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Title	Genetic structure of Vaccinium vitis-idaea in lowland cool spot and alpine populations : microrefugia of alpine plants in the midlatitudes
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1	Genetic structure of Vaccinium vitis-idaea in lowland cool spot and alpine populations:
2	Microrefugia of alpine plants in the mid-latitudes
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Abstract (246 words: 150–250 words)

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

- 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.
- 40

Introduction

42	Many species inhabiting mountainous areas have upwardly shifted their distributions
43	along with ongoing climate change (Walther et al. 2005; Forister et al. 2010). These
44	elevational shifts are large in alpine plants (Lenoir et al. 2008), and the future distributions of
45	these species are predicted to decline greatly due to the limited area for escape in alpine and
46	geographically isolated habitats (Thuiller et al. 2005; Thuiller 2007). Large-scale range shifts
47	associated with climate change have occurred in past glacial periods, and it has been
48	suggested that small populations persist in local suitable habitats within broadly unsuitable
49	geographical areas (Stewart and Lister 2001; McLachlan et al. 2005; Parducci et al. 2012).
50	These local refugia are called "microrefugia" in contrast to the large, continuous
51	"macrorefugia" (Rull 2009).
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60	Vaccinium vitis-idaea L. (Ericaceae) is a common alpine dwarf shrub inhabiting
61	mid-latitude alpine and subarctic regions. V. vitis-idaea also grow in lowland cool spots,
62	including wind-hole micro-topographies (Sato et al. 1993; Sato 1995; Růžička 1999). Wind
63	holes frequently occur at the bottom of talus slopes containing volcanic bedrock (Shimizu
64	2004), where cool conditions are maintained in the vicinity by the preferential flow of cool air
65	generated in interstitial spaces created by rock fragments or colluvia. We call such cool spots
66	"wind-hole sites." Wind-hole sites harbor various taxa of alpine species that are not found in
67	the surrounding areas (Růžička 1999; Shimokawabe et al. 2015). Shiboi (1975) speculated
68	that lowland V. vitis-idaea populations have been maintained in local wind-hole sites since the
69	last glacial period in Hokkaido, northern Japan. If this is true, not only alpine habitats
70	(macrorefugia) but also local wind-hole sites (microrefugia) are important habitats for the
71	maintenance of V. vitis-idaea population genetic diversity in mid-latitude regions.
72	Populations in small and isolated habitats are subject to heightened extinction risks
73	due to inbreeding depression, genetic drift, and demographic stochasticity (Lande 1988; Reed
74	and Frankham 2003). Several mechanisms contribute to the long-term maintenance of
75	microrefugia populations (Hampe and Jump 2011). One exogenous mechanism is migration
76	from the surrounding populations (Mosblech et al. 2011), and one endogenous mechanism is
77	purging deleterious alleles and self-fertilization/compatibility (Mee and Moore 2014).
78	Wind-hole populations are small and isolated from alpine populations, and the mechanisms of

population maintenance may be manifested in their genetic structure. 7980 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 81 measured the annual shoot growth to estimate the age of clonal patches. We discuss the 82 mechanism of population maintenance at wind-hole sites and its ecological significance under 83 ongoing climate change. 84 85 86 Methods 87 Species studied and field sampling 88 V. vitis-idaea (2n = 24) is an evergreen dwarf shrub (5-20 cm in height) with creeping 89 90 stems under the soil surface (Iwatsuki et al. 1993). Based on genetic analyses, Ikeda et al. 91(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 92143.0–143.4° E, respectively) were alpine zones and lowland wind-hole sites. This species is 93 also known to occur on acid soils in boreal forests and bogs (Garkava-Gustavsson et al. 2005). 94However, lowland V. vitis-idaea populations in Japan other than those at wind-hole sites are 9596 limited and fragmentally distributed only in the coastal wetlands of northern/eastern Hokkaido (Umezawa 2007). Flowering occurs in early summer; shrubs are pollinated mainly 97

98	by bees, and seeds are distributed by animals (Ritchie 1955; Iwatsuki et al. 1993). Upon
99	self-fertilization, partial self-sterility leads to reduced numbers of developed seeds due to
100	early acting inbreeding depression (Guillaume and Jacquemart 1999). V. vitis-idaea can form
101	large clonal patches (>30 m) by expanding via underground stems (Persson and Gustavsson
102	2001).
103	The study area was located in northeastern Hokkaido (Fig. 1). Alpine habitats
104	harboring V. vitis-idaea cover the western high elevation area (>1,500 m), and wind-hole sites
105	with V. vitis-idaea are scattered across the eastern lowland area. This area is topographically
106	complex and includes steep slopes and bedrock dominated by pumice flow deposits
107	(1:200,000 surface geology map by the Ministry of Land, Infrastructure, Transport, and
108	Tourism of Japan). The mean annual temperature during the past 30 years was 5.8°C (mean
109	temperatures of the coldest and warmest months were -8.3 and 19.9°C, respectively) and
110	annual precipitation was 794.5 mm (Japan Meteorological Agency,
111	http://www.jma.go.jp/jma/indexe.html). The temperature at ground level remains 7°C lower
112	than the air temperature during the summer (Shimokawabe et al. 2015).
113	We collected leaves in August-September 2013 from eight wind-hole sites and five
114	separate alpine habitats (Fig. 1). The sample size was 23-31 leaves from each site, totaling
115	393 samples (Table 1). No additional wind-hole sites known to harbor V. vitis-idaea are
116	present in our study area (Shimokawabe et al. 2015). We uniformly sampled leaves from all

117	areas of the wind-hole sites and from the ridge areas in the alpine habitats (sampling interval:
118	3-10 m for wind-hole sites, $5-10$ m for alpine habitats). We recorded the locations of the
119	sampling points using a global positioning system (Garmin GPSMAP 62SJ; Garmin
120	International, Inc., Olathe, KS, USA). Leaf samples were dried in silica gel and preserved at
121	room temperature until analysis.
122	
123	Microsatellite analysis
124	We extracted DNA using the cetyltrimethylammonium bromide (CTAB) method. In
125	brief, the sample was crushed in CTAB extraction buffer, chloroform-isoamyl alcohol was
126	added, the aqueous layer containing the DNA was separated, the DNA was purified with
127	isopropanol and ethanol, and the DNA was preserved in Tris-EDTA buffer. Seven
128	microsatellite loci (CA169F, CA236F, NA741, NA800, NA1040, VCC_I2 and VCC_K4:
129	Boches et al. 2005; Appendix A) were amplified and developed for a related species
130	(Vaccinium corymbosum L.) using polymerase chain reaction (PCR) and a thermal cycler
131	(Applied Biosystems 2720 Thermal Cycler; Applied Biosystems Inc., Foster City, CA, USA).
132	Following Boches et al. (2005), we used the following PCR program: 3 min at 94°C, followed
133	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
134	min at 72°C. Microsatellite fragments were analyzed using the Applied Biosystems 3130
135	Genetic Analyzer, and the genotypes were coded using Peak Scanner ver. 2.0 (Applied

- Biosystems). GeneScan 500 LIZ Size Standard (Applied Biosystems) was used as the sizestandard.
- 138
- 139Population genetics analysis140A total of 244 multi-locus genotypes in seven loci were identified, and 69 were141assigned to more than two different samples (17.6%). Samples with the same genotypes in all142loci were treated as the same individual (genet), and the genet was used as the analytical unit.143We calculated the probability that two different individuals would have the same genotype144(PI: probability of identity) using GenAlEx 6.501 (Peakall and Smouse 2006) to evaluate145misassignment 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.

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

155	examined the differences in genetic differentiation (F_{ST}) and geographic distances among
156	populations for (i) wind-hole and wind-hole pairs, (ii) wind-hole and alpine pairs, and (iii)
157	alpine and alpine pairs with multiple comparison tests (Steel–Dwass method). We obtained
158	the geographic distance as three-dimensional straight-line distances considering elevational
159	differences. The F_{ST} values were calculated, Mantel tests were conducted using GenAlEx
160	6.501, and multiple comparison tests were performed using R ver. 2.15.2 (R Core Team,
161	Vienna, Austria).
162	We calculated allelic richness and private allelic richness (the number of unique alleles
163	for each population, corrected for sample size) as genetic diversity indices (Kalinowski 2004)
164	using HP-Rare v. June-6-2006 (Kalinowski 2005). The diversity indices were compared
165	between wind-hole sites and alpine habitats using <i>t</i> -tests. The correlation coefficient between
166	allelic richness and the index of genetic isolation was determined as the mean F_{ST} value for
167	each population. The mean F_{ST} was obtained from the F_{ST} values of all other populations, and
168	the correlations were examined separately for the wind-hole sites and alpine habitats.
169	We conducted analyses of molecular variance (AMOVA) based on these F_{ST} values to
170	divide genetic variation into those occurring within populations, among populations, and
171	among habitats using GenAlEx 6.501. STRUCTURE 2.3.4 (Pritchard et al. 2000) was used to
172	examine individual based Bayesian clustering to estimate the most likely number of
173	genetically differentiated populations (K). An admixture model with the correlated allele

174	frequencies and informative locations was employed. The optimal number of clusters from 1
175	to 13 was inferred by 200,000 interactions following a 100,000 step burn-in with 10 replicate
176	runs for each K-value. STRUCTURE can identify the highest hierarchical level of a
177	population but often fails to detect hierarchical genetic structure at lower levels (Evanno et al.
178	2005). Therefore, subsequent runs were executed with the same method to reveal additional
179	hierarchical genetic clusters when $K > 1$ was identified at the initial run. The STRUCTURE
180	results and the online program STRUCTURE HARVESTER ver. 0.6.94 (Earl and vonHoldt
181	2012) were used to determine the best K number. This program infers the best K value using
182	the delta K based on the rate of change in the log probability of the data (Ln K) between
183	successive K values (the best K has the highest delta K value) (Evanno et al. 2005). Finally,
184	the online program CLUMPAK was used to visualize the spatial genetic structure estimated
185	by STRUCTURE (Kopelman et al. 2015).
186	
187	Annual growth measurements
188	We measured the annual growth of shoots and estimated their ages to infer the
189	persistence of individuals at the wind-hole sites. We measured annual growth length of the
190	previous year's shoots from 10 individuals each in the six wind-hole populations during May
191	2015. We thereby obtained a mean value for the 10 individuals in each population. The
192	longest length between ramets was obtained from genetic analyses. We treated this length as

193	the size of the oldest individual in the corresponding population, and estimated its age
194	assuming circular growth: Age = $(0.5 \times \text{distance between ramets})/\text{annual growth}$.
195	
196	Results
197	The mean PI across 13 populations, which indicates the probability of misassignment
198	of different individuals to genets, was very low (2×10^{-4}) . Therefore, we treated plants with
199	the same genotype as the same individual (genet). Clones were detected at all alpine and
200	wind-hole sites, except at Mt. Mur (Table 1). The longest distances within clones were 14.3–
201	101.8 m (Table 1). Large ramets had between-ramet lengths > 50 m (Appendix B). The mean
202	annual shoot growth of the six wind-hole populations was $3.90 \text{ cm year}^{-1}$. The largest clone
203	was estimated to be 594 years old, and four populations had clones that were > 500 years old
204	(Table 1). Note that our calculations may have underestimated the ages of the clones because
205	we assumed a uniform circular growth pattern. However, this result suggests that V.
206	vitis-idaea individuals have been maintained at the wind-hole sites for hundreds of years. We
207	observed deviations from Hardy–Weinberg equilibrium at a mean of two populations per
208	locus (adjusted P < 0.05). All wind-hole populations had negative F_{IS} values, and six
209	populations differed significantly from zero (P < 0.05 : Table 1). The existence of null alleles
210	was suggested for multiple loci and populations. However, their frequencies were small, and
211	we used all loci and genets (mean frequency per loci = 0.03).

212	The Mantel tests did not reveal any significant IBD among populations (r = -0.12 , P
213	= 0.23; Fig. 2) or within habitat types (wind-hole–wind-hole pairs: $r = 0.32$, $P = 0.16$; alpine–
214	alpine pairs: $r = 0.05$, $P = 0.54$; Fig. 2). However, the alpine–alpine pairs and the wind-hole–
215	wind-hole pairs had lower and higher F_{ST} values, respectively, than those obtained among
216	habitat pairs (Steel–Dwass method: $P < 0.05$; Fig. 3a). The wind-hole–alpine pairs had larger
217	geographic distances than the other two pairs (Steel–Dwass method: $P < 0.01$; Fig. 3b). These
218	results suggest that the wind-hole–wind-hole pairs had large F_{ST} values regardless of their
219	short geographic distances. Some wind-hole–alpine pairs had F_{ST} values similar to those of
220	alpine-alpine pairs (Fig. 2).
221	The wind-hole populations had lower allelic richness (<i>t</i> -test: $P < 0.01$; Table 1) and
222	larger variations in allelic richness (Fig. 3c) than the alpine populations. No differences in
223	private allelic richness were detected between the wind-hole and alpine populations, and both
224	populations had unique loci (<i>t</i> -test: $P = 0.09$; Fig. 3d, Table 1). Allelic richness and mean F_{ST}
225	values were negatively correlated in the wind-hole populations (r = -0.87 , P < 0.01), but not
226	in the alpine populations ($r = 0.13$, $P = 0.84$; Fig. 4).
227	All three levels (between habitats, among populations, and within populations)
228	significantly contributed to the genetic variation. Variations between habitat types were
229	smaller than those among and within populations, and within-population variations dominated
230	total genetic variation (Table 2).

231	STRUCTURE revealed two main genetic clusters with the highest delta K value (Fig.
232	5a). The alpine populations were assigned to cluster 1, and most genets of the wind-hole
233	populations were assigned to cluster 2. However, some genets of three wind-hole populations
234	(HiH, HiN, and Sin) were assigned to cluster 1. These populations were geographically closer
235	to the alpine sites than the other wind-hole sites (Fig. 1). Therefore, we separated 13
236	populations into two genetic groups with contrasting genetic composition and examined
237	subsequent STRUCTURE runs for each of the two groups (Fig. 5b, c). Subsequent analyses
238	revealed seven genetic clusters within the wind-hole and alpine populations ($K = 4$ for the
239	first group, including five wind-hole populations and $K = 3$ for the second group, including
240	three wind-hole and five alpine populations). Most populations formed unique genetic clusters,
241	indicating high genetic variation among populations, as suggested by AMOVA. However,
242	some populations, which were spatially close to each other, formed similar clusters (e.g., Iga
243	and Mir; HiH, HiN, and Sin; Mt. Hir and Mt. Mur).
244	
245	
246	Discussion
247	Population maintenance at the wind-hole sites
248	The existence of large clonal patches (>50 m spans) in the wind-hole and alpine
249	populations suggests that individual plants can persist for hundreds of years. Such long clonal

250	lifespans have also been reported in other ericaceous alpine species (Kameyama et al. 2008)
251	and was reported by another genetic study on V. vitis-idaea (Persson and Gustavsson 2001). A
252	long lifespan through clonal growth may be important for persistence of small populations,
253	such as wind-hole populations. A low level of heterozygosity is expected for small relict
254	populations if self-fertilization is common (Mee and Moore 2014). However, wind-hole
255	populations had negative F_{IS} values, indicating the existence of mechanisms for avoiding
256	self-fertilization (Wright 1965). This may be because of a very long generation time, which
257	would delay the genetic homogenization caused by inbreeding. On the other hand, early
258	acting inbreeding depression strictly prevents the formation of selfing seeds in V. vitis-idaea
259	(Guillaume and Jacquemart 1999). Although restricting seed production by selfing may
260	increase the risk of local extinction by decreasing mating opportunities (Byers and Meagher
261	1992), the existence of long-lived clones would alleviate the extinction risk.
262	Genetic diversity was lower in the wind-hole populations than that in the alpine
263	populations. Although we did not find IBD in the wind-hole populations, a negative
264	correlation between the level of genetic isolation (F_{ST}) and genetic diversity suggests that (1)
265	the wind-hole populations are isolated and subject to genetic drift (Hutchison and Templeton
266	1999) and (2) that gene flow between the wind-hole populations is not dependent on
267	geographic distance. The latter may be partly due to the existence of undiscovered wind-hole
268	populations in the study area. As genetic diversity of a small population can increase by rare

269	immigration of gametes (Ingvarsson 2001), genetic diversity of a wind-hole population would
270	strongly depend on genetic exchange with other populations. In our study, gene flow was
271	lower in the wind-hole–wind-hole pairs than that in the alpine–alpine pairs. Pollinators and
272	seed dispersers would infrequently visit small wind-hole sites surrounded by forest.
273	
274	Genetic diversity of the alpine populations
275	We did not find IBD among the alpine populations, and genetic diversity and
276	differentiation were higher and lower than those in in the wind-hole populations, respectively.
277	Although we selected alpine populations from separate mountains, these sites were
278	structurally connected by alpine habitats. Ikeda et al. (2015) also reported the lack of
279	nationwide genetic differentiation in V. vitis-idaea populations in Japan using nuclear and
280	chloroplast DNA markers. This finding suggests long-distance and efficient seed dispersal by
281	birds during the post-glacial period. Therefore, pollinators and seed dispersers may move
282	between alpine populations. High genetic diversity would have been maintained in large-sized
283	alpine populations with small effects from genetic drift and metapopulation structure among
284	populations (Billington 1991).
285	
286	Ecological significance of wind-hole populations
287	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).308

309

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321 **References**

322	Billington HL (1991) Effect of population size on genetic variation in a dioecious conifer.
323	Conserv Biol 5:115–119
324	Boches PS, Bassil NV, Rowland LJ (2005) Microsatellite markers for Vaccinium from EST
325	and genomic libraries. Mol Ecol Notes 5:657–660
326	Byers DL, Meagher TR (1992) Mate availability in small populations of plant species with
327	homomorphic sporophytic self-incompatibility. Heredity 68:353–359
328	Dobrowski SZ (2011) A climatic basis for microrefugia: the influence of terrain on climate.
329	Global Change Biol 17:1022–1035
330	Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for
331	visualizing STRUCTURE output and implementing the Evanno method. Conservation
332	Genetics Resources 4:359–361
333	Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using
334	the software structure: a simulation study. Mol Ecol 14:2611–2620
335	Forister ML et al. (2010) Compounded effects of climate change and habitat alteration shift
336	patterns of butterfly diversity. Proc Natl Acad Sci USA 107:2088–2092
337	Garkava-Gustavsson L, Persson HA, Nybom H, Rumpunen K, Gustavsson BA, Bartish IV
338	(2005) RAPD-based analysis of genetic diversity and selection of lingonberry
339	(Vaccinium vitis-idaea L.) material for ex situ conservation. Genet Resour Crop Evol
340	52:723-735
341	Gentili R, Baroni C, Caccianiga M, Armiraglio S, Ghiani A, Citterio S (2015) Potential
342	warm-stage microrefugia for alpine plants: Feedback between geomorphological and
343	biological processes. Ecol Complex 21:87–99
344	Guillaume P, Jacquemart A-L (1999) Early-inbreeding depression in Vaccinium myrtillus and
345	V. vitis-idaea. Protoplasma 208:107–114
346	Hampe A, Jump AS (2011) Climate relicts: past, present, future. Annu Rev Ecol Evol Syst
347	42:313–333
348	Hannah L, Flint L, Syphard AD, Moritz MA, Buckley LB, McCullough IM (2014) Fine-grain
349	modeling of species' response to climate change: holdouts, stepping-stones, and
350	microrefugia. Trends Ecol Evol 29:390–397
351	Hjort J, Gordon JE, Gray M, Hunter ML (2015) Why geodiversity matters in valuing nature's
352	stage. Conserv Biol 29:630–639
353	Hutchison DW, Templeton AR (1999) Correlation of pairwise genetic and geographic
354	distance measures: inferring the relative influences of gene flow and drift on the
355	distribution of fenetic variability. Evolution 53:1898–1914
356	Hylander K, Ehrlén J, Luoto M, Meineri E (2015) Microrefugia: not for everyone. Ambio
357	44:60–68

Ikeda H et al. (2015) Persistent history of the bird-dispersed arctic-alpine plant Vaccinium 358359vitis-idaea L. (Ericaceae) in Japan. J Plant Res 128:437-444 Ingvarsson PK (2001) Restoration of genetic variation lost – the genetic rescue hypothesis. 360 Trends Ecol Evol 16:62-63 361362Iwatsuki K, Yamazaki T, Boufford DE, Ohba H (1993) Flora of Japan. Volume. IIIa. Angiospermae Dicotyledoneae Sympetalae(a). Kodansya, Tokyo 363 Kalinowski ST (2004) Counting alleles with rarefaction: private alleles and hierarchical 364365sampling designs. Conserv Genet 5:539-543 Kalinowski ST (2005) hp-rare 1.0: a computer program for performing rarefaction on 366 measures of allelic richness. Mol Ecol Notes 5:187-189 367Kameyama Y, Kasagi T, Kudo G (2008) A hybrid zone dominated by fertile F₁s of two alpine 368 369 shrub species, Phyllodoce caerulea and Phyllodoce aleutica, along a snowmelt 370 gradient. J Evol Biol 21:588-597 Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I (2015) CLUMPAK: a 371program for identifying clustering modes and packaging population structure 372inferences across K. Mol Ecol Resour 15:1179–1191 373374Lande R (1988) Genetics and demography in biological conservation. Science 241:1455-3751460 Lenoir J, Gegout JC, Marquet PA, de Ruffray P, Brisse H (2008) A significant upward shift in 376 plant species optimum elevation during the 20th century. Science 320:1768-1771 377 Lindenmayer DB, Franklin JF (2002) Conserving forest biodiversity: a comprehensive 378379 multiscaled approach. Island Press, Washington, D.C. 380 Marko PB, Hart MW (2011) The complex analytical landscape of gene flow inference. Trends Ecol Evol 26:448-456 381382 McLachlan JS, Clark JS, Manos PS (2005) Molecular indicators of tree migration capacity under rapid climate change. Ecology 86:2088-2098 383 Mee JA, Moore J-S (2014) The ecological and evolutionary implications of microrefugia. J 384385Biogeogr 41:837-841 386 Mosblech NAS, Bush MB, van Woesik R (2011) On metapopulations and microrefugia: palaeoecological insights. J Biogeogr 38:419-429 387Parducci L et al. (2012) Glacial survival of boreal trees in northern Scandinavia. Science 388 335:1083-1086 389390 Peakall ROD, Smouse PE (2006) genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288-295 391Persson HA, Gustavsson BA (2001) The extent of clonality and genetic diversity in 392lingonberry (Vaccinium vitis-idaea L.) revealed by RAPDs and leaf-shape analysis. 393 394 Mol Ecol 10:1385–1397 395Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using

396	multilocus genotype data. Genetics 155:945–959
397	Reed DH, Frankham R (2003) Correlation between ftness and genetic diversity. Conserv Biol
398	17:230–237
399	Ritchie JC (1955) Vaccinium vitis-idaea L. J Ecol 43:701-708
400	Rousset F (2008) genepop'007: a complete re-implementation of the genepop software for
401	Windows and Linux. Mol Ecol Resour 8:103–106
402	Rull V (2009) Microrefugia. J Biogeogr 36:481–484
403	Růžička V (1999) The freezing scree slopes and their arachnofauna. Decheniana-Beihefte
404	37:141–147
405	Růžička V, Zacharda M, Šmilauer P, Kučera T (2015) Can paleorefugia of cold-adapted
406	species in talus slopes resist global warming? Boreal Environ Res 20:403-412
407	Sato K (1995) An outline of the cool-spots site vegetation of Hokkaido, Japan. Bull Higashi
408	Taisetsu Museum Nat Hist 17:107–115
409	Sato K, Kudo G, Uemura S (1993) Cool-spots site vegetation in Izariiri-Heide, northern Japan.
410	Jpn J Ecol 43:91–98
411	Shiboi T (1975) Supplementally report on the periglacial phenomena observed in Kitami
412	District. Mem Kitami Inst Techn 7:163–194
413	Shimizu C (2004) An information on the cool air blow holes (wind-holes) including the ice
414	caves in Japan: references to the surrounding landforms and the existence of sporadic
415	perm afrost. Komazawa Geogr 40:121–148
416	Shimokawabe A, Yamaura Y, Akasaka T, Sato T, Shida Y, Yamanaka S, Nakamura F (2015)
417	The distribution of cool spots as microrefugia in a mountainous area. PLoS ONE
418	10:e0135732
419	Stewart JR, Lister AM (2001) Cryptic northern refugia and the origins of the modern biota.
420	Trends Ecol Evol 16:608–613
421	Thuiller W (2007) Climate change and the ecologist. Nature 448:550–552
422	Thuiller W, Lavorel S, Araujo MB, Sykes MT, Prentice IC (2005) Climate change threats to
423	plant diversity in Europe. Proc Natl Acad Sci USA 102:8245-8250
424	Umezawa S (2007) Wild flowers of Hokkaido. New edn. Hokkaido University Press, Sapporo
425	Walther G-R, Beißner S, Burga CA (2005) Trends in the upward shift of alpine plants. J Veg
426	Sci 16:541–548
427	Wright S (1943) Isolation by distance. Genetics 28:114–138
428	

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	$3.62~\pm~1.20$	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	$3.44~\pm~0.61$	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.

Scale	d.f. ¹	SS^2	Est. var. ³	% variation	Р
Between habitat types	1	26.5	0.04	1 /	0.001
(wind-holes and high mountains)	1	20.3	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

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

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



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

446





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.









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.



