This indicates that reproductive activity is maintained in isolated populations at lower elevations. However, flower number and fruit-set rates varied greatly among populations. These variations may be caused by pollinator abundance, weather conditions during the flowering and fruiting, and other characteristics specific to each population. Since fruit-set rate was counted at only one population (TA) in central Japan, a significant decrease in fruit-set rate in central Japan might be caused by specific characteristic of TA population. A significant decrease in seed number per fruit in the tetraploid populations might reflect low seed fertility in polyploids (Ramsey and Schemske 1998) because ovule number per flower was similar between the diploid and tetraploid populations.

The evolution of selfing ability in isolated populations has been predicted (Mee and Moore 2014) and reported in previous studies (García-Fernández *et al.* 2012; Rosche *et al.* 2017). Also in the case of *V. vitis-idaea*, selfing ability increased both at the alpine populations of central Japan and the low-elevational populations in northern area. The pollination experiment also indicated high selfing ability in three low-elevational populations in Hokkaido (both in diploid and tetraploid populations). In addition, pollen tube extension in self-pollinated flower was observed in all populations. Considering these results, the partial self-sterility in this species may not be universal. It may be not regulated by typical self-incompatibility systems, such as gametophytic or sporophytic self-incompatibility, the system that self-fertilizations are inhibited by the suspension of pollen germination or extension of pollen-tube (Hiscock *et al.* 2003; Tao and Iezzoni 2010).

The lack of self-fertilized seeds in the alpine diploid populations may be caused by early-acting inbreeding depression, in that fertilization occurs normally but seeds derived from selfing are not fully developed (Husband and Schemske 1996). Early-acting inbreeding depression is reported in other *Vaccinium* species in arctic area (Krebs and Hancock 1990; Nuortila *et al.* 2006) and in *V. vitis-idaea* (Guillaume and Jacquemart 1999). If self-incompatibility of *V. vitis-idaea* is caused by early-acting inbreeding depression, the variation in selfing rates can be explained by two aspects. First, small, isolated populations might have experienced a strong bottleneck event in the past (Kawecki and Ebert 2004). Actually, most tetraploid populations showed high selfing ability had low clonal diversity, only a few large genets dominated (Table 1-2, 2-1). In this situation, opportunities for outcrossing are highly restricted, and selfing ability is advantageous as a reproductive assurance (Pannell 2015). Because purging of recessive deleterious alleles occurs rapidly in such population, inbreeding depression become weakened (Hedrick and Garcia-Dorado 2016). Although clonal diversity was not so small in the diploid populations of central Japan, strong bottleneck might occur during the long history in these populations. Also, some diploid populations of northern

Japan showing relatively high selfing rates might have experienced strong bottleneck in the past. Another explanation of high selfing ability is linked to whole-genome duplication. Because tetraploids have twice as many alleles than diploids, homozygosity of deleterious alleles rarely occurs even with repeated selfing or inbreeding (Soltis and Soltis 2000). Thus, tetraploids can compensate for the genetic load caused by selfing with high allele diversity and heterozygosity.

Populations maintained by selfing tend to have less attractive floral traits, such as smaller flower size and reduced investment in male function (Sicard and Lenhard 2011). In these measurements, however, corolla size and flower number per shoot in the self-compatible low-elevational populations were even greater than in the self-incompatible alpine populations. In addition, distance between stigma and anther was larger in the low-elevational populations. Therefore, the low-elevational populations may maintain a mixed-mating system of outcrossing and selfing. Actually, the out-crossing rate was maintained at least 50% even in the self-compatible populations. In addition, *Vaccinium vitis-idaea* can live for several hundreds of years by clonal growth (Persson and Gustavsson 2001). Existence of huge clones over 30 m in diameter were reported by Shimokawabe *et al.* (2016), and large-sized clones were detected in several populations also in this study. Thus, the exuberant clonal growth may play an important role for population maintenance, especially in the tetraploid populations.

The alpine populations showed smaller morphological traits than the low-elevational populations, and diploids generally showed smaller morphological values than tetraploids. This may be due to the mixed effect of ploidy level and environmental conditions. Small shoot elongation in the alpine populations reflects harsh climatic conditions, characterized by cool summer temperatures, strong winds, and a short growing season (Bliss 1962). Enhancement of annual growth of *V. vitis-idaea* is reported by the experimental warming in the alpine population (Kudo and Suzuki 2003), indicating that the annual growth of this species is sensitive to climate conditions. Larger morphology in tetraploids may be resulted from effect of larger ploidy. Polyploid individuals generally tend to have larger morphology and greater growth activity than diploids (Otto 2007) because of their larger cell size resulting from their increased DNA content (Ramsey and Ramsey 2014). Additionally, larger leaf size in the forest habitats might be caused by acclimation to shaded condition (Poorter *et al.* 2009). Overall, tetraploids had high potential for vegetative growth and morphological regulation in response to the environments; in particular, large leaf size might be important for colonization in forest habitats. On the other hand, large annual shoot growth and low LMA were detected in the populations of central Japan. LMA is affected by various environmental factors, such as temperature, light environment, and humidity (Niinemets 2001; Wright *et al.* 2005; Poorter *et al.* 2009). Some of these factors might decrease the LMA in the central populations, such as large precipitation in central Japan.

This chapter revealed that *V. vitis-idaea* populations at low-elevational habitat possess different mating properties and ecological traits from typical alpine populations, associated with environmental variation and the effect of polyploidization. The diploid populations in central Japan also differed in the selfing ability and morphological traits from the alpine populations of northern Japan. It was also revealed that this species has flexible variations in the morphological traits responding to various environmental conditions. Taken together, this species is widely adaptable to various climatic and environmental conditions at marginal habitats, and that the ability of adaptation is facilitated by ploidy duplication.

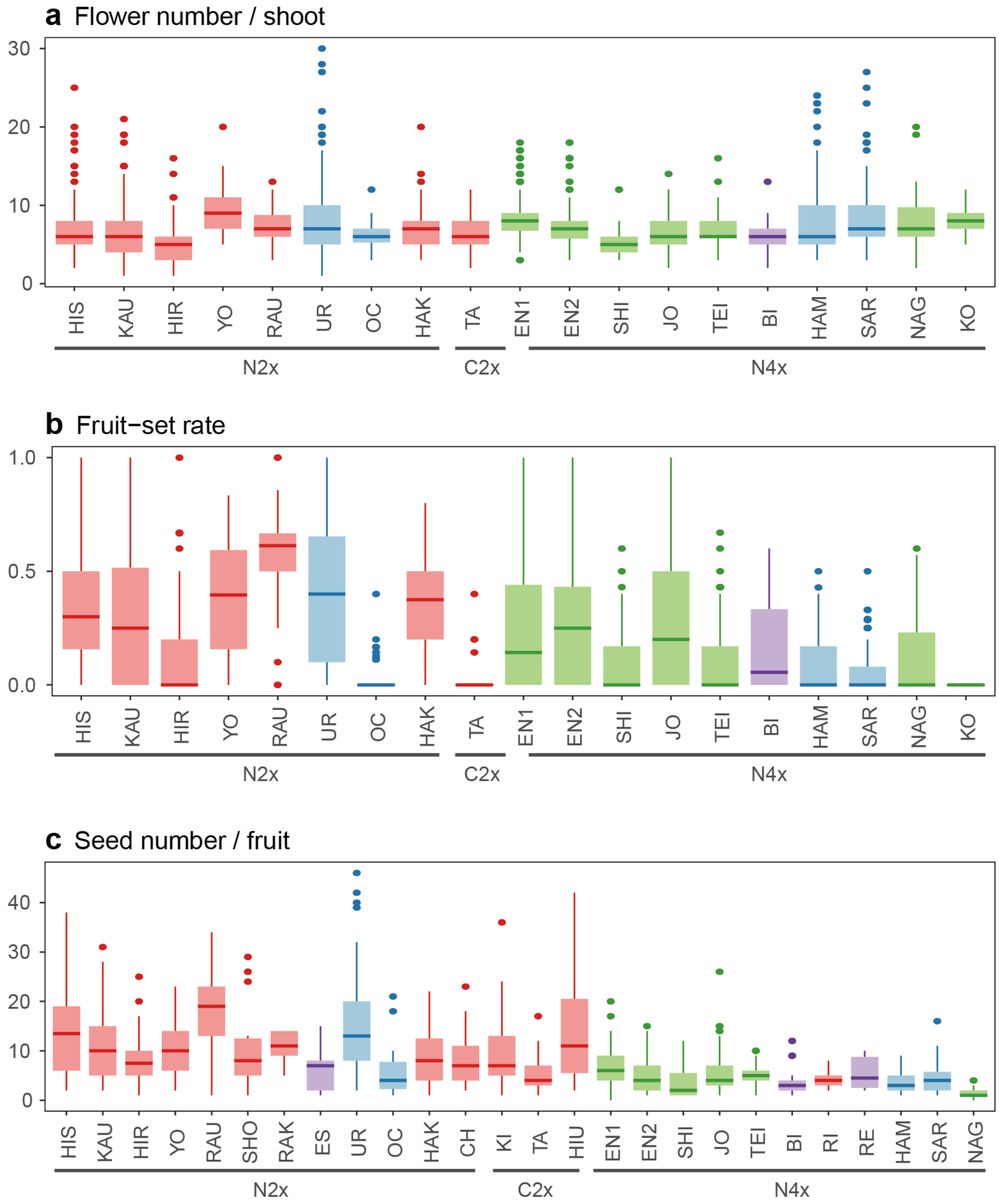
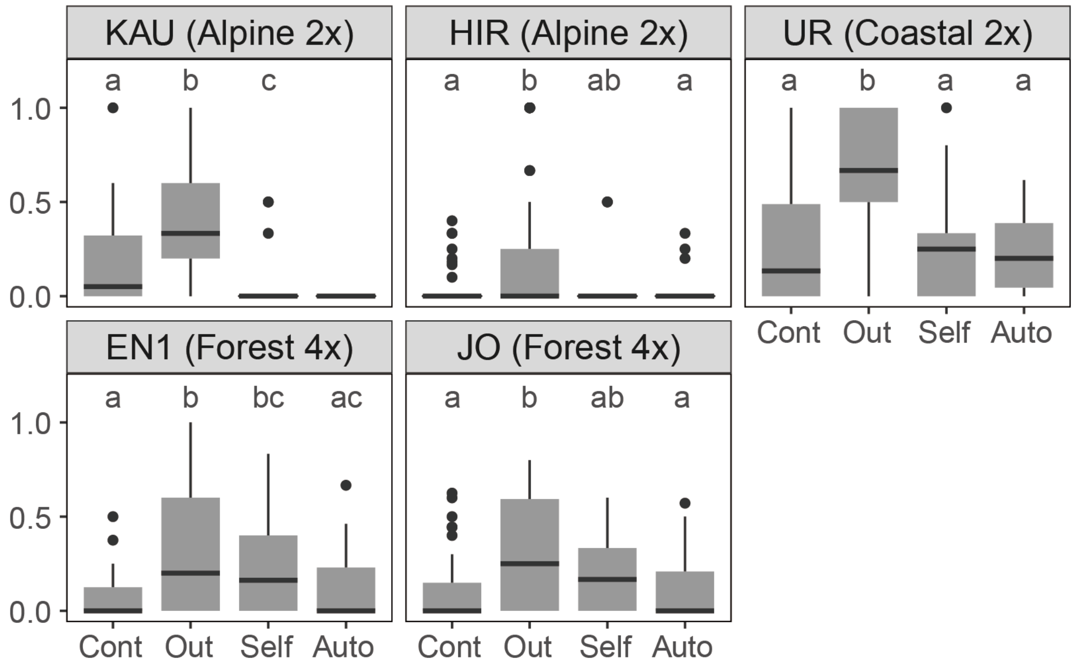
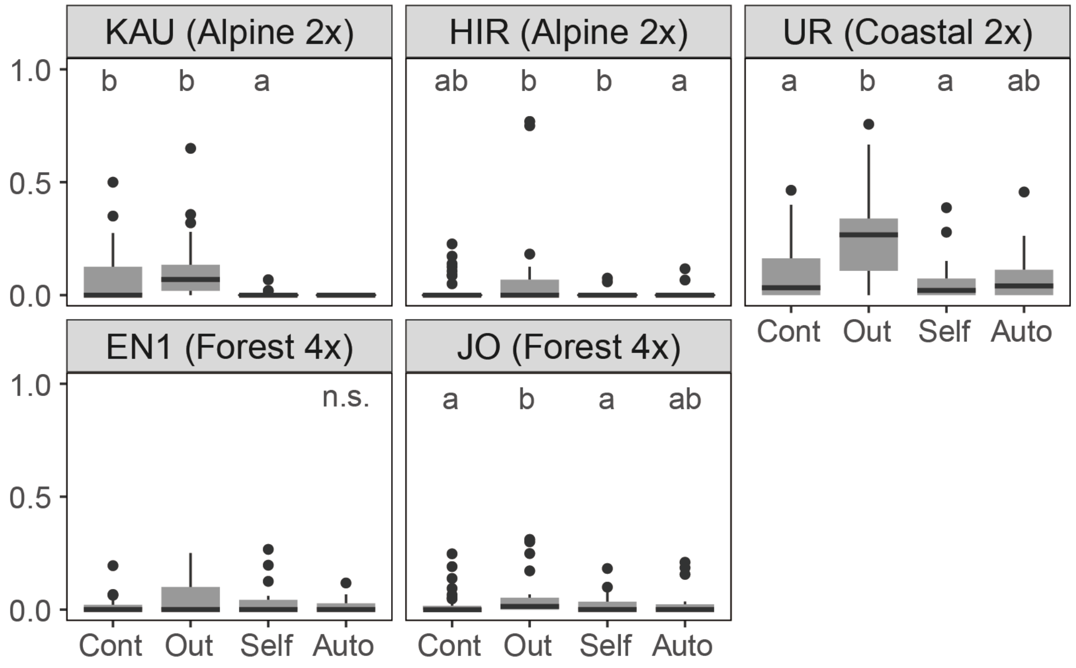


Fig. 2-1. Reproductive performance of *V. vitis-idaea* populations: (a) flower number per shot, (b) fruit-set rate, (c) seed number per fruit. Population IDs correspond to Table 1-1 and Fig. 1-2. N2x: diploid populations in northern Japan, C2x: diploid populations in central Japan, N4x: tetraploid populations in northern Japan. Colors of box plots represent different habitat types (red: alpine, purple: open montane, green: montane forest, blue: coastal mire)

a Fruit-set rate



b Relative seed-set rate



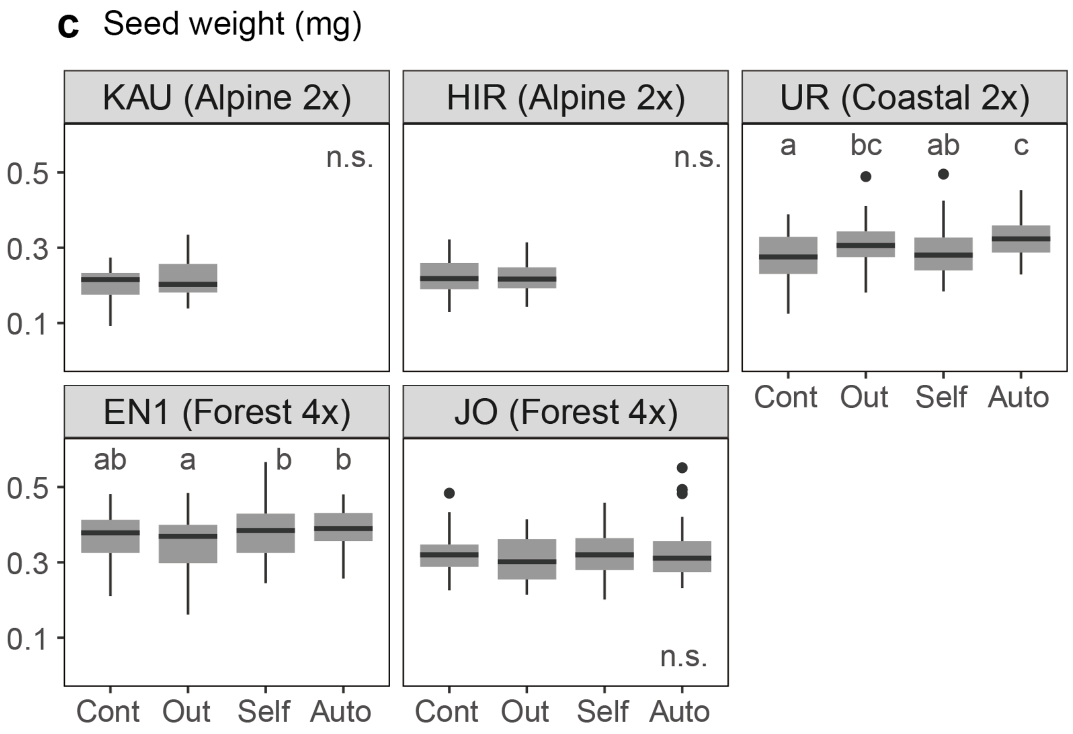


Fig. 2-2. Comparisons of (a) fruit-set rate, (b) relative seed-set rate, and (c) seed weight among four treatments in each population.

Cont: control, Cros: cross-pollination, Self: self-pollination, Auto: automatic-selfing

Different letters: *P* < 0.05 according to GLM and Tukey's post-hoc test

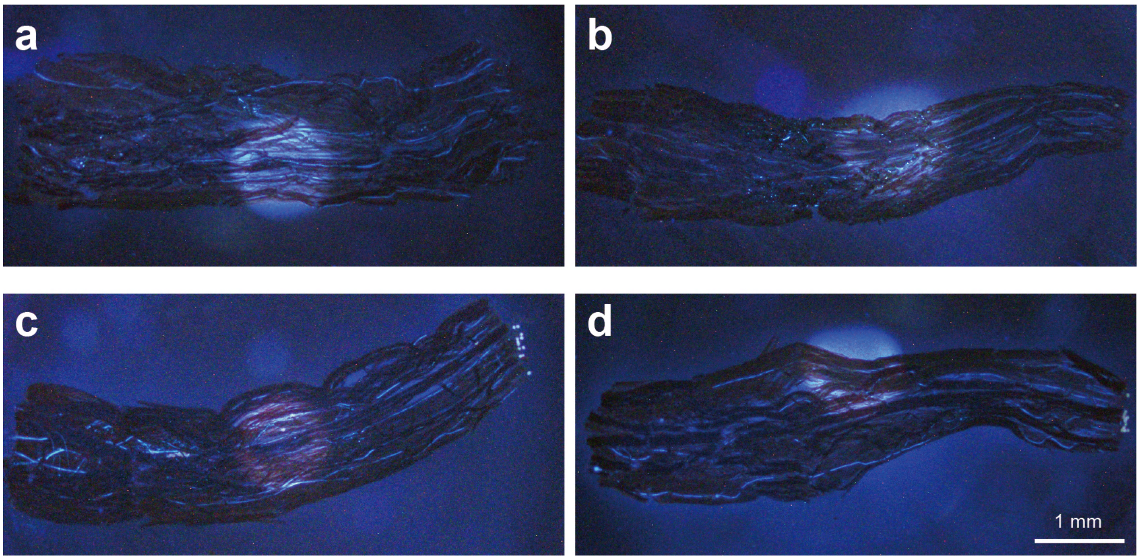


Fig. 2-3. Pollen tube growth of *V. vitis-idaea*. (a) cross-pollinated flower in the alpine population (HIR), (b) self-pollinated flower in the alpine population (HIR), (c) cross-pollinated flower in the low-elevational population (EN1), and (d) self-pollinated flower in the low-elevational population (EN1).

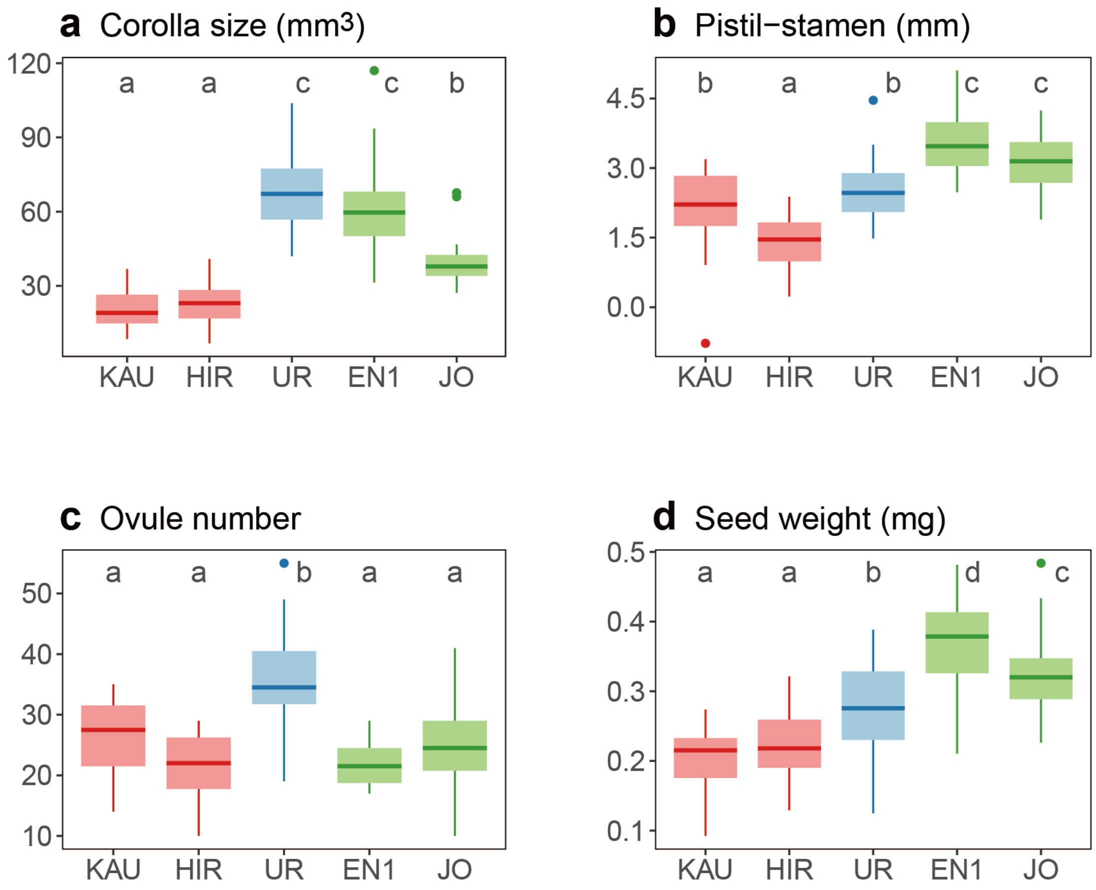


Fig. 2-4. Comparisons of reproductive traits among five populations: (a) corolla size, (b) pistil length-stamen length, (c) ovule number, and (d) seed weight.
Different letters: $P < 0.05$ according to GLM and Tukey's post-hoc test
Colors of box plots represent different habitat types (red: alpine, purple: open montane, green: montane forest, blue: coastal mire)

Table 2-1. Comparisons of reproductive traits among ploidy levels, habitat types, and geographic locations under natural pollination based on GLMM.
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Intercept = Ploidy 2*x*, Alpine, Northern Japan

		Estimate	Std. Error	z value	*P*	
Flower number / shoot	(Intercept)	1.95	0.11	18.4	< 0.001	***
	Ploidy 4*x*	0.10	0.19	0.53	0.60	
	Open montane	0.02	0.15	0.10	0.92	
	Montane forest	-0.09	0.21	-0.42	0.68	
	Coastal mire	-0.26	0.28	-0.93	0.35	
	Central Japan	0.07	0.21	0.34	0.74	
Fruit-set rate	(Intercept)	-1.25	0.47	-2.64	0.008	**
	Ploidy 4*x*	-0.86	0.82	-1.05	0.30	
	Open montane	0.05	1.20	0.05	0.96	
	Montane forest	-0.14	0.94	-0.15	0.87	
	Coastal mire	-1.06	0.67	-1.59	0.11	
	Central Japan	-2.21	0.98	-2.25	0.02	*
Seed number / fruit	(Intercept)	2.36	0.11	20.8	< 0.001	***
	Ploidy 4*x*	-0.80	0.22	-3.56	< 0.001	***
	Open montane	-0.23	0.27	-0.84	0.40	
	Montane forest	-0.15	0.28	-0.56	0.58	
	Coastal mire	-0.16	0.23	-0.69	0.49	
	Central Japan	-0.14	0.23	-0.62	0.53	

Table. 2-2. Estimated selfing rare ($s = 1 - tm$) of 26 *V. vitis-idaea* populations. Population: population IDs correspond to Table 1-1 and Fig. 1-2, Ploidy: ploidy level (2*x*: diploid, 4*x*: tetraploid), Location: geographic location (North: northern Japan, Central: central Japan), Year: the year sampling conducted

Population	Habitat	Ploidy	Location	Year	*s*
HIS	Alpine	2*x*	North	2016–2017	0
KAU	Alpine	2*x*	North	2016–2017	0.07
HIR	Alpine	2*x*	North	2016–2017	0
YO	Alpine	2*x*	North	2017	0.24
RAU	Alpine	2*x*	North	2017	0.08
SHO	Alpine	2*x*	North	2019	0.06
RAK	Alpine	2*x*	North	2019	0
ES	Open montane	2*x*	North	2018	0.06
UR	Coastal mire	2*x*	North	2016–2017	0.30
OC	Coastal mire	2*x*	North	2016–2017	0
HAK	Alpine	2*x*	North	2019	0.02
CH	Alpine	2*x*	North	2019	0
KI	Alpine	2*x*	Central	2019	0.22
TA	Alpine	2*x*	Central	2019	0.37
HIU	Alpine	2*x*	Central	2019	0.27
RI	Alpine	4*x*	North	2019	0.30
EN1	Montane forest	4*x*	North	2016–2017	0.37
EN2	Montane forest	4*x*	North	2016–2017	0.47
SHI	Montane forest	4*x*	North	2016	0.53
JO	Montane forest	4*x*	North	2016–2017	0.33
TEI	Montane forest	4*x*	North	2016–2017	0.28
BI	Open montane	4*x*	North	2017	0.19
RE	Open montane	4*x*	North	2019	0.29
HAM	Coastal mire	4*x*	North	2016–2017	0.49
SAR	Coastal mire	4*x*	North	2016–2017	0.37
NAG	Montane forest	4*x*	North	2019	0.35

Table 2-3. Comparisons of vegetative traits among ploidy levels, habitat types, and geographic locations across 30 populations based on GLMM.
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Intercept = Ploidy 2*x*, Alpine, Northern Japan

		Estimate	Std. Error	t value	*P*	
Annual shoot growth	(Intercept)	2.83	0.15	18.5	< 0.001	***
	Ploidy 4*x*	0.30	0.17	1.74	0.08	
	Open montane	0.36	0.19	1.85	0.06	
	Montane forest	0.65	0.21	3.15	0.00	**
	Coastal mire	0.42	0.19	2.21	0.03	*
	Central Japan	0.34	0.18	1.89	0.06	
Leaf area	(Intercept)	-0.63	0.17	-3.74	0.00	***
	Ploidy 4*x*	0.73	0.15	4.78	0.00	***
	Open montane	0.05	0.18	0.28	0.78	
	Montane forest	0.44	0.19	2.35	0.02	*
	Coastal mire	0.22	0.18	1.22	0.22	
	Central Japan	0.08	0.16	0.49	0.62	
LMA	(Intercept)	5.00	0.12	43.3	< 0.001	***
	Ploidy 4*x*	-0.12	0.09	-1.31	0.19	
	Open montane	0.11	0.10	1.07	0.28	
	Montane forest	0.04	0.11	0.39	0.70	
	Coastal mire	0.04	0.11	0.38	0.70	
	Central Japan	-0.20	0.10	-2.10	0.04	*
Annual leaf production	(Intercept)	-2.62	0.13	-19.9	< 0.001	***
	Ploidy 4*x*	0.67	0.25	2.73	0.01	**
	Open montane	0.29	0.28	1.03	0.30	
	Montane forest	0.57	0.29	1.95	0.05	
	Coastal mire	0.17	0.25	0.66	0.51	
	Central Japan	0.00	0.24	0.01	0.99	

Chapter 3: Variations in genetic structure, mating system, and morphological characteristics of *Rhododendron diversipilosum* populations in Japan

3.1 Introduction

In general, isolated populations at the distributional margin tend to possess unique genotypes, such as low genetic diversity, high selfing ability, and ecological characteristics adapted for local environments (Hampe and Petit 2005). However, some studies reported that not a few species had unique genetic and ecological characteristics different from the general pattern of marginal populations, such as high genetic diversity and high outcrossing rate (Yakimowski and Eckert 2007, 2008; Eckert *et al.* 2008). Phylogenetic background, mating system, and dispersal mode of individual species can affect the genetic and ecological characteristics of local populations (Hamrick *et al.* 1992), thus intra-specific comparisons is crucial to understand the historical background and maintenance mechanisms of marginal populations (Abeli *et al.* 2014).

As I mentioned before, alpine flora in Japan are composed of plants having diverse geographic distribution patterns (Shimizu 1982). In Chapters 1 and 2, I compared genetic traits and reproductive system of *V. vitis-idaea* populations and detected clear differences in genetic structure, mating system, and morphological traits between alpine and low-elevational populations. These results indicate the evidence of local adaptation in Arctic-alpine plants to various habitats in Hokkaido. The next question is that what kinds of genetic and ecological variations are seen in other species, having different origin and reproductive traits from *V. vitis-idaea*.

In northern Japan, *Rhododendron diversipilosum* (synonym: *Ledum palustre* ssp. *diversipilosum*, Ericaceae) often cooccur with *V. vitis-idaea*, both at the alpine and low-elevational habitats (Sato 2007). However, these two species have large differences in the geographic distribution ranges and reproductive traits. Geographical distribution of *R. diversipilosum* is restricted in Eastern Asia, mainly in boreal forests of eastern Siberia, while *V. vitis-idaea* is distributed widely across the northern hemisphere and its main habitat is tundra (Hulten 1968). *Vaccinium vitis-idaea* has pink-colored bell-shaped flowers visited by bees (Ritchie 1955), whereas *R. diversipilosum* flowers are white-colored and open-shaped, and visited by various insects including bees, flies, and butterflies (Hébert *et al.* 2010). Fleshy and red-colored sap fruits of *V. vitis-idaea* are dispersed by animals, whereas dry and dehiscent capsule fruits of *R. diversipilosum* are dispersed by gravity and wind (Campbell *et al.* 2003).

Because *R. diversipilosum* is absent in North America, it is highly possible that all populations distributed in Japan are originated in eastern Siberia. In other words, *R. diversipilosum* in Japan is considered to be a single origin and might have been dispersed to various environments after migration to Japan. In contrast, *V. vitis-idaea* populations in Japan have two different origins and they inhabit in different environments in northern Japan, as shown in Chapters 1 and 2. Therefore, differences in the original environment and reproductive characteristics may cause different patterns of population maintenance and local adaptation between the two species. In this Chapter, I focused on the genetic and ecological characteristics of *R. diversipilosum* with reference to the results of *V. vitis-idaea* (Chapters 1 and 2), to reveal general and/or species-specific patterns in the migration history and ecological adaptation of Japanese alpine plant.

3.2 Methods

Study species and study sites

Rhododendron diversipilosum (synonym: *Ledum palustre* ssp. *diversipilosum*) (Fig. 3-1) is a common ericaceous evergreen shrub in subalpine to alpine regions of Hokkaido and Tohoku areas in Japan (Shimizu 1982). This species grows also in coniferous forests on algific-talus slopes, coastal grasslands, high-moors, and sulfurous wilderness in Hokkaido. Outside Japan, the distribution of *R. diversipilosum* is restricted to eastern Siberia, Sakhalin, and the Southern Kuril Islands (Shimizu 1982), although there are several closely related species (the *Ledum* sect. in the genus *Rhododendron*) in the northern-hemisphere (Dampc and Luczkiewicz 2013; Hart *et al.* 2017). In the alpine region of Hokkaido, one related species *Rhododendron subarcticum* (synonym: *Ledum palustre* ssp. *decumbens*) exists, whose leaves and plant height are smaller than *R. diversipilosum*, and that species is widely distributed in the circumpolar regions (Shimizu 1982). Ploidy level of *R. diversipilosum* is unknown, although *R. subarcticum* is known as diploid ($2n = 2x = 26$, Hart *et al.* 2017).

The inflorescence (corymb) of *R. diversipilosum* is composed of many white flowers that are visited by various insects, and seeds are mainly dispersed by gravity from dehisced capsules but also by wind to some extent because of small sized seeds (Campbell *et al.* 2003). The mating system of *R. diversipilosum* is unclear, although self-compatibility has been reported in related species in North America (Hébert and Thiffault 2011).

Field survey in Japan was conducted for 18 populations, 17 populations in Hokkaido and one population in Tohoku (Table 3-1, Fig. 3-2). These populations are classified into four habitat types; alpine, open montane, montane forest, and coastal mire, in the same way as the populations of *V. vitis-idaea* (see Chapter 1). *Rhoddendron diversipilosum* coexisted with *V. vitis-idaea* in all study sites except for one site (KAW), where only *R. diversipilosum* occurred.

Analysis of microsatellite fragments and ploidy variation

During 2018–2019, one leaf and a few fruits per plant were sampled from 5–30 patches in each of 19 *R. diversipilosum* populations (17 in Hokkaido, one in Honshu, and one in eastern Siberia; Table 3-1, Fig. 3-2). All sampling points within Japan were mapped using a GPS (GPSmap 64s, Garmin). In addition, leaves of specimens collected outside Japan were sampled from herbarium of Hokkaido University Museum (SAPS); 9 samples from Sakhalin, and 12 samples from the Kuril Islands, respectively. DNA extraction and fragment analysis of microsatellite loci were conducted in the same way as *V. vitis-idaea* (Chapter 1). Because microsatellite arrays of *R. diversipilosum* were absent, I developed eight microsatellite markers (Table 3-2). In total, 1303 samples (535 leaves and 768 seeds) were analyzed.

Because the ploidy level of *R. diversipilosum* is unknown, I conducted a ploidy analysis based on the amount of DNA using a flow cytometer (Partec PA; Partec GmbH, Münster, Germany). One to five flesh leaves from 14 populations (KAU, HIS, HIR, YO, HAK, RAU, ES, KAW, EN, JO, TEI, SHI, SAR, RI; Fig. 3-2) were used for the ploidy analysis. Leaves of *R. subarcticum* were used as a standard because this species is known as a diploid ($2n = 2x = 26$; Hart *et al.* 2017).

Genetic differentiation among populations and genetic diversity within populations

I assessed genetic similarity between the populations by discriminant analysis of principal components (DAPC) to reveal the genetic structure of *R. diversipilosum*. As with *V. vitis-idaea*, Bruvo distances (Bruvo *et al.* 2004) were used for this analysis. In order to reveal the spatial genetic structures, furthermore, I calculated the extent of isolation-by-distance (IBD) across the populations using *Rho* value and geographic distance (incorporating elevation difference) of population pairs by Mantel test.

Clonal diversity was calculated based on Shannon-Wiener diversity (*H*) for 18 populations in Japan. Allelic richness (*Ar*), expected heterozygosity (*He*), and observed heterozygosity (*Ho*) were calculated as genetic diversity indices using SPAGeDi 1.5a.

Mating system

To examine the mating system of *R. diversipilosum* populations, I estimated selfing rates for each population based on multilocus outcrossing rates (*tm*) using MLTET (Ritland 1990; Murawski *et al.* 1994), and selfing rates (*s*) were calculated as $s = 1 - tm$. Calculations were performed with 1,000 bootstraps with families as resampling units.

In 2019, I conducted a pollination experiment in four populations, including both alpine and low-elevational populations (KAU, HIR, EN, JO; Fig. 3-2). In early summer, I selected 35 individuals with floral buds randomly, marked on one inflorescence, and recorded the flower number to measure fruit-set success under natural conditions. For 20 of selected individuals, furthermore, three more inflorescences were chosen for pollination treatments: one for a cross-pollination, one for a self-pollination, and one for an automatic-selfing pollination. All pollination treatments were conducted in the same way with *V. vitis-idaea*. The number of treated flowers was recorded, and fruit set was calculated in autumn. At fruiting period, a single fruit per inflorescence was sampled to count developed seeds and unfertilized ovules to calculate the seed-set rate per fruit. In addition, I calculated relative seed-set rate as fruit-set rate multiplied by seed-set rate.

Measurements of floral morphology and vegetative morphology

Corolla width was measured as flower size and distance between stigma and anther was estimated from the length of pistil and stamen in four populations (KAU, HIR, EN, JO). I randomly selected 30 flowers from different individuals in each population for the measurements. For the measurements of vegetative morphology, annual shoot growth and the number of current leaves were recorded for 30 shoots in all of 18 populations in Japan. One leaf was sampled in each of 30 shoots, and leaf area and dry mass were measured. Then, leaf mass per unit area (LMA) was calculated. Annual leaf production in each population was estimated from annual leaf number and dry mass of a single leaf.

Statistical analysis

ANOVA comparisons among different habitat types were conducted for allelic richness, expected heterozygosity, observed heterozygosity and selfing rate. For the comparisons of reproductive success among four populations, I used generalized linear models (GLMs) and Tukey's post-hoc test. Fruit-set rate (with negative binomial error distribution), and relative seed-set rate per shoot (with negative binomial error distribution) were compared among pollination treatments in each population. Relative seed-set rate per shoot of each pollination treatment (with negative binomial error distribution) was also compared among populations. Additionally, comparisons of flower number per shoot (with poisson error distribution), corolla width (with gamma error distribution) and distance between stigma and anther (with gamma error distribution) were conducted among populations. To compare the vegetative traits among populations, I used generalized linear mixed models (GLMMs). GLMMs (with gamma error distribution) were created for annual shoot growth, leaf area, LMA, and annual leaf production. Habitat type (intercept = alpine) was set as an explanatory variable and population ID was set as a random effect.

3.3 Results

Ploidy levels and genetic structure

A flow-cytometry analysis revealed that *R. diversipilosum* had twice as much DNA content as *R. subarcticum* in all populations. Because *R. subarcticum* is known to be a diploid ($2n = 2x = 26$), all *R. diversipilosum* populations are considered to be a tetraploid ($2n = 4x = 52$). Although I did not analyze the samples of the populations outside Japan (eastern Siberia, Sakhalin, Kuril), they were judged to be tetraploid, on the basis of the number of microsatellite peaks (see Chapter 1).

In the microsatellite analysis, I recorded a total of 176 alleles. Among the Japanese populations, nine populations carried one to four unique alleles each, and eight populations carried no unique allele (Table 3-3). Among 535 leaf samples from all populations, 358 genets were recognized. The number of genets varied from four to 30 per population. Most populations included diverse genotypes, except for two populations (RE and HAK). DAPC showed small separation of populations along the first two axes, where the three sites in eastern Hokkaido and Russian samples (EN, RAU, OC, SAK, KUR, SIB) formed a group separated from other populations, and RI formed a single genetic group that did not overlap with other populations (Fig. 3-3). Mantel tests revealed a significant IBD across populations ($r = 0.32$, $P < 0.05$; Fig. 3-4).

There was no significant difference in clonal diversity (*H*) among habitat types and latitudes ($P > 0.1$). In addition, the indices of genetic diversity (*Ar*, *He*, *Ho*) were not varied significantly among habitat types and latitudes ($P > 0.1$).

Mating system and reproductive characteristics

Multilocus estimates of selfing rates were very low in all populations, ranging from 0 to 0.16 (Table 3-3). In the pollination experiment, fruit-set rates of the cross-pollinated inflorescences were not significantly higher than the fruit-set rates of the naturally pollinated inflorescences in every population (Fig. 3-5a), indicating that pollen limitation was absent for fruit production of this species. In all populations, fruit-set rates in the cross-pollinated and self-pollinated groups were equivalent. The bagged inflorescences showed lower fruit-set rates than other treatments in all populations, although significant difference was not detected in KAU.

Relative seed-set rates of the cross-pollinated inflorescences were higher than those of the naturally pollinated inflorescences in all populations except for JO (Fig. 3-5b). The self-pollinated inflorescences resulted in lower relative seed-set rates than the cross-pollinated inflorescences, and the bagged inflorescences (automatic-selfing treatment) showed lowest values in all populations. In the comparison among populations, the low-elevational populations showed higher relative seed-set rates in all treatments (Fig. 3-6).

In the measurement of floral morphology, the montane-forest populations showed significantly larger flower size and flower number per shoot than the alpine populations. Flower number per shoot was the largest in EN and flower size was the largest in JO (Fig. 3-7-ab). In *R. diversipilosum*, stamens were longer than pistils, with a difference of more than 2 mm on average (Fig. 3-7c).

Vegetative traits

All vegetative traits varied significantly among habitat types (Table 3-4). In the comparisons of annual shoot growth, annual leaf production, and leaf area, the montane-forest populations showed the largest values, and the alpine populations showed the smallest values. Annual shoot growth of the montane-forest populations (49.2 ± 23.8 mm) was significantly larger than that of the alpine populations (24.8 ± 10.5 mm) ($P <$ 0.01). Leaf area was significantly greater in the open-montane populations (2.15 ± 0.86 cm^2), the montane-forest populations (4.86 ± 1.96 cm^2), and the coastal-mire populations (2.37 ± 0.86 cm^2) than in the alpine populations (1.25 ± 0.86 cm^2; $P <$ 0.001). LMA was significantly lower in the montane-forest populations (99.7 ± 24.4 gm^{-2}) than in the alpine populations (138.5 ± 51.4 gm^{-2}, $P < 0.05$). Annual leaf production varied significantly larger in the open-montane populations (0.439 ± 0.188 g), the montane-forest populations (0.544 ± 0.266 g), and the coastal-mire populations (0.432 ± 0.266 g) than in the alpine populations (0.141 ± 0.065 g; $P < 0.001$).

3.4 Discussion

Different from *V. vitis-idaea* populations, genetic differences between alpine and low-elevational populations were not clear in *R. diversipilosum*. Ploidy level of *R. diversipilosum* was tetraploid in all populations, regardless of habitat types. In the DAPC analysis, genetic differentiation was not found among the alpine and low-elevational habitat types in Japanese populations. Current distribution of *R. diversipilosum* is limited to northern Japan, Sakhalin, south Kuril Islands, and eastern

Siberia (Shimizu 1982). Considering the current distribution range, *R. diversipilosum* probably migrated from eastern Siberia via Sakhalin. Japanese populations were largely classified into three groups. Populations in three sites of eastern Hokkaido formed a same genetic group with Russian samples from Siberia, Sakhalin and Kuril, and Rishiri Island (RI) formed a separate genetic group from other populations. It is possible that the three populations in eastern Hokkaido might have migrated later than other populations. Because RI is in the isolated island, it might have become genetically distinct due to a strong bottleneck and/or genetic drift in the past.

Regarding the seed dispersal mode of the two species, *R. diversipilosum* is expected to have a shorter dispersal distance than *V. vitis-idaea*, and genetic differentiation between populations would be greater in *R. diversipilosum* (Hamrick *et al.* 1992). However, the genetic differentiation among populations was small in *R. diversipilosum*. In one possibility, *R. diversipilosum* might have migrated to Japan in relatively recent years, resulting in the small genetic differentiation among populations. As mentioned before, many alpine plants in Japan are divided into two lineages between the central Japan (southern lineage) and northern Japan from Tohoku to Hokkaido (northern lineage) (e.g. Ikeda *et al.* 2008, 2009). It is considered that northern lineage migrated to Japan in the last glacial period, while the southern lineage had migrated in the earlier glacial periods. Because *R. diversipilosum* is not distributed in central Japan, *R. diversipilosum* populations in Japan might migrate during the last glacial period like northern lineage of other alpine plants. In addition, because significant IBD was detected, there might occur gene flow between populations, which are geographically located close each other.

Clonal diversity was generally high in most populations, except for HAK and RE.

This result is unexpected from the property of *R. diversipilosum*, which can expand clonally by branching of prostrate stolon. It was reported that a congeneric species *R. groenlandicum*, which is distributed in North America, propagates vegetatively only when aboveground parts are destroyed by disturbance, while populations are mainly maintained by sexual reproduction (Hébert and Thiffault 2011). Because *R. groenlandicum* and *R. diversipilosum* are closely related species with similar morphological structures (Hart *et al.* 2017), *R. diversipilosum* may also be less likely to expand vegetatively in the absence of disturbance of habitat. RE population is located in an isolated island and HAK population is located at the southern limit of the distribution range, and their population sizes are quite small. Therefore, small habitat areas might result in low clonal diversity and these populations may be maintained by clonal growth.

Although *R. diversipilosum* have selfing ability both in the alpine and low-elevational populations, lower relative seed-set rate by self-pollination indicates a partial self-incompatibility in this species as reported in many other ericaceous species (Hokanson and Hancock 2000; Nuortila *et al.* 2006; Kudo *et al.* 2011). Some ericaceous plants have the property called cryptic self-incompatibility, which selectively develop seeds derived from cross-pollination, when both outcross-pollen and self-pollen are deposited on stigmas at the same time (Kameyama and Kudo 2009). It is possible that *R. diversipilosum* may have the same mating system to promote outcrossing. In addition, it is considered that cross-pollination likely to occur in *R. diversipilosum*, because many kinds of insects visit the flowers and clonal diversity is high within each population. Furthermore, automatic-selfing may not occur frequently because *R. diversipilosum* flowers are open-shaped and pistils and stamens are separately arranged, thus self-pollen rarely deposits on stigmas.

High heterozygosity and allelic richness were maintained in all populations both at the alpine and low-elevational habitats. This may reflect prevailing outcrossing and increased allele number associated with polyploidization (Soltis and Soltis 2000). The mechanism of population maintenance in *R. diversipilosum* may not vary between the alpine populations and the low-elevational populations. Differences in flower production, flower size, and relative seed-set success between the habitats might result from the milder and resource-rich environments at the low-elevational habitats.

Although there are no ploidy and genetic variations between the alpine and low-elevational populations in *R. diversipilosum*, morphological traits were significantly larger at the low-elevational populations, especially at the montane-forest habitat. As mentioned in Chapter 2, tall stature and large leaf area are advantageous for light capture in understory environment (Poorter *et al.* 2009). Although tetraploids have a tendency to grow larger than diploids, a few studies reported that some plant species possess the quite large morphological plasticity regardless of the ploidy level (Mráz *et al.* 2012; Rosche *et al.* 2018). In addition, morphological plasticity tends to be greater in polyploids than diploids (Otto 2007). Therefore, morphological variations between the alpine and low-elevational populations might reflect acclimation to local environments regardless of genetic background.

In summary, *R. diversipilosum* showed small variations in genetic composition and mating system but large variation in vegetative traits among habitat types. It is considered that *R. diversipilosum* populations in Japan have the same origin and expanded to the alpine and low-elevational habitats after migration. *Rhododendron groenlandicum*, a closely related species of *R. diversipilosum*, is also known to have large morphological variation and distributed across various environments, acclimating to wide range of temperature and humidity conditions (Hébert and Thiffault 2011). Availability of diverse pollinators for outcrossing and large morphological plasticity might have enabled these species to survive in various environments.

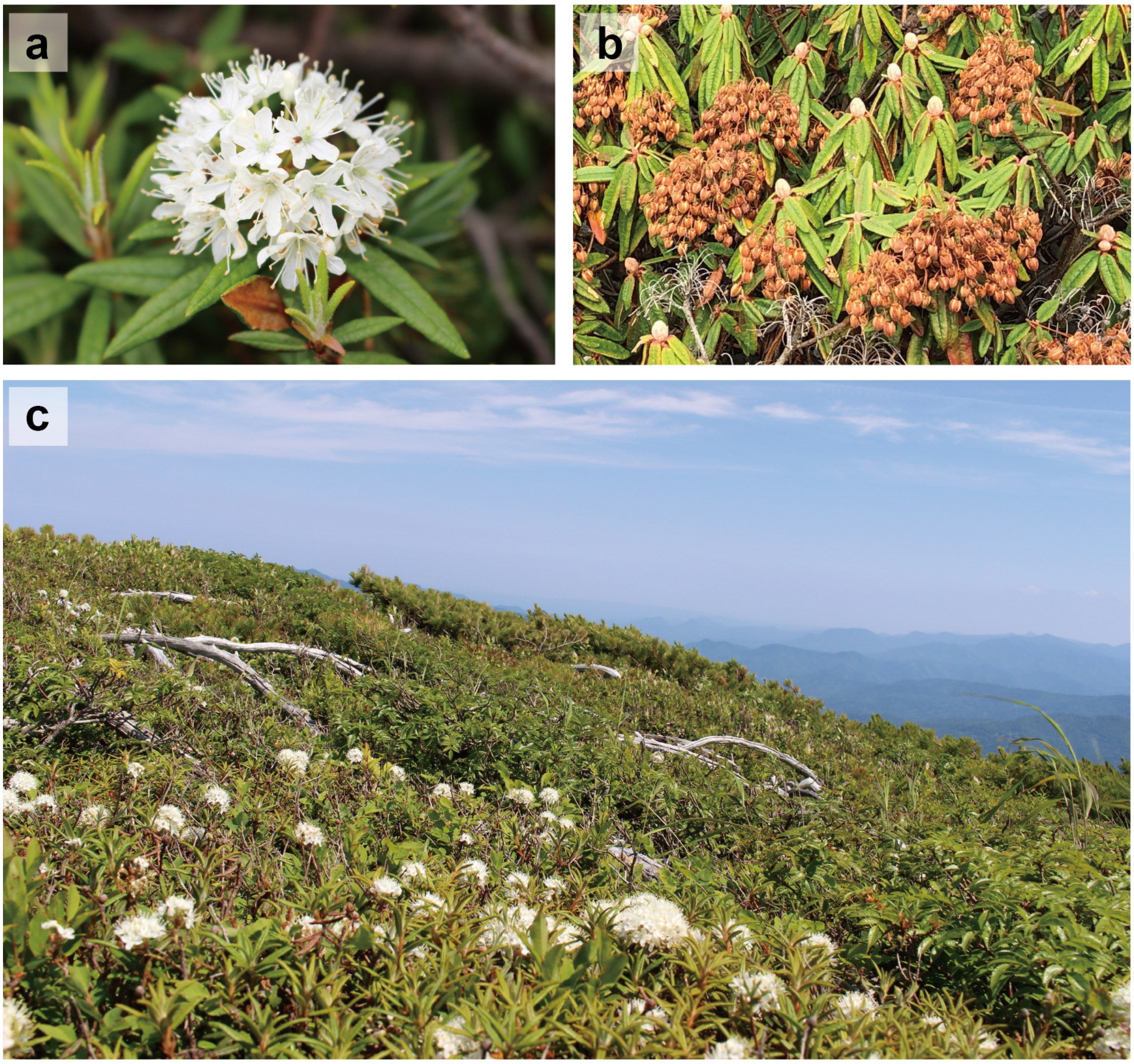


Fig. 3-1. *Rhododendron diversipilosum*. (a) Inflorescence composed of open-shaped flowers, (b) ripen capsules, and (c) population in flowering season.

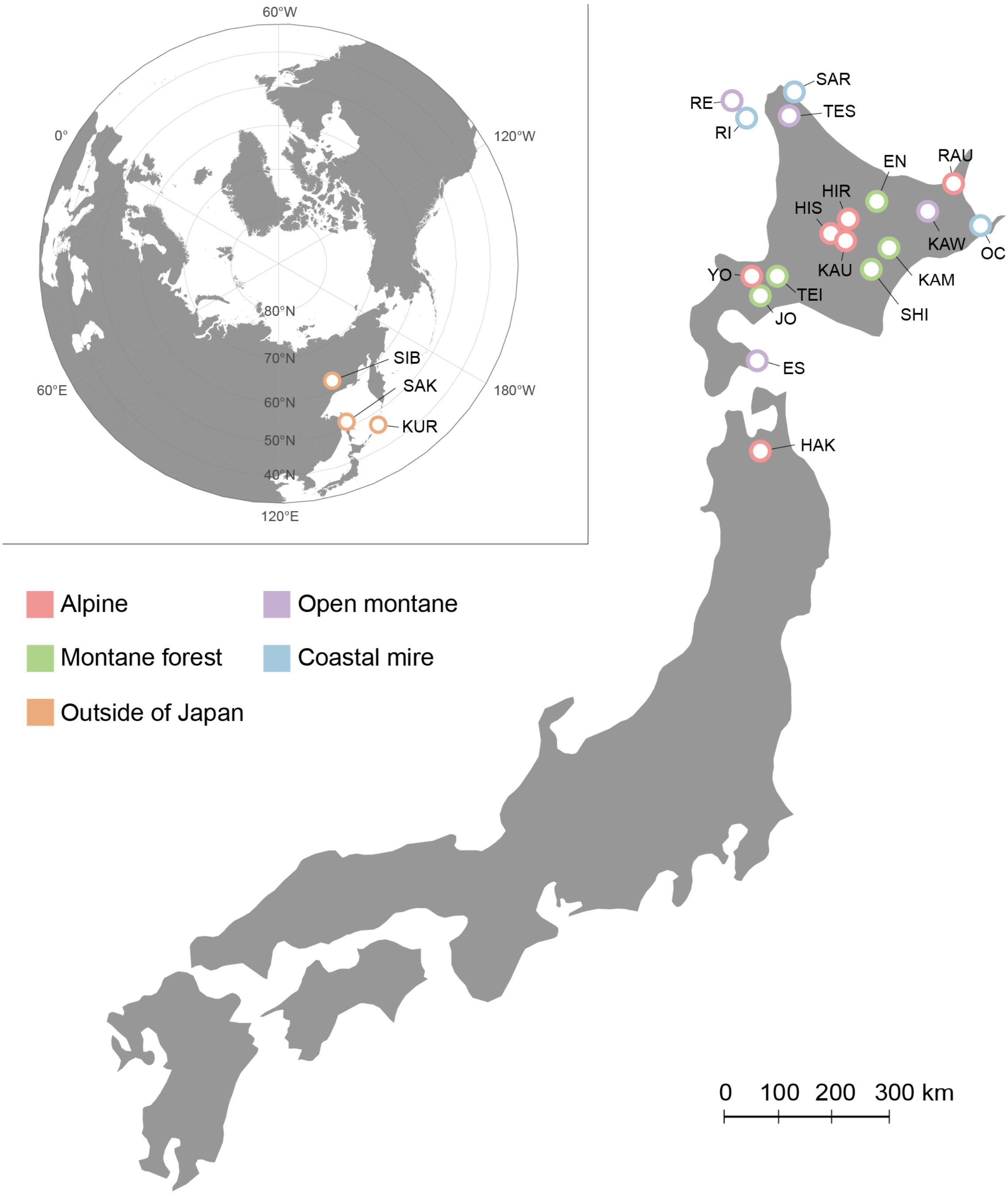


Fig. 3-2. Distribution and habitat type of sampling sites. Red circles represent alpine habitat, purple circles represent open-montane habitat, green circles represent montane-forest habitat, blue circles represent coastal-mire habitat, orange circles represent Russian sites. Population IDs correspond to those listed in Table 3-1.

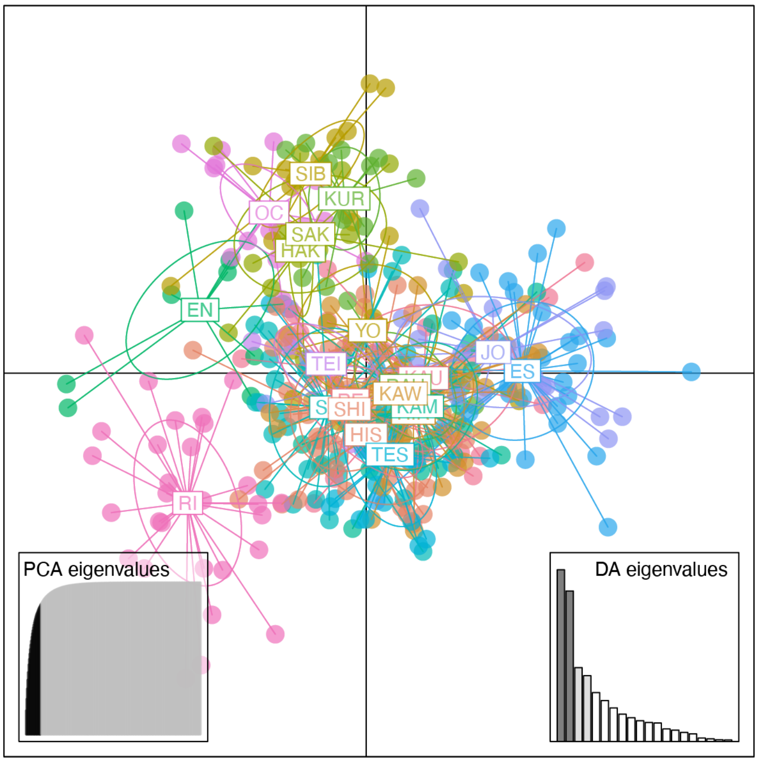


Fig. 3-3. Results of discriminant analysis of principal components (DAPC). Each point represents one genotype. Population IDs correspond to those listed in Table 3-1.

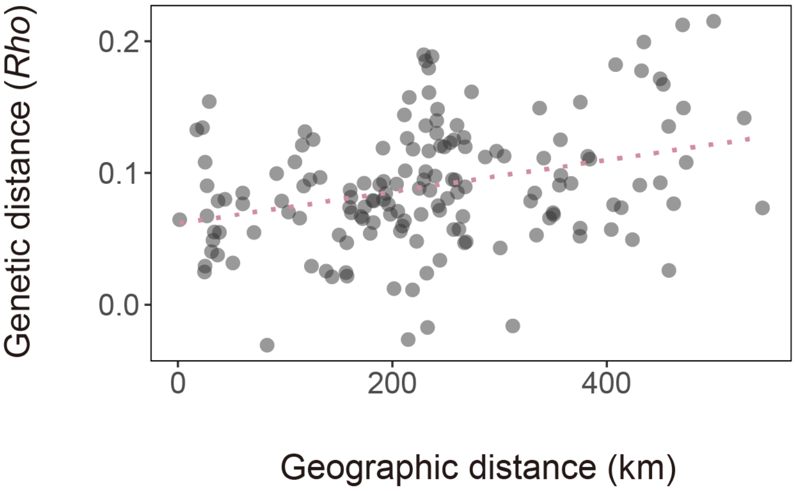
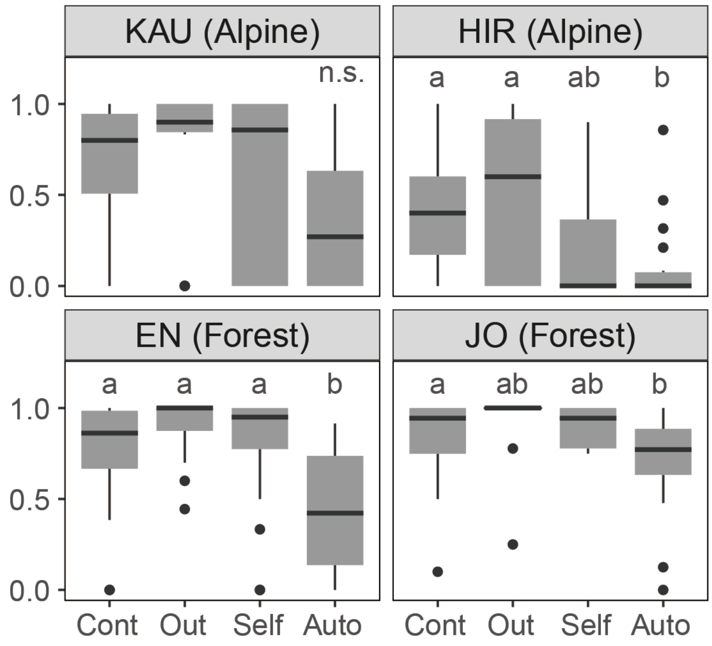


Fig. 3-4. Relationship between geographic distance (km) and genetic distance *Rho*. Geographic distance was calculated using QGIS, and *Rho* values (Ronfort *et al*. 1998) were calculated as genetic distance using SPAGeDi 1.5a.

a Fruit-set rate



b Relative seed-set rate

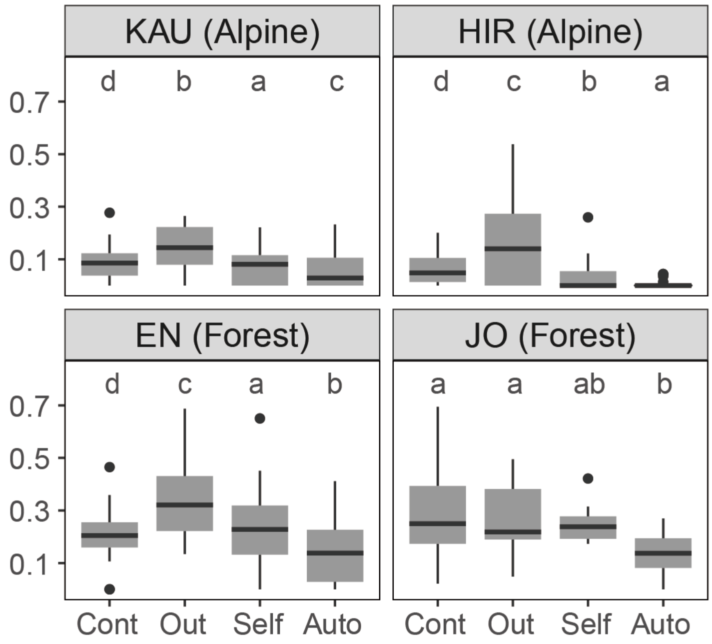


Fig. 3-5. Comparisons of (a) fruit-set rates and (b) relative seed-set rates among four pollination treatments in each population.

Cont: control, Cros: cross-pollination, Self: self-pollination, Auto: automatic-selfing

Different letters: $P < 0.05$ according to GLM and Tukey's post-hoc test

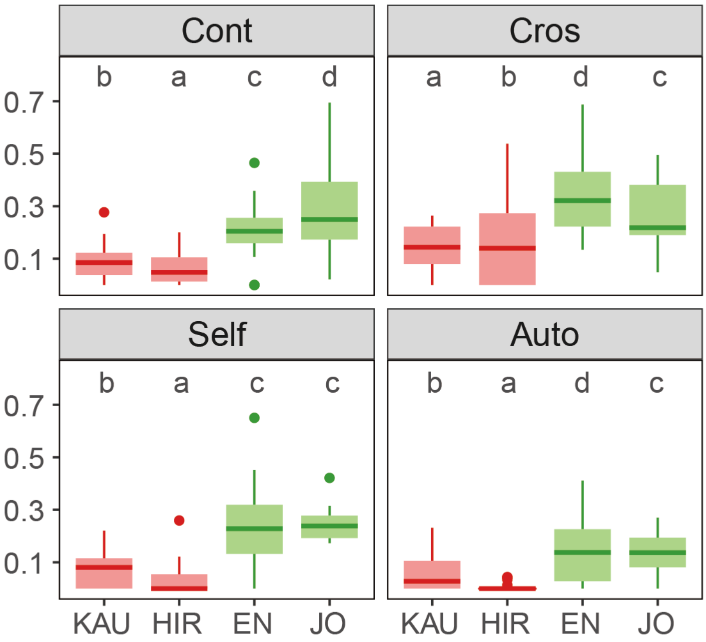


Fig. 3-6. Comparisons of relative seed-set rates among four populations in each pollination treatment.

Cont: control, Cros: cross-pollination, Self: self-pollination, Auto: automatic selfing

Different letters: $P < 0.05$ according to GLM and Tukey's post-hoc test

Colors of box plots represent different habitat types (red: alpine, green: montane forest)

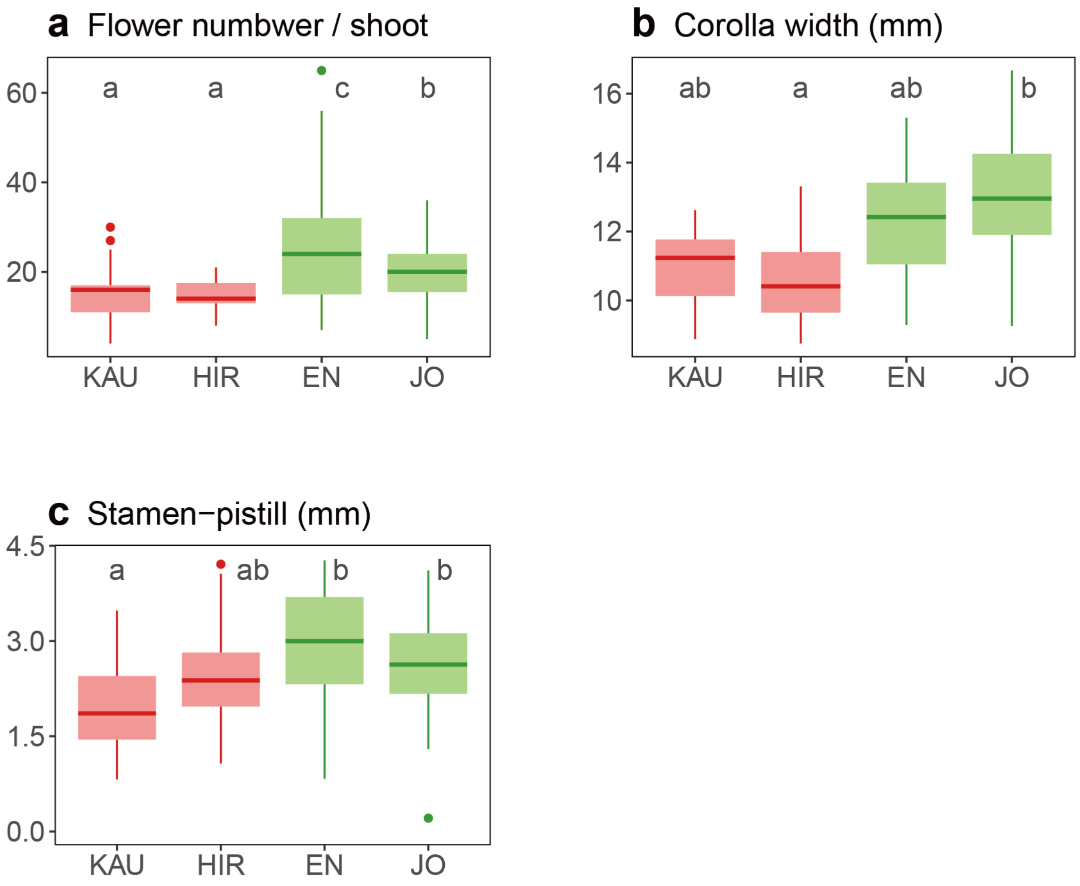


Fig. 3-7. Comparisons of floral traits among four populations: (a) flower number per shoot, (b) corolla width, and (c) stamen length-pistil length.
Different letters: $P < 0.05$ according to GLM and Tukey's post-hoc test
Colors of box plots represent different habitat types (red: alpine, green: montane forest)

Table 3-1. Geographic details of 21 sampling sites. See Fig. 3-2 for locations.

Population	ID	Habitat type	Region	Latitude	Longitude	Elevation (m)
Mt. Kaun	KAU	Alpine	Hokkaido	43° 34' 35"	142° 51' 06"	1907
Hisago Lake	HIS	Alpine	Hokkaido	43° 32' 04"	142° 53' 20"	1671
Mt. Hira	HIR	Alpine	Hokkaido	43° 47' 10"	143° 00' 37"	1721
Mt.Yoichi	YO	Alpine	Hokkaido	43° 02' 32"	141° 01' 06"	1485
Mt. Hakkoda	HAK	Alpine	Tohoku	40° 40' 16"	140° 48' 25"	1538
Mt. Rausu	RAU	Alpine	Hokkaido	43° 51' 45"	144° 52' 31"	1445
Teshio	TES	Open montane	Hokkaido	44° 56' 56"	142° 01' 06"	434
Esan	ES	Open montane	Hokkaido	41° 48' 12"	141° 09' 52"	352
Rebun	RE	Open montane	Hokkaido	45° 23' 07"	141° 01' 24"	422
Kawayu	KAW	Open montane	Hokkaido	43° 37' 20"	144° 26' 22"	160
Engaru	EN	Montane forest	Hokkaido	43° 55' 48"	143° 20' 45"	371
Kamishihoro	KAM	Montane forest	Hokkaido	43° 22' 31"	143° 10' 52"	828
Jozankei	JO	Montane forest	Hokkaido	42° 51' 33"	141° 09' 23"	720
Teine	TEI	Montane forest	Hokkaido	43° 04' 30"	141° 11' 27"	725
Shikaribetsu	SHI	Montane forest	Hokkaido	43° 11' 12"	142° 59' 18"	948

Continued

Sarufutsu	SAR	Coastal mire	Hokkaido	45° 16' 10"	142° 13' 15"	8
Ochiishi	OC	Coastal mire	Hokkaido	43° 12' 01"	145° 30' 37"	62
Rishiri	RI	Coastal mire	Hokkaido	45° 06' 41"	141° 16' 45"	6
Eastern Siberia	SIB	Outside of Japan	Russia			
Sakhalin	SAK	Outside of Japan	Russia			
Kuril	KUR	Outside of Japan	Russia			

Table 3-2. DNA sequences of eight newly developed SSR markers for *R. diversipilosum*: type of repeat, forward and reverse primer sequences, expected allele size from the sequencing data, and the primer annealing temperatures.

Primer name	Repeat motif	Forward / Reverse (5'-3')	Size	Tm (°C)
LP_SSR_004	(CCG)$_{8}$	GCGTTCGAATTTCGGATCGT / CCTCGCAGAAGATCCAGCAT	130	69.41
LP_SSR_047	(AAG)$_{8}$	GCAACCATACTATCCAGATGCC / CGTCCAACGGATAGTACGAGG	292	69.15
LP_SSR_016	(AG)$_{10}$	CCCAATGGCCGACAATCTGT / TCGAAATTCTCTGAGCTGGTT	229	66.42
LP_SSR_034	(AG)$_{12}$	GCGAGTTAGCAACCTGAGGA / TGAACAAATTGCCCTGCTGG	90	63.89
LP_SSR_003	(AG)$_{17}$	ACTTTCCTTCACCTTAACAAAGCA / AGCCTTGCCTTCGGAATGAA	120	67.51
LP_SSR_023	(AG)$_{21}$	CAATAATGAGTAACTGCCAATGCG / ATCGAAAGACACCAGATACAGCA	290	66.61
LP_SSR_028	(AAG)$_{8}$	CAACACAAACTTCTTTGAGCCT / TCTTTCCCTACAGGATATAACCGAA	139	66.27
LP_SSR_008	(AG)$_{11}$	TGCTCCTGTTGATACTCGAACT / CCAGCTTCTTCAATTCTGAGAGC	271	68.49

Table 3-3. Genetic characteristics of 18 populations in Japan. See Fig. 3-2 for locations. N: sample size, Genet: number of genets, P_A: number of private alleles, H: clonal diversity based on Shannon-Wiener's index, Ar: allelic richness, He: expected heterozygosity, Ho: observed heterozygosity, s: multilocus selfing rate

ID	Habitat	N	Genet	P_A	H	Ar	He	Ho	s
KAU	Alpine	30	18	2	2.84	4.81	0.83	0.79	0.09
HIS	Alpine	30	14	0	2.48	4.49	0.81	0.67	0.16
HIR	Alpine	30	20	1	2.90	4.59	0.82	0.66	0.09
YO	Alpine	30	11	2	2.34	4.56	0.81	0.77	-
HAK	Alpine	30	4	0	1.24	4.35	0.77	0.72	0
RAU	Alpine	5	5	0	1.61	5.36	0.87	0.87	-
TES	Open montane	30	21	1	2.98	4.55	0.81	0.64	0
ES	Open montane	30	30	4	3.40	4.89	0.83	0.75	0
RE	Open montane	30	4	1	0.66	3.54	0.73	0.78	-
KAW	Open montane	30	30	2	3.40	5.23	0.86	0.79	0
EN	Montane forest	30	10	2	2.25	4.29	0.78	0.81	0
KAM	Montane forest	30	23	2	3.05	4.51	0.81	0.69	-
JO	Montane forest	30	21	3	2.97	4.72	0.82	0.78	0
TEI	Montane forest	30	8	0	2.01	4.22	0.78	0.79	0
SHI	Montane forest	30	30	0	3.40	4.97	0.84	0.74	0
SAR	Coastal mire	30	30	1	3.40	4.95	0.83	0.73	0.1
OC	Coastal mire	30	15	1	2.69	3.92	0.74	0.73	-
RI	Coastal mire	30	29	0	3.35	4.37	0.79	0.75	0

Table 3-4. Comparisons of vegetative traits among habitat types across 18 populations based on GLMM.

****P* < 0.001, ***P*< 0.01, **P* < 0.05. Intercept = Alpine

		Estimate	Std. Error	t value	*P*	
Annual shoot growth	(Intercept)	3.21	0.20	15.99	< 0.001	***
	Open montane	0.10	0.20	0.47	0.64	
	Montane forest	0.60	0.20	3.07	0.002	**
	Coastal mire	0.23	0.22	1.03	0.3	
Leaf area	(Intercept)	0.21	0.11	2.04	0.04	*
	Open montane	0.53	0.17	3.21	0.001	**
	Montane forest	1.34	0.16	8.48	< 0.001	***
	Coastal mire	0.62	0.21	2.96	0.003	**
LMA	(Intercept)	4.86	0.11	43.32	< 0.001	***
	Open montane	0.21	0.12	1.75	0.08	
	Montane forest	-0.24	0.12	-2.05	0.04	*
	Coastal mire	0.22	0.15	1.44	0.15	
Annual leaf production	(Intercept)	-2.00	0.15	-13.64	< 0.001	***
	Open montane	1.15	0.22	5.24	< 0.001	***
	Montane forest	1.38	0.21	6.59	< 0.001	***
	Coastal mire	1.12	0.27	4.08	< 0.001	***

General discussion

The present study revealed three major conclusions regarding the evolutionary aspects of alpine plants in Japan. First, *Vaccinium vitis-idaea* populations at the alpine and low-elevational habitats were composed of different ploidy levels (diploids and tetraploids) derived from different origins (Chapter 1). Second, *V. vitis-idaea* populations at low elevations had higher selfing ability and larger plant size than the alpine diploid populations (Chapter 2). Third, all populations of *Rhododendron diversipilosum*, a sympatric species of *V. vitis-idaea*, were monophyletic having similar mating system across the habitat types from low elevations to alpine regions, but they had high morphological variations (Chapter 3). In this section, I discuss how the current distribution patterns of alpine plants have been formed in northern Japan, and how alpine plants from different origins established in different habitats and maintained the populations in various environments from low elevations to alpine regions.

Migration history of alpine plants in Japan

Japanese alpine plants are closely related to the species distributed in Beringia region (Shimizu 1982), the region from northeastern Asia to northwestern America that was not covered by ice sheets during the last glacial period (Frenzel 1968). Phylogenetic analyses were conducted for many alpine plant species in Japan that revealed the existence of unique haplotypes in the mountain regions of central Japan (Ikeda *et al.* 2008, 2009). On the other hand, the populations in northern Japan are mostly recognized as the same lineage with the populations in Beringia region. In this study, I found tetraploid populations of *V. vitis-idaea* at low-elevational local habitats, which was not previously recognized (Chapter 1). My analyses suggest that the diploid populations originated from North America and the tetraploid populations originated from eastern Asia. Also in the previous study, *V. vitis-idaea* populations in eastern Siberia and Alaska were classified into different genetic groups although the comparison of ploidy levels was not conducted (Eidesen *et al.* 2013). Therefore, the two genetic groups having different geographic distributions might have migrated to Hokkaido, via different land bridges, Sakhalin and the Kuril Islands. Such patterns are likely to occur due to the geographic location of Hokkaido, but there are no reports suggesting that different phyletic lineages of same species have migrated to Japan separately via Sakhalin and the Kuril Islands.

In the case of *R. diversipilosum*, all populations in Japan, Sakhalin, and the Kuril Islands were composed of tetraploids that were supposed to be originated in eastern Siberia (Chapter 3). According to the current distributions of *R. diversipilosum* in the Kuril Islands, this species is distributed only at the northern and southern parts of the Kuril Islands, and absent in the central part of the Kuril Islands (Takahashi 2015). Although Sakhalin had been connected to Japan during the last glacial period, Japan and Kamchatka have never been completely connected, due to the existence of deep channels between the Kuril Islands (Ono 1990). The difference in seed dispersal mode may be related to the diversity of lineage migrated to Japan. It is considered that *R. diversipilosum*, whose seeds are mostly dispersed by gravity, might be difficult to cross the straits of the Kuril Islands, whereas *V. vitis-idaea*, whose seeds are dispersed by birds, could migrate to Japan via the Kuril Island by long-distance seed dispersal. Therefore, there may be more diverse phyletic lineages of alpine plants than currently recognized in Hokkaido, especially for species that are distributed in both North

America and eastern Siberia, and possess high dispersal ability.

Population maintenance mechanism of alpine plants in Japan

Although distribution and phylogenetic patterns of alpine plants are well documented, there are few ecological studies focusing on how species originating in higher latitudes maintain populations in Japan. For *V. vitis-idaea*, this study revealed that the diploids in alpine environment and the tetraploids in low-elevational environments have different origins having different mating systems and morphological traits (Chapter 2). The diploids showed similar ecological characteristics to that reported in the tundra populations (i.e. self-incompatibility and small plant size; Landolt 1996; Guillaume and Jacquemart 1999). Because the biome of westernmost Alaska and the central part of the Kuril Islands is mostly like tundra without forest (Takahashi 2015), alpine environments in Japan may be a suitable habitat for the diploids. The tetraploids possessed characteristics of high selfing ability and large plant size. The tetraploids of *V. vitis-idaea* are considered to be originated in taiga biome in eastern Siberia, where coniferous forests spread widely and *V. vitis-idaea* grows in the understory. Therefore, the original habitats of the diploids and the tetraploids are very different, and low-elevational environments in Japan may be more favorable for the tetraploids. Several previous studies on polyploid complex reported distinct habitat segregation between ploidy levels (Rivero-Guerra 2008; Sonnleitner *et al.* 2010; Kudo and Hirao 2020), and the habitat segregation was related to the differences in mating systems and tolerance ability to environmental conditions between ploidy levels (Cosendai *et al.* 2013; Thompson *et al.* 2014; Karunarathne *et al.* 2018). A clear habitat separation of the diploid and tetraploid populations in *V. vitis-idaea* may reflect the different phylogenetic background and adaptation to different environments.

In contrast to *V. vitis-idaea*, *R. diversipilosum* populations did not show any clear genetic differentiation and mating system variation among habitat types, and they were considered to maintain the populations by the morphological regulation to the specific environments (Chapter 3). Considering that *R. diversipilosum* is originated in taiga biome in eastern Siberia, it is likely that this species might preadapt to understory environments, and have expanded the distribution to alpine environments after the migration to Hokkaido. On the other hand, *Rhoddendron subarcticum*, a diploid species closely related to *R. diversipilosum*, is distributed in tundra biome across circum-arctic area and in alpine regions in mid-latitudes. In the Taisetsu Mountains, *R. subarcticum* commonly grows at the exposed habitat near the mountain summits and ridges, while *R. diversipilosum* grows on leeward slopes and close to the shrubs of *Pinus pumila*, where the intensity of solar radiation and wind are moderate (unpublished information). Therefore, habitat differentiation between diploid *R. subarcticum* and tetraploid *R. diversipilosum* reflects the original environments of individual species, just like to the habitat differentiation between the ecotypes of *V. vitis-idaea*.

Rhododendron subarcticum is self-incompatible and has smaller morphological characteristics compared with *R. diversipilosum* (unpublished information). Taken together, both the tetraploid *V. vitis-daea* and *R. diversipilosum* possess relatively high selfing ability and large morphological variations, and grow in diverse environments in Japan. Therefore, tetraploid *V. vitis-idaea* and *R. diversipilosum* are ecologically equivalent, as well as diploid *V. vitis-idaea* and *R. subarcticum*. In general, tetraploids have higher adaptability to various environments in comparison with diploids (Soltis and Soltis 2000), and polyploids are considered to be advantageous in marginal populations (Abeli *et al.* 2014). The results obtained in this study are largely consistent with the general advantages of polyploidization.

Generally, the frequency of polyploid plants tends to increase toward polar regions or harsh environments (Rice *et al.* 2019). In contrast, polyploid populations existed in climatically milder environments in this study. Polyploid plants are advantageous in expanding to new environments, as they can reproduce quickly by selfing or clonal propagation (Rosche *et al.* 2017; Van Drunen and Husband 2018). It is considered that there are many polyploids in polar regions, as it was important for tundra plants to colonize quickly when ice sheets melt (Brochmann *et al.* 2004). On the other hand, since Beringia region was free from ice sheets, diploid ecotypes might be maintained. Conversely, if tetraploid *V. vitis-idaea* and *R. diversipilosum* evolved from diploid ancestors in their original distributional regions, polyploid evolution might be advantageous for Arctic plants to adapt to forest ecosystems, and it can be a preadaptation to the understory habitats for the maintenance of isolated populations in the southern distribution margins.

Conclusion

In this study, I discovered diploid and tetraploid populations of *V. vitis-idaea*, of which growing habitat types and ecological characteristics were different, reflecting the original environments. Because Hokkaido was the gateway for the most northern species, which migrated from Eurasia and North America to Japan, existences of much more phylogenetic lineages and ecotypes of alpine species are expected that have been overlooked in the previous studies. By the comparison between *V. vitis-idaea* and *R. diversipilosum*, I clarified that polyploidization has a potential to enhance the local adaptation of plants by the improvements of selfing ability and/or morphological plasticity. This finding indicates evolutionary importance of polyploid evolution in plants in terms of the exploitation of new habitats, expansion of distribution range, and ecotypic diversification or speciation.

In conclusion, Japanese alpine plants may contain more complex lineages and diverse adaptability to local habitats than previously recognized. Isolated populations in the local environments have the potential to accumulate genetic and ecological diversity and can be a driving force for speciation. In future studies, fine-scale phylogenetic and ecological studies including small populations at local habitat may provide new insights into the history of migration and local evolution of alpine plants in mid-latitudes.

Acknowledgements

I am deeply grateful to my supervisor Assoc. Prof. Gaku Kudo for his helpful discussions and suggestions throughout this study. I would thank to Prof. Yoichiro Hoshino, Prof. Shin-ichiro Aiba, and Prof. Shiro Tsuyuzaki for their kind advice and comments for this study. I appreciate Asst. Prof. Takuya Kubo for his suggestions on statistical analysis and Dr. Tetsuo Kohyama for his kind advice on genetic analysis.

I am grateful to Research Asst. Prof. Go Iwahana of University of Alaska Fairbanks, Prof. Naoya Wada of Toyama University, Prof. Elisabeth Cooper of University of Toromso, Assoc. Prof. Kenji Narita of Akita University, and Assoc. Prof. Hajime Kobayashi of Shinsyu University for their kind supports for sampling of plants in the fields. I appreciate Asst. Prof. Kohtaroh Shutoh of Hokkaido University Museum, for providing herbarium specimens for DNA analysis. I also appreciate all the officers and residents who provided information of my object species and guided me to the survey site. This work was partly supported by the Grant-in-Aid for JSPS Research Fellows [18J20218] from the Japan Society for the Promotion of Science (JSPS) and funding from Shirataki Geopark Promotion Council, Engaru-town, Hokkaido.

I thank Akari Shibata, Nozomi Oshima, Akira Matsuura, Yuki Shiotani, Jun Shigeeda and Yohei Ito, for their kind assistance in field work and helpful discussions and comments. I thank the member of Biodiversity Course, Graduate school of Environmental Science, Hokkaido University, for their helpful discussions and comments. Finally, I would like to thank Dr. Masashi Nakata and all staff of Botanic Gardens of Toyama, for helping chromosome observation and every kind encouragement in writing this thesis.

References

Abbott RJ, Smith LC, Milne RI, Crawford RMM, Wolff K, Balfour J. **2000**. Molecular analysis of plant migration and refugia in the Arctic. *Science* **289**: 1343–1346.

Abeli T, Gentili R, Mondoni A, Orsenigo S, Rossi G. **2014**. Effects of marginality on plant population performance. *Journal of Biogeography* **41**: 239–249.

Abeli T, Vamosi JC, Orsenigo S. **2018**. The importance of marginal population hotspots of cold-adapted species for research on climate change and conservation. *Journal of Biogeography* **45**: 977–985.

Alsos IG, Engelskjon T, Gielly L, Taberlet P, Brochmann C. **2005**. Impact of ice ages on circumpolar molecular diversity: insights from an ecological key species. *Molecular Ecology* **14**: 2739–2753.

Arnaud-Haond S, Teixeira S, Massa SI, *et al.* 2006. Genetic structure at range edge: Low diversity and high inbreeding in Southeast Asian mangrove (*Avicennia marina*) populations. *Molecular Ecology* **15**: 3515–3525.

Bliss L. 1962. Adaptations of arctic and alpine plants to environmental conditions. *Arctic* **15**: 117 – 144.

Brochmann C, Brystring A, Alsos IG, *et al.* 2004. Polyploidy in arctic plants. *Biological Journal of the Linnean Society* **82**: 521–536.

Bruvo R, Michiels NK, D'Souza TG, Schulenburg H. **2004**. A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Molecular Ecology* **13**: 2101–2106.

Campbell DR, Rochefort L, Lavoie C. **2003**. Determining the immigration potential of plants colonizing disturbed environments: The case of milled peatlands in Quebec. *Journal of Applied Ecology* **40**: 78–91.

Carbognani M, Piotti A, Leonardi S, *et al.* 2019. Reproductive and genetic consequences of extreme isolation in *Salix herbacea* L. at the rear edge of its distribution. : 849–860.

Choler P. **2005**. Consistent shifts in alpine plant traits along a mesotopographical gradient. *Arctic, Antarctic, and Alpine Research* **37**: 444–453.

Clark LV., Jasieniuk M. **2011**. Polysat: An R package for polyploid microsatellite analysis. *Molecular Ecology Resourses* **11**: 562–566.

Cosendai AC, Wagner J, Ladinig U, Rosche C, Hörandl E. **2013**. Geographical parthenogenesis and population genetic structure in the alpine species *Ranunculus kuepferi* (Ranunculaceae). *Heredity* **110**: 560–569.

Dampc A, Luczkiewicz M. **2013**. *Rhododendron tomentosum* (*Ledum palustre*). A review of traditional use based on current research. *Fitoterapia* **85**: 130–143.

DeChaine EG, Martin AP. **2005**. Marked genetic divergence among sky island populations of *Sedum lanceolatum* (Crassulaceae) in the Rocky Mountains. *American Journal of Botany* **92**: 477–486.

Van Drunen WE, Husband BC. **2018**. Immediate vs. evolutionary consequences of polyploidy on clonal reproduction in an autopolyploid plant. *Annals of Botany* **122**: 195–205.

Dufresne F, Stift M, Vergilino R, Mable BK. **2014**. Recent progress and challenges in population genetics of polyploid organisms: An overview of current state-of-the-art molecular and statistical tools. *Molecular Ecology* **23**: 40–69.

Eckert CG, Samis KE, Lougheed SC. **2008**. Genetic variation across species' geographical ranges: The central-marginal hypothesis and beyond. *Molecular Ecology* **17**: 1170–1188.

Eidesen PB, Ehrich D, Bakkestuen V, *et al.* **2013**. Genetic roadmap of the Arctic: Plant dispersal highways, traffic barriers and capitals of diversity. *New Phytologist* **200**: 898–910.

Frenzel B. **1968**. The Pleistocene vegetation of northern Eurasia. *Science* **161**: 637–649.

García-Fernández A, Iriondo JM, Escudero A. **2012**. Inbreeding at the edge: Does inbreeding depression increase under more stressful conditions? *Oikos* **121**: 1435–1445.

Garkava-Gustavsson L, Persson HA, Nybom H, Rumpunen K, Gustavsson BA, Bartish I V. 2005. RAPD-based analysis of genetic diversity and selection of lingonberry (*Vaccinium vitis-idaea* L.) material for ex situ conservation. *Genetic Resources and Crop Evolution* **52**: 723–735.

Guillaume P, Jacquemart A. **1999**. Early-inbreeding depression in*Vaccinium myrtillus* and *V. vitis-idaea*. *Protoplasma* **208**: 107–114.

Hampe A, Jump AS. **2011**. Climate relicts: past, present, future. *Annual Review of Ecology, Evolution, and Systematics* **42**: 313–333.

Hampe A, Petit RJ. **2005**. Conserving biodiversity under climate change: The rear edge matters. *Ecology Letters* **8**: 461–467.

Hamrick JL, Godt MJW, Sherman-Broyles SL. **1992**. Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6**: 95–124.

Hardy OJ, Vekemans X. **2002**. Spagedi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* **2**: 618–620.

Hart A, Kron K, Emily G. **2017**. Molecular phylogenetic analysis of the north-temperate labrador teas (Ericaceae: *Rhododendron* subsect. *Ledum*) suggests a complex genetic history. *Journal of the Botanical Research Institute of Texas* **11**: 53–65.

Hébert F, Thiffault N. **2011**. The biology of canadian weeds. 146. *Rhododendron groenlandicum* (Oeder) kron and judd. *Canadian Journal of Plant Science* **91**: 725–738.

Hébert F, Thiffault N, Ruel JC, Munson AD. **2010**. Comparative physiological responses of *Rhododendron groenlandicum* and regenerating *Picea mariana* following partial canopy removal in northeastern Quebec, Canada. *Canadian Journal of Forest Research* **40**: 1791–1802.

Hedrick PW, Garcia-Dorado A. **2016**. Understanding inbreeding depression, purging, and genetic rescue. *Trends in Ecology and Evolution* **31**: 940–952.

Herbert TJ. **2003**. A latitudinal cline in leaf inclination of *Dryas octopetala* and implications for maximization of whole plant photosynthesis. *Photosynthetica* **41**: 631–633.

Hewitt GM. **2000**. The genetic legacy of the quaternary ice ages. *Nature* **405**: 907–913.

Hewitt GM. **2004**. Genetic consequences of climatic oscillations in the Quaternary. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **359**: 183–195; discussion 195.

Hirao AS, Watanabe M, Tsuyuzaki S, *et al.* 2017. Genetic diversity within populations of an arctic–alpine species declines with decreasing latitude across the Northern Hemisphere. *Journal of Biogeography* **44**: 2740–2751.

Hiscock SJ, Tabah DA, Charlesworth D, Stephenson AG. **2003**. The different mechanisms of sporophytic self-incompatibility. *Philosophical Transactions of the Royal Society B: Biological Sciences* **358**: 1037–1045.

Hokanson K, Hancock J. **2000**. Early-acting inbreeding depression in three species of *Vaccinium* (Ericaceae). *Sex Plant Reprod* **13**: 145–150.

Hulten E. **1968**. *Flora of Alaska and Neighboring Territories: A Manual of the Vascular Plants*. Redwood, Canada: Stanford University Press.

Husband CB, Schemske DW. **1996**. Evolution of the magnitude and timing of inbreeding. *Evolution* **50**: 54–70.

Ikeda H, Senni K, Fujii N, Setoguchi H. **2008**. Survival and genetic divergence of an arctic-alpine plant, *Diapensia lapponica* subsp. *obovata* (Fr. Schm.) Hulten (Diapensiaceae), in the high mountains of central Japan during climatic oscillations.

Plant Systematics and Evolution **272**: 197–210.

Ikeda H, Senni K, Fujii N, Setoguchi H. **2009**. High mountains of the Japanese archipelago as refugia for arctic-alpine plants: Phylogeography of *Loiseleuria procumbens* (L.) Desvaux (Ericaceae). *Biological Journal of the Linnean Society* **97**: 403–412.

Ikeda H, Yoneta Y, Higashi H, *et al.* 2015. Persistent history of the bird-dispersed arctic–alpine plant *Vaccinium vitis-idaea* L. (Ericaceae) in Japan. *Journal of Plant Research* **128**: 437–444.

Jombart T. **2008**. Adegenet: A R package for the multivariate analysis of genetic markers. *Bioinformatics* **24**: 1403–1405.

Kameyama Y, Kudo G. **2009**. Flowering phenology influences seed production and outcrossing rate in populations of an alpine snowbed shrub, *Phyllodoce aleutica*: Effects of pollinators and self-incompatibility. *Annals of Botany* **103**: 1385–1394.

Kamvar ZN, Tabima JF, Grünwald NJ. **2014**. Poppr : An R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* **2**: e281.

Karunarathne P, Schedler M, Martínez EJ, Honfi AI, Novichkova A, Hojsgaard D. **2018**. Intraspecific ecological niche divergence and reproductive shifts foster cytotype displacement and provide ecological opportunity to polyploids. *Annals of Botany* **121**: 1183–1196.

Kawano S. **1971**. Studies on the alpine floral of Hokkaido Japan. *The Journal of the College of Liberal Arts, Toyama University, Japan* **4**: 13–96.

Kawecki TJ. **2008**. Adaptation to marginal habitats. *Annual Review of Ecology, Evolution, and Systematics* **39**: 321–342.

Kawecki TJ, Ebert D. **2004**. Conceptual issues in local adaptation. *Ecology Letters* **7**: 1225–1241.

Krebs SL, Hancock JF. **1990**. Early-acting inbreeding depression and reproductive success in the highbush blueberry, *Vaccinium corymbosum* L. *Theoretical and Applied Genetics* **79**: 825–832.

Kudo G, Hirao AS. **2020**. Geographical distribution, genetic diversity, and reproductive traits of mixed polyploid populations in *Parasenecio kamtschaticus* (Senecioneae; Asteraceae). *Plant Systematics and Evolution* **306**: 86.

Kudo G, Hirao AS, Kawai Y. **2011**. Pollination efficiency of bumblebee Queens and workers in the alpine shrub *Rhododendron aureum*. *International Journal of Plant Sciences* **172**: 70–77.

Kudo G, Suzuki S. **2003**. Warming effects on growth, production, and vegetation structure of alpine shrubs: a five-year experiment in northern Japan. *Oecologia* **135**: 280–287.

Landolt E. **1996**. *Vaccinium vitis-idaea* L. subsp. *minus* (G. Lodd.) Hultén (Ericaceae), an overlooked circumpolar-arctic taxon of the alps. *Anales del Jardín Botánico de Madrid* **54**: 277–284.

Mee JA., Moore JS. **2014**. The ecological and evolutionary implications of microrefugia. *Journal of Biogeography* **41**: 837–841.

Mimura M, Aitken SN. **2007**. Increased selfing and decreased effective pollen donor number in peripheral relative to central populations in *Picea sitchensis* (Pinaceae). *American Journal of Botany* **94**: 991–998.

Mishiba KI, Ando T, Mii M, *et al.* **2000**. Nuclear DNA content as an index character discriminating taxa in the genus *Petunia sensu Jussieu* (Solanaceae). *Annals of Botany* **85**: 665–673.

Moracho E, Moreno G, Jordano P, Hampe A. **2016**. Unusually limited pollen dispersal and connectivity of *Pedunculate oak* (Quercus robur) refugial populations at the species' southern range margin. *Molecular ecology* **25**: 3319–3331.

Mráz P, Španiel S, Keller A, *et al.* **2012**. Anthropogenic disturbance as a driver of microspatial and microhabitat segregation of cytotypes of *Centaurea stoebe* and cytotype interactions in secondary contact zones. *Annals of Botany* **110**: 615–627.

Murawski DA, Fleming TH, Ritland K, Hamrick JL. **1994**. Mating system of pachycereus pringlei: An autotetraploid cactus. *Heredity* **72**: 86–94.

Niinemets Ü. **2001**. Global-scale climatic controls of leaf dry mass per area, density, and thickness in trees and shrubs. *Ecology* **82**: 453–469.

Nuortila C, Tuomi J, Aspi J, Laine K. **2006**. Early-acting inbreeding depression in a clonal dwarf shrub, *Vaccinium myrtillus*, in a northern boreal forest. *Annales Botanici Fennici* **43**: 36–48.

Ono Y. **1990**. The northern landbridge of Japan. *Quaternary Research (Tokyo)* **29**: 183–192.

Otto SP. **2007**. The evolutionary consequences of polyploidy. *Cell* **131**: 452–462.

Pannell JR. **2015**. Evolution of the mating system in colonizing plants. *Molecular Ecology* **24**: 2018–2037.

Persson HA, Gustavsson BA. **2001**. The extent of clonality and genetic diversity in lingonberry (*Vaccinium vitis-idaea* L.) revealed by RAPDs and leaf-shape analysis. *Molecular Ecology* **10**: 1385–1397.

Plue J, Kimberley A, Slotte T. **2018**. Interspecific variation in ploidy as a key plant trait outlining local extinction risks and community patterns in fragmented landscapes. *Functional Ecology* **32**: 2095–2106.

Poorter H, Niinemets Ü, Poorter L, *et al.* **2009**. Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New phytologist* **182**: 565–588.

Popp M, Mirré V, Brochmann C. **2011**. A single mid-Pleistocene long-distance dispersal by a bird can explain the extreme bipolar disjunction in crowberries (*Empetrum*). *Proceedings of the National Academy of Sciences of the United States of America* **108**: 6520–6525.

Ramsey J, Ramsey TS. **2014**. Ecological studies of polyploidy in the 100 years following its discovery. *Philosophical Transactions of the Royal Society B: Biological Sciences* **369**: 15–19.

Ramsey J, Schemske DW. **1998**. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* **29**: 467–501.

Rice A, Šmarda P, Novosolov M, *et al.* **2019**. The global biogeography of polyploid plants. *Nature Ecology and Evolution* **3**: 265–273.

Ritchie JC. **1955**. *Vaccinium vitis-idaea* L. *Journal of Ecology* **43**: 701–708.

Ritland K. **1990**. Inferences about inbreeding depression based on changes of the inbreeding coefficient. *Evolution* **44**: 1230–1241.

Ritland K. **2002**. Extensions of models for the estimation of mating systems using independent loci. *Heredity* **88**: 221–228.

Rivero-Guerra AO. **2008**. Phenotypic differentiation of peripheral populations of *Santolina rosmarinifolia* (Asteraceae). *Botanical Journal of the Linnean Society* **158**: 650–668.

Ronfort J, Jenczewski E, Bataillon T, Rousset F. **1998**. Analysis of population structure in autotetraploid species. *Genetics* **150**: 921–930.

Rosche C, Durka W, Hensen I, *et al.* **2016**. The population genetics of the fundamental cytotype-shift in invasive *Centaurea stoebe* s.l.: genetic diversity, genetic differentiation and small-scale genetic structure differ between cytotypes but not between ranges. *Biological Invasions* **18**: 1895–1910.

Rosche C, Hensen I, Lachmuth S. **2018**. Local pre-adaptation to disturbance and inbreeding – environment interactions affect colonisation abilities of diploid and tetraploid *Centaurea stoebe*. **20**: 75–84.

Rosche C, Hensen I, Mráz P, Durka W, Hartmann M, Lachmuth S. **2017**. Invasion success in polyploids: the role of inbreeding in the contrasting colonization abilities of diploid versus tetraploid populations of *Centaurea stoebe* s.l. *Journal of Ecology* **105**: 425–435.

Sato K. **1995**. An outline of the cool-spots site vegetation of Hokkaido, Japan. *Bulletin of the Higashi Taisetsu Museum of Natural History* **17**: 107–115.

Sato K. **2007**. *Geobotanical study on the alpine vegetation of Hokkaido, Japan*. Hokkaido University Press.

Sato K, Kudo G, Uemura S. **1993**. Cool-spots site vegetation in Izariiri-Heide, northern Japan. *Japanese Journal of Ecology* **43**: 91–98.

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.

Shimizu T. **1982**. *The new alpine flora of Japan in color. Vol.1*. Hoikusha Publishing Co. Ltd, Osaka, Japan.

Shimizu C. **2004**. An information on the cool air blow holes (wind-holes) including the ice caves in Japan: References to the surrounding landforms and the existence of sporadic permafrost. *Komazawa Geography* **40**: 121–148.

Shimokawabe A, Yamaura Y, Akasaka T, *et al.* **2015**. The distribution of cool spots as microrefugia in a mountainous area. *PLOS ONE* **10**: e0135732.

Shimokawabe A, Yamaura Y, Sueyoshi M, Kudo G, Nakamura F. **2016**. Genetic structure of *Vaccinium vitis-idaea* in lowland cool spot and alpine populations: microrefugia of alpine plants in the midlatitudes. *Alpine Botany* **126**: 143–151.

Sicard A, Lenhard M. **2011**. The selfing syndrome: A model for studying the genetic and evolutionary basis of morphological adaptation in plants. *Annals of Botany* **107**: 1433–1443.

Soltis DE, Soltis PS. **1999**. Polyploidy: recurrent formation and genome evolution. *Science* **14**: 348–352.

Soltis PS, Soltis DE. **2000**. The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences* **97**: 7051–7057.

Soltis DE, Soltis PS, Tate JA. **2004**. Advances in the study of polyploidy since plant speciation. *New Phytologist* **161**: 173–191.

Sonnleitner M, Flatscher R, Escobar García P, *et al.* **2010**. Distribution and habitat segregation on different spatial scales among diploid, tetraploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps. *Annals of Botany* **106**: 967–977.

Takahashi H. **2015**. *Plants of the Kuril Islands*. Sapporo: Hokkaido University Press.

Tao R, Iezzoni AF. **2010**. The S-RNase-based gametophytic self-incompatibility system in *Prunus* exhibits distinct genetic and molecular features. *Scientia Horticulturae* **124**: 423–433.

Thompson KA, Husband BC, Maherali H. **2014**. Climatic niche differences between diploid and tetraploid cytotypes of *Chamerion angustifolium* (Onagraceae). *American Journal of Botany* **101**: 1868–1875.

Varshney RK, Graner A, Sorrells ME. **2005**. Genic microsatellite markers in plants: Features and applications. *Trends in Biotechnology* **23**: 48–55.

Vucetich JA, Waite TA. **2003**. Spatial patterns of demography and genetic processes across the species' range: Null hypotheses for landscape conservation genetics. *Conservation Genetics* **4**: 639–645.

Wada N. **2008**. Ecology of *Dryas octopetala sensu lato*, a relict plant in the Tateyama Mountains in the North Alps of Japan: Comparison between mid-latitude alpine and arctic tundra populations. *Japanese Journal of Ecology* **58**: 205–212.

Wakui A, Sueyoshi M, Shimokawabe A, Kudo G, Morimoto J, Nakamura F. **2017**. Environmental factors determining the distribution of highland plants at low-altitude algific talus sites. *Ecological Research* **32**: 183–191.

Winkler M, Tribsch A, Schneeweiss GM, *et al.* 2012. Tales of the unexpected: Phylogeography of the arctic-alpine model plant *Saxifraga oppositifolia* (Saxifragaceae) revisited. *Molecular Ecology* **21**: 4618–4630.

Wright S. **1943**. Isolation by distance. *Genetics* **28**: 114–138.

Wright IJ, Reich PB, Cornelissen JHC, *et al.* 2005. Modulation of leaf economic traits and trait relationships by climate. *Global Ecology and Biogeography* **14**: 411–421.

Yakimowski SB, Eckert CG. **2007**. Threatened peripheral populations in context: Geographical variation in population frequency and size and sexual reproduction in a clonal woody shrub. *Conservation Biology* **21**: 811–822.

Yakimowski SB, Eckert CG. **2008**. Populations do not become less genetically diverse or more differentiated towards the northern limit of the geographical range in clonal *Vaccinium stamineum* (Ericaceae). *New Phytologist* **180**: 534–544.

Zhu H, Senalik D, McCown BH, *et al.* 2012. Mining and validation of pyrosequenced simple sequence repeats (SSRs) from American cranberry (*Vaccinium macrocarpon* Ait.). *Theoretical and Applied Genetics* **124**: 87–96.