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Genetic structure of *Vaccinium vitis-idaea* in lowland cool spot and alpine populations:

Microrefugia of alpine plants in the mid-latitudes

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Abstract (246 words: 150–250 words)

Local cool spots (wind-holes) in lowland areas of mid-latitudes may act as microrefugia for cold-adapted species outside of their typical alpine habitats. We examined the genetic structure of *Vaccinium vitis-idaea*, a common alpine species in Japan, in eight lowland wind-hole and five surrounding alpine populations. We collected leaf samples and genotyped seven microsatellite loci. Clonal patches (genets) were common in almost all populations. An analysis of annual shoot growth suggested that individuals in the wind-hole populations were long-lived (>500 years old). Genetic diversity (allelic richness) and differentiation (F_{ST}) of the wind-hole populations were lower and higher than those of the alpine populations, respectively. No significant isolation-by-distance trend in the genetic structure was detected for the wind-hole or alpine populations. All wind-hole populations had negative inbreeding coefficients (F_{IS}), suggesting no tendency toward homozygosity due to inbreeding, regardless of the small populations geographically isolated from the large alpine populations. Therefore, wind-holes may harbor genetically isolated but stable populations due to clonal growth, limited gene flow, and abortion of selfed seeds by early acting inbreeding depression. Analysis of molecular variance demonstrated that genetic variations among and within populations contributed more to regional genetic diversity than those between wind-hole and alpine populations, suggesting that the wind-hole and alpine populations are important for maintaining the genetic diversity of mid-latitude *V. vitis-idaea* populations. On

37 the other hand, Bayesian clustering showed that some wind-hole populations geographically
38 close to the alpine populations had mixed genetic compositions of the alpine and wind-hole
39 populations.

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Introduction

Many species inhabiting mountainous areas have upwardly shifted their distributions along with ongoing climate change (Walther et al. 2005; Forister et al. 2010). These elevational shifts are large in alpine plants (Lenoir et al. 2008), and the future distributions of these species are predicted to decline greatly due to the limited area for escape in alpine and geographically isolated habitats (Thuiller et al. 2005; Thuiller 2007). Large-scale range shifts associated with climate change have occurred in past glacial periods, and it has been suggested that small populations persist in local suitable habitats within broadly unsuitable geographical areas (Stewart and Lister 2001; McLachlan et al. 2005; Parducci et al. 2012). These local refugia are called “microrefugia” in contrast to the large, continuous “macrorefugia” (Rull 2009).

Microrefugia are characterized by locally specialized microclimates that are only loosely linked to the surrounding macroclimate. As such, they can provide long-lasting small habitats for specialist species (Dobrowski 2011). In the current interglacial period, alpine habitats are macrorefugia, whereas cool spots situated in topographic depressions, heavily incised valley bottoms, or debris-covered glaciers can be microrefugia (Dobrowski 2011; Gentili et al. 2015). As these small cool spots can be distributed over a broad geographical range (Shimokawabe et al. 2015), microrefugia may play important roles in the response of many species to climate change (Mosblech et al. 2011; Hannah et al. 2014).

Vaccinium vitis-idaea L. (Ericaceae) is a common alpine dwarf shrub inhabiting mid-latitude alpine and subarctic regions. *V. vitis-idaea* also grow in lowland cool spots, including wind-hole micro-topographies (Sato et al. 1993; Sato 1995; Růžicka 1999). Wind holes frequently occur at the bottom of talus slopes containing volcanic bedrock (Shimizu 2004), where cool conditions are maintained in the vicinity by the preferential flow of cool air generated in interstitial spaces created by rock fragments or colluvia. We call such cool spots “wind-hole sites.” Wind-hole sites harbor various taxa of alpine species that are not found in the surrounding areas (Růžicka 1999; Shimokawabe et al. 2015). Shiboi (1975) speculated that lowland *V. vitis-idaea* populations have been maintained in local wind-hole sites since the last glacial period in Hokkaido, northern Japan. If this is true, not only alpine habitats (macrorefugia) but also local wind-hole sites (microrefugia) are important habitats for the maintenance of *V. vitis-idaea* population genetic diversity in mid-latitude regions.

Populations in small and isolated habitats are subject to heightened extinction risks due to inbreeding depression, genetic drift, and demographic stochasticity (Lande 1988; Reed and Frankham 2003). Several mechanisms contribute to the long-term maintenance of microrefugia populations (Hampe and Jump 2011). One exogenous mechanism is migration from the surrounding populations (Mosblech et al. 2011), and one endogenous mechanism is purging deleterious alleles and self-fertilization/compatibility (Mee and Moore 2014). Wind-hole populations are small and isolated from alpine populations, and the mechanisms of

population maintenance may be manifested in their genetic structure.

In the present study, we examined the genetic structure of *V. vitis-idaea* populations in northeastern Hokkaido, including alpine and lowland wind-hole populations. We also measured the annual shoot growth to estimate the age of clonal patches. We discuss the mechanism of population maintenance at wind-hole sites and its ecological significance under ongoing climate change.

Methods

Species studied and field sampling

V. vitis-idaea ($2n = 24$) is an evergreen dwarf shrub (5–20 cm in height) with creeping stems under the soil surface (Iwatsuki et al. 1993). Based on genetic analyses, Ikeda et al. (2015) suggested that Japanese *V. vitis-idaea* populations have persisted since before the last glacial period. The major habitats in our study area (Engaru and Kitami: 43.7–43.9° N and 143.0–143.4° E, respectively) were alpine zones and lowland wind-hole sites. This species is also known to occur on acid soils in boreal forests and bogs (Garkava-Gustavsson et al. 2005). However, lowland *V. vitis-idaea* populations in Japan other than those at wind-hole sites are limited and fragmentally distributed only in the coastal wetlands of northern/eastern Hokkaido (Umezawa 2007). Flowering occurs in early summer; shrubs are pollinated mainly

by bees, and seeds are distributed by animals (Ritchie 1955; Iwatsuki et al. 1993). Upon self-fertilization, partial self-sterility leads to reduced numbers of developed seeds due to early acting inbreeding depression (Guillaume and Jacquemart 1999). *V. vitis-idaea* can form large clonal patches (>30 m) by expanding via underground stems (Persson and Gustavsson 2001).

The study area was located in northeastern Hokkaido (Fig. 1). Alpine habitats harboring *V. vitis-idaea* cover the western high elevation area (>1,500 m), and wind-hole sites with *V. vitis-idaea* are scattered across the eastern lowland area. This area is topographically complex and includes steep slopes and bedrock dominated by pumice flow deposits (1:200,000 surface geology map by the Ministry of Land, Infrastructure, Transport, and Tourism of Japan). The mean annual temperature during the past 30 years was 5.8°C (mean temperatures of the coldest and warmest months were −8.3 and 19.9°C, respectively) and annual precipitation was 794.5 mm (Japan Meteorological Agency, <http://www.jma.go.jp/jma/indexe.html>). The temperature at ground level remains 7°C lower than the air temperature during the summer (Shimokawabe et al. 2015).

We collected leaves in August–September 2013 from eight wind-hole sites and five separate alpine habitats (Fig. 1). The sample size was 23–31 leaves from each site, totaling 393 samples (Table 1). No additional wind-hole sites known to harbor *V. vitis-idaea* are present in our study area (Shimokawabe et al. 2015). We uniformly sampled leaves from all

areas of the wind-hole sites and from the ridge areas in the alpine habitats (sampling interval: 3–10 m for wind-hole sites, 5–10 m for alpine habitats). We recorded the locations of the sampling points using a global positioning system (Garmin GPSMAP 62SJ; Garmin International, Inc., Olathe, KS, USA). Leaf samples were dried in silica gel and preserved at room temperature until analysis.

Microsatellite analysis

We extracted DNA using the cetyltrimethylammonium bromide (CTAB) method. In brief, the 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. Seven microsatellite loci (CA169F, CA236F, NA741, NA800, NA1040, VCC_I2 and VCC_K4: Boches et al. 2005; Appendix A) were amplified and developed for a related species (*Vaccinium corymbosum* L.) using polymerase chain reaction (PCR) and a thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems Inc., Foster City, CA, USA). Following Boches et al. (2005), we used the following PCR program: 3 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 60°C or 62°C, and 40 s at 72°C with a final rest for 30 min at 72°C. Microsatellite fragments were analyzed using the Applied Biosystems 3130 Genetic Analyzer, and the genotypes were coded using Peak Scanner ver. 2.0 (Applied

136 Biosystems). GeneScan 500 LIZ Size Standard (Applied Biosystems) was used as the size
137 standard.

138

139 Population genetics analysis

140 A total of 244 multi-locus genotypes in seven loci were identified, and 69 were
141 assigned to more than two different samples (17.6%). Samples with the same genotypes in all
142 loci were treated as the same individual (genet), and the genet was used as the analytical unit.
143 We calculated the probability that two different individuals would have the same genotype
144 (PI: probability of identity) using GenAlEx 6.501 (Peakall and Smouse 2006) to evaluate
145 misassignment of different individuals to genets. We tested departures from Hardy–Weinberg
146 equilibrium based on the chi-square (χ^2) test using GenAlEx 6.501 and obtained the
147 inbreeding coefficient (F_{IS}). We examined the presence of null alleles for each population
148 using GENEPOP 4.3 (Rousset 2008). The Bonferroni correction was used to determine the
149 significance levels of the equilibrium tests.

150 We obtained F_{ST} values as genetic differentiation indices for each pair of populations.

151 The value of this index can be affected by population history, mutation, gene flow, and genetic
152 drift (Marko and Hart 2011). The correlation between the F_{ST} value and geographic distance
153 (isolation by distance, IBD: Wright 1943) was examined based on the Mantel tests for (i) all
154 populations, (ii) wind-hole and wind-hole pairs, and (iii) alpine and alpine pairs. We also

examined the differences in genetic differentiation (F_{ST}) and geographic distances among populations for (i) wind-hole and wind-hole pairs, (ii) wind-hole and alpine pairs, and (iii) alpine and alpine pairs with multiple comparison tests (Steel–Dwass method). We obtained the geographic distance as three-dimensional straight-line distances considering elevational differences. The F_{ST} values were calculated, Mantel tests were conducted using GenAlEx 6.501, and multiple comparison tests were performed using R ver. 2.15.2 (R Core Team, Vienna, Austria).

We calculated allelic richness and private allelic richness (the number of unique alleles for each population, corrected for sample size) as genetic diversity indices (Kalinowski 2004) using HP-Rare v. June-6-2006 (Kalinowski 2005). The diversity indices were compared between wind-hole sites and alpine habitats using *t*-tests. The correlation coefficient between allelic richness and the index of genetic isolation was determined as the mean F_{ST} value for each population. The mean F_{ST} was obtained from the F_{ST} values of all other populations, and the correlations were examined separately for the wind-hole sites and alpine habitats.

We conducted analyses of molecular variance (AMOVA) based on these F_{ST} values to divide genetic variation into those occurring within populations, among populations, and among habitats using GenAlEx 6.501. STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to examine individual based Bayesian clustering to estimate the most likely number of genetically differentiated populations (*K*). An admixture model with the correlated allele

frequencies and informative locations was employed. The optimal number of clusters from 1 to 13 was inferred by 200,000 interactions following a 100,000 step burn-in with 10 replicate runs for each K -value. STRUCTURE can identify the highest hierarchical level of a population but often fails to detect hierarchical genetic structure at lower levels (Evanno et al. 2005). Therefore, subsequent runs were executed with the same method to reveal additional hierarchical genetic clusters when $K > 1$ was identified at the initial run. The STRUCTURE results and the online program STRUCTURE HARVESTER ver. 0.6.94 (Earl and vonHoldt 2012) were used to determine the best K number. This program infers the best K value using the delta K based on the rate of change in the log probability of the data ($\text{Ln } K$) between successive K values (the best K has the highest delta K value) (Evanno et al. 2005). Finally, the online program CLUMPAK was used to visualize the spatial genetic structure estimated by STRUCTURE (Kopelman et al. 2015).

Annual growth measurements

We measured the annual growth of shoots and estimated their ages to infer the persistence of individuals at the wind-hole sites. We measured annual growth length of the previous year's shoots from 10 individuals each in the six wind-hole populations during May 2015. We thereby obtained a mean value for the 10 individuals in each population. The longest length between ramets was obtained from genetic analyses. We treated this length as

the size of the oldest individual in the corresponding population, and estimated its age assuming circular growth: $\text{Age} = (0.5 \times \text{distance between ramets}) / \text{annual growth}$.

Results

The mean PI across 13 populations, which indicates the probability of misassignment of different individuals to genets, was very low (2×10^{-4}). Therefore, we treated plants with the same genotype as the same individual (genet). Clones were detected at all alpine and wind-hole sites, except at Mt. Mur (Table 1). The longest distances within clones were 14.3–101.8 m (Table 1). Large ramets had between-ramet lengths > 50 m (Appendix B). The mean annual shoot growth of the six wind-hole populations was $3.90 \text{ cm year}^{-1}$. The largest clone was estimated to be 594 years old, and four populations had clones that were > 500 years old (Table 1). Note that our calculations may have underestimated the ages of the clones because we assumed a uniform circular growth pattern. However, this result suggests that *V. vitis-idaea* individuals have been maintained at the wind-hole sites for hundreds of years. We observed deviations from Hardy–Weinberg equilibrium at a mean of two populations per locus (adjusted $P < 0.05$). All wind-hole populations had negative F_{IS} values, and six populations differed significantly from zero ($P < 0.05$; Table 1). The existence of null alleles was suggested for multiple loci and populations. However, their frequencies were small, and we used all loci and genets (mean frequency per loci = 0.03).

The Mantel tests did not reveal any significant IBD among populations ($r = -0.12$, $P = 0.23$; Fig. 2) or within habitat types (wind-hole–wind-hole pairs: $r = 0.32$, $P = 0.16$; alpine–alpine pairs: $r = 0.05$, $P = 0.54$; Fig. 2). However, the alpine–alpine pairs and the wind-hole–wind-hole pairs had lower and higher F_{ST} values, respectively, than those obtained among habitat pairs (Steel–Dwass method: $P < 0.05$; Fig. 3a). The wind-hole–alpine pairs had larger geographic distances than the other two pairs (Steel–Dwass method: $P < 0.01$; Fig. 3b). These results suggest that the wind-hole–wind-hole pairs had large F_{ST} values regardless of their short geographic distances. Some wind-hole–alpine pairs had F_{ST} values similar to those of alpine–alpine pairs (Fig. 2).

The wind-hole populations had lower allelic richness (t -test: $P < 0.01$; Table 1) and larger variations in allelic richness (Fig. 3c) than the alpine populations. No differences in private allelic richness were detected between the wind-hole and alpine populations, and both populations had unique loci (t -test: $P = 0.09$; Fig. 3d, Table 1). Allelic richness and mean F_{ST} values were negatively correlated in the wind-hole populations ($r = -0.87$, $P < 0.01$), but not in the alpine populations ($r = 0.13$, $P = 0.84$; Fig. 4).

All three levels (between habitats, among populations, and within populations) significantly contributed to the genetic variation. Variations between habitat types were smaller than those among and within populations, and within-population variations dominated total genetic variation (Table 2).

STRUCTURE revealed two main genetic clusters with the highest delta K value (Fig. 5a). The alpine populations were assigned to cluster 1, and most genets of the wind-hole populations were assigned to cluster 2. However, some genets of three wind-hole populations (HiH, HiN, and Sin) were assigned to cluster 1. These populations were geographically closer to the alpine sites than the other wind-hole sites (Fig. 1). Therefore, we separated 13 populations into two genetic groups with contrasting genetic composition and examined subsequent STRUCTURE runs for each of the two groups (Fig. 5b, c). Subsequent analyses revealed seven genetic clusters within the wind-hole and alpine populations ($K = 4$ for the first group, including five wind-hole populations and $K = 3$ for the second group, including three wind-hole and five alpine populations). Most populations formed unique genetic clusters, indicating high genetic variation among populations, as suggested by AMOVA. However, some populations, which were spatially close to each other, formed similar clusters (e.g., Iga and Mir; HiH, HiN, and Sin; Mt. Hir and Mt. Mur).

Discussion

Population maintenance at the wind-hole sites

The existence of large clonal patches (>50 m spans) in the wind-hole and alpine populations suggests that individual plants can persist for hundreds of years. Such long clonal

lifespans have also been reported in other ericaceous alpine species (Kameyama et al. 2008) and was reported by another genetic study on *V. vitis-idaea* (Persson and Gustavsson 2001). A long lifespan through clonal growth may be important for persistence of small populations, such as wind-hole populations. A low level of heterozygosity is expected for small relict populations if self-fertilization is common (Mee and Moore 2014). However, wind-hole populations had negative F_{IS} values, indicating the existence of mechanisms for avoiding self-fertilization (Wright 1965). This may be because of a very long generation time, which would delay the genetic homogenization caused by inbreeding. On the other hand, early acting inbreeding depression strictly prevents the formation of selfing seeds in *V. vitis-idaea* (Guillaume and Jacquemart 1999). Although restricting seed production by selfing may increase the risk of local extinction by decreasing mating opportunities (Byers and Meagher 1992), the existence of long-lived clones would alleviate the extinction risk.

Genetic diversity was lower in the wind-hole populations than that in the alpine populations. Although we did not find IBD in the wind-hole populations, a negative correlation between the level of genetic isolation (F_{ST}) and genetic diversity suggests that (1) the wind-hole populations are isolated and subject to genetic drift (Hutchison and Templeton 1999) and (2) that gene flow between the wind-hole populations is not dependent on geographic distance. The latter may be partly due to the existence of undiscovered wind-hole populations in the study area. As genetic diversity of a small population can increase by rare

immigration of gametes (Ingvarsson 2001), genetic diversity of a wind-hole population would strongly depend on genetic exchange with other populations. In our study, gene flow was lower in the wind-hole–wind-hole pairs than that in the alpine–alpine pairs. Pollinators and seed dispersers would infrequently visit small wind-hole sites surrounded by forest.

Genetic diversity of the alpine populations

We did not find IBD among the alpine populations, and genetic diversity and differentiation were higher and lower than those in the wind-hole populations, respectively. Although we selected alpine populations from separate mountains, these sites were structurally connected by alpine habitats. Ikeda et al. (2015) also reported the lack of nationwide genetic differentiation in *V. vitis-idaea* populations in Japan using nuclear and chloroplast DNA markers. This finding suggests long-distance and efficient seed dispersal by birds during the post-glacial period. Therefore, pollinators and seed dispersers may move between alpine populations. High genetic diversity would have been maintained in large-sized alpine populations with small effects from genetic drift and metapopulation structure among populations (Billington 1991).

Ecological significance of wind-hole populations

The AMOVA results suggested that genetic variation within and among populations

288 made greater contributions to regional genetic diversity than did differences between the
289 alpine and wind-hole populations. We found unique genetic clustering between most of the
290 wind-hole populations and unique loci in almost all populations, suggesting that the alpine
291 and wind-hole populations are important to regional genetic diversity of *V. vitis-idaea*.
292 Although the wind-hole populations had lower genetic diversity than that of the alpine
293 populations, some wind-hole populations had relatively high genetic diversity and were likely
294 subject to gene flow from surrounding populations. Our results also convincingly demonstrate
295 the long-term persistence and genetic isolation of the wind-hole populations.

296 Microrefugia (wind-hole sites, in our case) are local small habitats, but can be
297 long-lived and span a broad geographical area, at least in our study area (Shimokawabe et al.
298 2015). Růžička et al. (2015) suggested that wind-hole sites in the Czech Republic will
299 maintain cool conditions even in warmer climates. The *V. vitis-idaea* wind-hole populations
300 geographically close to the alpine populations included the genetic composition of the alpine
301 populations, suggesting that wind-hole habitats act as safe sites for wind-hole and alpine
302 populations of cold-adapted species under global warming conditions. Not all cold-adapted
303 species would benefit from microrefugia (Hylander et al. 2015). Nevertheless, because
304 localized unique geographic features, which can be the bases of many microrefugia (Hjort et
305 al. 2015), occur in economically unproductive sites (Lindenmayer and Franklin 2002), we
306 have suggested previously that identifying and protecting potential microrefugia is a robust

and cost-effective way to mitigate the impact of climate change (Shimokawabe et al. 2015).

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430 Table 1. Habitat types and characteristics of *Vaccinium vitis-idaea* populations. Size and perimeter were not measured for the high mountain
431 population. Asterisks (*) on the F_{IS} value indicates a significant difference from zero (<0.05) based on mean and standard deviation across seven
432 loci.

Site	Habitat ¹	Ele. ²	Pop. area ³	Peri ⁴	Dist. alp. ⁵	Growth ⁶	Dist. ramets ⁷	Age ⁸	N^9	# genet ¹⁰	AR ¹¹	Pr. AR ¹²	Mean F_{ST}	F_{IS}
Ara	WH	309	2022	278	13.5	6.26 ± 1.66	74.4	594.2	31	10	2.45	0.53	0.19	-0.67*
Goj	WH	519	516	252	18.0	3.46 ± 1.04	32.2	465.3	31	13	2.93	0.05	0.12	-0.45*
Mir	WH	359	555	765	15.2		72.3		31	19	4.07	0.23	0.10	-0.20
Iga	WH	290	866	283	14.5		43.9		30	14	4.35	0.00	0.10	-0.12
HiH	WH	456	248	130	10.8	3.43 ± 1.41	36.2	528.1	31	15	4.81	0.16	0.08	-0.31*
HiN	WH	486	1321	546	10.5	3.62 ± 1.20	37.8	521.4	30	24	4.91	0.25	0.08	-0.17*
Set	WH	635	111	152	19.5	3.16 ± 1.10	14.3	226.3	31	17	4.35	0.38	0.12	-0.33*
Sin	WH	495	671	150	7.2	3.44 ± 0.61	38.8	564.0	31	8	2.43	0.25	0.14	-0.67*

Mt.Ari	HM	1635	-	-	-	55.6	31	27	5.75	0.64	0.08	0.13
Mt.Hir	HM	1771	-	-	-	86.2	31	22	5.39	0.37	0.08	0.04
Mt.Shi	HM	1688	-	-	-	50.7	23	17	5.36	0.42	0.08	-0.03
Mt.Mur	HM	1876	-	-	-	-	31	31	6.64	1.01	0.08	0.21*
Mt.Muk	HM	1759	-	-	-	101.8	31	26	5.57	0.24	0.07	-0.07

433 ¹Habitat: WH (wind-hole); HM (high mountain). ²Elevation (m). ³Population area (m²). ⁴Perimeter of population (m). ⁵Distance from alpine
434 vegetation (km). ⁶Mean growth rate ($\pm$ standard deviation; cm year⁻¹). ⁷Longest distance between ramets (m). We did not detect ramets with the
435 same multi-genotype on Mt. Mur. ⁸Estimated age (years). ⁹Number of individuals analyzed. ¹⁰Number of genets. ¹¹Allelic richness. ¹²Private
436 allelic richness.

437

Table 2. Analysis of molecular variance for the *Vaccinium vitis-idaea* samples.

Scale	d.f. ¹	SS ²	Est. var. ³	% variation	<i>P</i>
Between habitat types (wind-holes and high mountains)	1	26.5	0.04	1.4	0.001
Among populations	11	163.0	0.34	12.0	0.001
Within population	475	1129.5	2.48	86.6	0.001

¹Degrees of freedom. ²Sum of squares. ³Estimated variance.

Fig. 1. Wind-hole and high mountain population sampling locations.

448

449 Fig. 2. Relationships between pairwise F_{ST} values and geographical distance. H-H, high

450 mountain pairs; W-W, wind-hole pairs; W-H, wind-hole–high mountain pairs. None of the

451 relationships were significant correlations based on the Mantel test.

452

Fig. 3. Genetic distances (a) and geographical distances (b) among habitat types and allelic richness (c) and private allelic richness (d) of *Vaccinium vitis-idaea* populations at the wind-hole and high mountain sites. W, wind-holes; H, high mountains. Different letters within box plots indicate significant differences ($P < 0.05$) based on the Steel–Dwass test for Fig. 3a, b and the t -test for Fig. 3c, d.

461

462 Fig. 4. Relationships between allelic richness and mean F_{ST} values of the wind-hole and high

463 mountain populations. Only the wind-hole populations were significantly correlated based on

464 Pearson's correlation analysis.

465

Fig. 5. Genetic clusters of *Vaccinium vitis-idaea* populations in the Engaru and Kitami regions.

(a) The initial analysis of all populations showed two genetic clusters ($K = 2$). (b) A subsequent analysis of five wind-hole populations showed four genetic clusters ($K = 4$). (c) A subsequent analysis using three wind-hole populations and five alpine populations showed three genetic clusters ($K = 3$). Colors indicate different genetic clusters within each analysis.