



# Population genetic diversity and conservation priority of prince's pine *Chimaphila umbellata* populations around the south margin of their distribution

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## Abstract

How genetic diversity is maintained within a range of species is one of the crucial pieces of information in species conservation. Although the classical central-marginal hypothesis assumes genetic paucity in peripheral populations, this pattern is not always the case. The semi-shrub *Chimaphila umbellata* subsp. *umbellata*, population of which are located in the southernmost part of the subspecies distribution, is considered to be a threatened species in Japan. Thus, this study aimed to examine which populations should be preferentially conserved and if the central-marginal hypothesis can be applied to this case. Genetic diversity was examined in 15 populations of *C. umbellata* subsp. *umbellata* in Japan using 16 nuclear simple sequence repeat markers. Overall, the genetic diversity values within the populations did not correlate with the latitude of the population locality, although those of the southern marginal populations tended to be lower than those of populations in other regions. While the populations are genetically differentiated from each other, recent population size declines were only detected in a few cases. Bayesian inference of population structure revealed three genetically distinct groups. An approximate Bayesian computation revealed that these three genetic groups were derived from the ancient population in a recent times. The contribution of each population to total genetic diversity was estimated by removing a given population and recalculating the total genetic diversity. Only one population contributed to both gene diversity and allelic diversity, while several populations contributed to one or the other. Considering genetic diversity and structure, the conservation priority of Japanese populations of *C. umbellata* to preserve genetic diversity is discussed.

**Keywords** *Chimaphila umbellata* subsp. *umbellata* · Conservation priority · Genetic diversity · Nuclear simple sequence repeat (nSSR) · Peripheral population · Rear edge

## Introduction

Climate is one of the most crucial factors determining plant species distribution. The view that climate oscillation during the Quaternary profoundly influenced the present distribution of vegetation is now commonly accepted (Comes and Kadereit 1998; Hewitt 2004). Plant species distributions contract or expand in response to climate change; the ranges of plant species contracted toward lower-latitude areas during the ice ages and moved north (poleward) in subsequent warmer ages. In some cases, plant species survived ice ages

by inhabiting refugia (climatically milder areas), even at relatively high latitudes (Comes and Kadereit 1998; Hewitt 2004). Range dynamics have significant impacts on genetic variation within populations and on genetic differentiation among populations, and can be traced by determining geographical patterns of genetic diversity and genetic constitution within populations (Petit et al. 2003; Lascoux et al. 2004; Provan and Bennett 2008).

According to the classical central-marginal hypothesis, populations located at the margins of a distribution are expected to suffer genetic paucity and show marked genetic differentiation from other populations because of their small effective population size, geographical isolation, and different selection regimes (Lesica and Allendorf 1995; Eckert et al. 2008; Qiang and Fu 2011). This hypothesis is known to be the case for some plant species (Liu et al. 2019; Kitamura et al. 2020); in these cases, the populations located in the

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center of the species distribution tended to have more genetic diversity than those at the periphery. Rear-edge populations of cold-adapted species are expected to be small and isolated from populations within the core distribution of the species (Hampe and Petit 2005). Accordingly, populations around the southern margin of a species distribution in the Northern Hemisphere are expected to have lower genetic diversity and be genetically differentiated from other populations, resulting in a positive correlation between genetic diversity and the latitude of the population locality. Conversely, genetic diversity within a plant species often decreased with increasing latitude (Zeng et al. 2015; Yang et al. 2016), although this is not always the case (Bai et al. 2010; Hirao et al. 2017).

However, because the central-marginal hypothesis does not consider many factors that affect genetic variations within a population (Vucetich and Waire 2003; Hampe and Petit 2005; Pironon et al. 2016), studies do not consistently show lower intrapopulation genetic diversity and higher interpopulation genetic differentiation in marginal populations (Kennedy et al. 2020). In fact, there are many examples that do not meet this hypothesis (Pironon et al. 2016). Recent molecular phylogenetic studies have found that rear-edge populations, which currently inhabit the lowest latitude margin of species distributions, often have large genetic variability compared to central populations, and/or show genetic uniqueness (Hampe and Petit 2005). This is probably because these populations were once in the center of the species distribution and had harbored greater genetic variation in the last glacial era. Thus, rear-edge populations may be worthy of conservation efforts to maintain the genetic diversity of the species (Yakimowski and Eckert 2008; Hampe and Jump 2011; Wagner et al. 2011; Diekmann and Serrao 2012; Assis et al. 2013; Pironon et al. 2015; Sugahara et al. 2017). One of the reasons for this discrepancy may be differences in the time elapsed from the isolation of the rear-edge populations from the core distribution of the species examined. However, a limited number of studies have examined the timing of the isolation of rear edge populations from the core distribution (Carbognani et al. 2019).

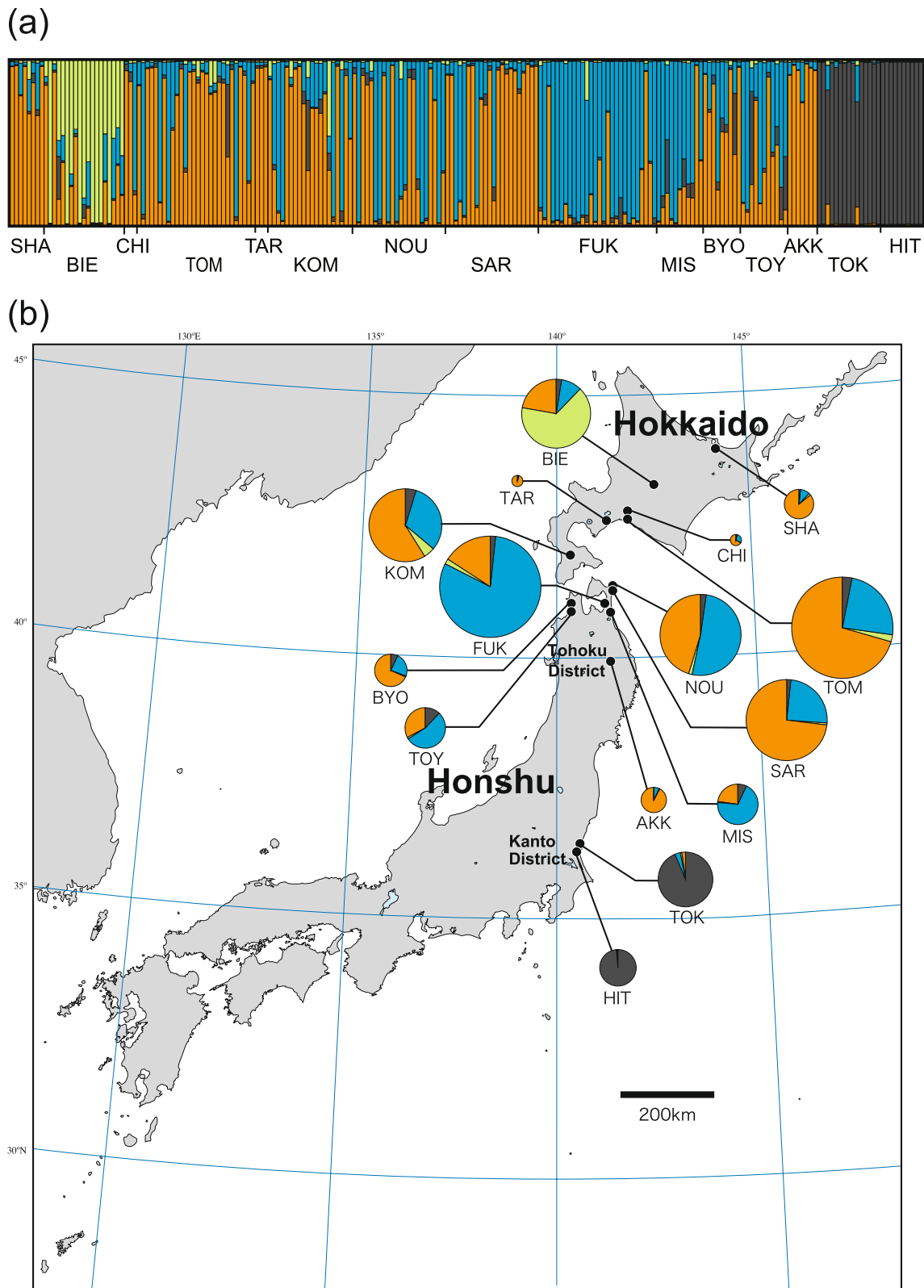
One of the major goals of conservation genetics is to determine which populations should be prioritized as targets of in situ and/or ex situ conservation from a genetic point of view. For this purpose, molecular markers have been employed to estimate genetic variation within populations and genetic differentiation among populations (Newton et al. 1999; Allendorf 2017). High genetic diversity is considered to ensure the persistence of a species, by increasing its adaptive potential to face environmental changes (Barrett and Kohn 1991; Booy et al. 2000). At the same time, the genetic uniqueness of a population may be as important in terms of conservation units (Petit et al. 1998; Funk et al. 2012).

*Chimaphila umbellata* (L.) W.P.C.Barton (Ericaceae), prince's pine, is a circumboreal semi-shrub species

distributed across Eurasia, East Asia, and North America. Five intraspecific taxa are generally recognized, although their taxonomic rank (i.e., subspecies or variety) depends on the taxonomist (Kortnas 1972; Hitchcock and Cronquist 1973; Takahashi 1987). In this study, we followed the taxonomic treatment of Takahashi (1987). Of the five subspecies, *C. umbellata* subsp. *umbellata* is patchily distributed from central Europe to Siberia, the East Asian area (including Manchuria, Sakhalin, and the South Kuriles), and the Japanese archipelago. The populations in the Japanese archipelago are located in the southernmost part of the distribution of *C. umbellata* subsp. *umbellata*.

In the Japanese archipelago, *C. umbellata* occurs mainly in the understory of cool-temperate or semi-boreal coniferous forests. The southernmost populations in Japan are geographically isolated from other populations (Fig. 1). The habitats of the populations of the Japanese mainland Honshu (Fig. 1) are located in the understory of coastal black pine forests. Although black pine forests frequently occur in the coastal areas between the southernmost populations and the more northern populations of *C. umbellata* and seem to offer preferred habitat for the species, they host no known populations of this species. One possible explanation for this disjunction is that in the last glacial era, *C. umbellata* may have been distributed more widely and continuously at lower latitudes than at present, and its distribution shifted northward after the ice ages. As a result, during the shift, the distribution may have been fragmented and only a few populations were left behind in the southern area of the current distribution. The historical populations that occurred between the southernmost population and the more northern populations may have been extirpated because of climate warming during the postglacial period. Even in the northern part of the archipelago, populations of this species are highly sporadic. This pattern may have been set during the north-poleward shift of the species distribution during the postglacial era and may be an idiosyncratic feature of the rear edge of the distribution of cold-adapted species. In some temperate plants in Japan, distribution expansions after the last glacier age have been inferred from phylogeographic studies (e.g. Kikuchi et al. 2010; Iwasaki et al. 2012). However, whether post-glacial migration is responsible for the range disjunction of *C. umbellata* in Japan is unknown.

In Japan, *C. umbellata* is found in only three prefectures in the northern part of Honshu (the Japanese mainland), and on Hokkaido Island. The national Red Data Book of vascular plants in Japan (Japanese Ministry of Environment 2015) categorized the species as nearly threatened (NT), and the local Red Data Books of the three prefectures in Honshu listed it as a threatened species, that is, nearly threatened (Aomori Prefecture), vulnerable to extinction (Iwate Prefecture), and endangered (Ibaragi Prefecture). Hokkaido Prefecture does not treat the species as threatened. Unfortunately



**Fig. 1** Bayesian inference of *Chimaphila umbellata* population structure using STRUCTURE. **a** Bar graph of clusters for each individual. **b** Cluster constitution of each population. Sizes of the pie charts indicate the sample size of each population

there are no data available on the recent population size declines of the species, although the recent sharp reduction seems unlikely in view of the species habitat stability. Although the species is threatened in many regions, the levels of genetic diversity and strategies to conserve it are lacking.

In this paper, we address the following questions by estimating genetic diversity and structure among populations of *C. umbellata* in the Japanese archipelago, which is the southern limit of the species' distribution, using nuclear simple sequence repeat (nSSR) markers; (i) consistent with the central-marginal hypothesis, does genetic diversity increase with latitude from the southern rear-edge distributional margin toward the distributional center of this species? (ii) how long have the rear populations been isolated from the core distribution of the species?; (iii) how is the genetic variation structured in the species? This question is relevant to the next question. (iv) which populations should be prioritized for conservation, based on the strategy that populations contributing greatly to the total genetic diversity of the species should be given preferential conservation value?

## Materials and methods

### Study organism and sampling

*Chimaphila umbellata* is a small semi-shrub, up to 20 cm tall, that propagates clonally via rhizomes in addition to sexual reproduction by seeds (Lundell et al. 2015). The seeds of the species are small and dust-like and are considered to have a symbiotic relationship with fungi during germination (Eriksson and Kainulainen 2011), although adults of the species are likely to be autotrophic (Hynson et al. 2012; Johansson et al. 2015). The flowers of *C. umbellata* are light-pink colored and 8–10 mm in diameter, and flowering populations are mainly visited by bees such as *Bombus* in early summer (M. Maki, personal observation in Aomori Prefecture). The species tend to occur in understories of black pine (*Pinus thunbergii*) forests on sand dunes near the seashore on the Japanese mainland (Honshu), although it also occurs in the understories of shrub forests in alpine zones on volcanoes, or in those of coniferous forests comprising species other than black pine, such as *Abies* and/or *Picea*, in Hokkaido. Sometimes it occurs in the understory of deciduous forests in both Honshu and Hokkaido (M. Maki, personal observation).

In 2009, 2011, and 2017, a total of 15 populations were sampled to cover the overall distribution of the species in Japan (Table 1); six populations (SHA, BIE, CHI, TOM, TAR, and KOM, all in Hokkaido Prefecture) from Hokkaido Island, seven (BYO, TOY, NOU, SAR, FUK, and MIS in Aomori Prefecture, and AKK in Iwate Prefecture) from Tohoku District on the main island Honshu, and two

(TOK and HIT, both in Ibaragi Prefecture) from the Kanto District in Honshu (Fig. 1). The habitats of the populations were categorized into three types: coniferous forest, deciduous forest, and deciduous shrub forest in an alpine region (Table 1). For each sample, a few mature leaves were collected from a shoot and dried with silica gel, and then preserved at room temperature until DNA extraction. The shoots were chosen randomly (at least 2-m intervals) to avoid sampling from the same genet. We tried to collect the shoots from each entire population as widely as possible. However, because many populations were relatively small in size, we could not sample many shoots in such populations. In two populations in the Kanto District, although a relatively large number of ramets occurred, we sampled only a subset of them population's protection. The average number of the shoots per population collected was 17.07, but only three shoots were sampled for the two populations (CHI and TAR).

### DNA extraction and nSSR analysis

The nSSR genotyping (see below) revealed apparently repeated sampling of the same genets in several populations. Therefore, the clonality of the samples in each population was tested using the program GenClone 2.0 (Arnaud-Haond and Belkhir 2007), and redundant samples were excluded from further analyses. The numbers of samples genotyped ( $N_g$ ) and used for the analyses ( $N_c$ ) are given in Table 1. The average number of the genets collected per population was 14.33 (S.E. = 2.15).

Total DNA was extracted using the CTAB method slightly modified from Doyle and Doyle (1990). The 16 PCR primer pairs developed by Kikuchi et al. (2018) were used to amplify the nSSR loci. PCR amplification was conducted for each of the 16 loci in 4- $\mu$ L volumes containing approximately 60 ng of genomic DNA, 2  $\mu$ L of 2 $\times$ Type-it Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.075  $\mu$ M of forward primer with a universal tail attached at its 5' end (Blacket et al. 2012), 0.25  $\mu$ M of reverse primer with a pigtail (5'-GTTCTT-3'; Brownstein et al. 1996), and 0.1  $\mu$ M of fluorescent-labeled universal primers, as used by Blacket et al. (2012). The PCR amplification program was as follows: initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 90 s, and 72 °C for 45 s, and a final extension at 60 °C for 30 min. The amplified products were electrophoresed with a GeneScan 600 LIZ internal size standard (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 DNA analyzer (Applied Biosystems), and their genotypes were determined using the GeneMapper software v4.0 (Applied Biosystems). We estimated null allele frequencies for each locus using the FreeNA program (Chapuis and Estoup 2007).

**Table 1** Details of the populations examined in this study and population genetic parameters based on 16SSR loci for each population and each genetic group

District	Code (Genetic group)	Coordinate	Habitat type <sup>a</sup>	<i>N<sub>s</sub></i>	<i>N<sub>c</sub></i>	GG	<i>A</i>	<i>P<sub>R</sub></i>	<i>A<sub>R</sub></i>	<i>H<sub>O</sub></i>	<i>H<sub>E</sub></i>	<i>F<sub>IS</sub></i> <sup>b</sup>
Population												
Hokkaido	SHA (2)	43° 54' N, 144° 41' E	C	25	8	2	2.313	0	2.046	0.357	0.248	− 0.405*
	BIE (1)	43° 28' N, 142° 37' E	C	20	19	1	2.875	5	1.947	0.375	0.367	− 0.034
	CHI (2)	42° 48' N, 141° 41' E	D	3	3	2	2.375	0	2.208	0.542	0.434	− 0.238*
	TOM (2)	42° 41' N, 141° 43' E	D	32	28	2	3.313	0	1.386	0.382	0.417	0.10
	TAR (2)	42° 42' N, 141° 21' E	A	3	3	2	1.500	0	1.983	0.438	0.233	− 0.838
	KOM (2)	42° 03' N, 141° 41' E	A	20	20	2	3.250	0	2.190	0.297	0.455	0.328*
Tohoku	BYO (2)	40° 58' N, 140° 21' E	C	14	9	2	1.781	0	2.375	0.372	0.394	− 0.048
	TOY (2)	40° 58' N, 140° 21' E	C	12	11	2	2.625	0	1.530	0.341	0.384	0.081
	NOU (2)	41° 21' N, 141° 21' E	C	22	22	2	3.000	1	1.609	0.318	0.455	0.285
	SAR (2)	41° 17' N, 141° 24' E	C	26	22	2	3.438	3	2.355	0.438	0.443	0.040
	FUK (2)	41° 01' N, 141° 15' E	C	28	28	2	3.118	2	1.500	0.408	0.507	0.258
	MIS (2)	40° 49' N, 141° 23' E	C	11	11	2	2.500	2	2.203	0.199	0.403	0.511*
Kanto	AKK (2)	42° 42' N, 141° 21' E	D	11	7	2	1.750	0	2.006	0.357	0.230	− 0.406
	TOK (3)	36° 28' N, 140° 36' E	C	17	15	3	1.875	1	1.530	0.229	0.275	0.176
	HIT (3)	36° 24' N, 140° 35' E	C	12	9	3	1.500	0	2.064	0.119	0.160	0.217
		Mean (S.E.)		17.07 (2.24)	14.33 (2.16)		2.480 (0.175)	0.93 (0.38)	1.929 (0.384)	0.345 (0.086)	0.367 (0.027)	− 0.034 (0.086)
Genetic group												
	1					Mean (S.E.)	2.875 (−)	5.00 (−)	1.947 (−)	0.375 (−)	0.367 (−)	− 0.034 (−)
	2					Mean (S.E.)	2.580 (0.199)	0.67 (0.32)	1.949 (0.106)	0.377 (0.026)	0.384 (0.028)	− 0.065 (0.114)
	3					Mean (S.E.)	1.688 (0.188)	0.50 (0.50)	1.797 (0.267)	0.174 (0.055)	0.218 (0.058)	0.197 (0.021)

<sup>a</sup>C, coniferous forest; A, deciduous shrub forest in alpine region; D, deciduous forest

<sup>b</sup>Asterisks denote significant deviation from Hardy–Weinberg equilibrium (\**P* < 0.05)

*N<sub>s</sub>*, number of samples examined; *N<sub>c</sub>*, number of clones included in the analyses; GG, genetic groups based on the STRUCTURE analysis; *A*, number of alleles per locus; *P<sub>R</sub>*, number of private alleles; *A<sub>R</sub>*, allelic richness; *H<sub>O</sub>*, observed heterozygosity; *H<sub>E</sub>*, expected heterozygosity; *F<sub>IS</sub>*, fixation index

### Data analysis

The level of polymorphism at each nSSR locus was evaluated by calculating the mean number of alleles per locus (*A*), observed heterozygosity (*H<sub>O</sub>*), expected heterozygosity within a population (*H<sub>E</sub>*), and expected heterozygosity in the total population (*H<sub>T</sub>*) using the program GenAlEx 6.501 (Peakall and Smouse 2006). In addition, *F*-statistics (*F<sub>IS</sub>* and *F<sub>ST</sub>*) and significant deviations from zero were also calculated using GenAlEx 6.501 based on 999 permutations. We also tested for linkage disequilibrium among the nSSR loci using Genepop (Rousset 2008).

The following six population genetic parameters were calculated to characterize each population: number of alleles per locus (*A*), number of private alleles (*P<sub>R</sub>*), allelic richness (*A<sub>R</sub>*), observed heterozygosity (*H<sub>O</sub>*), expected heterozygosity

(*H<sub>E</sub>*), and fixation index (*F<sub>IS</sub>*). Except for *A<sub>R</sub>* and *F<sub>ST</sub>*, which were calculated using FSTAT 2.9.3 (Goudet 1995), the remaining parameters were calculated using GenAlEx 6.501 (Peakall and Smouse 2006). Significant deviations of the fixation indices (*F<sub>ST</sub>*) from zero were tested using FSTAT 2.9.3. The statistical significance of correlations between the latitude of the population locality and genetic parameters (*A<sub>R</sub>*, *H<sub>O</sub>*, and *H<sub>E</sub>*) was tested with Kendall’s rank correlation test using R version 3.4.0 (R Development Core Team 2017).

To estimate the genetic structures of the populations examined, the model-based Bayesian program STRUCTURE 2.3.4 (Pritchard et al. 2000), which determines the number of clusters and assigns individuals to *K* clusters, was employed. In the program settings, a correlated allele frequency, an admixture model, a burn-in period of 100,000 iterations, and 500,000 Markov chain Monte Carlo



simulations were selected, and 10 independent runs were performed for each  $K$  (range: 1–10). To determine the best number of clusters, both the  $\Delta K$  values and  $L(K)$  were visualized using Structure Harvester software (Earl and Holdt 2012; Janes et al. 2017). (2005). Ten simulation runs with the highest modal value of  $\Delta K$  were aligned in Clumpp 1.1 (Jakobsson and Rosenberg 2004) and visualized as a bar graph in Distruct 1.1 (Rosenberg 2004).

To illustrate genetic similarity among the populations, a neighbor-joining tree with 1000 bootstrap replications was constructed using POPTREE2 (Takezaki et al. 2010), and a principal coordinates analysis (PCoA) was performed using Past 3.22 (Hammer et al. 2001). Both the analyses were conducted based on Nei's corrected genetic distance ( $D_A$ ) (Nei et al. 1983), which was calculated using Populations 1.2.32 (Langella 1999).

To partition the genetic variation among the groups (see below), among populations within the groups, and among all the populations, an analysis of molecular variance (AMOVA) was performed with a two-factorial design using Arlequin 3.5.2.2 (Excoffier et al. 2005), for the following two population groupings: (1) three genetic groups based on the STRUCTURE results (see Results; group 1: only BIE; group 3: two populations in the Kanto District, TOK and HIT; group 2: all remaining populations) and (2) those distinguished by district (the Hokkaido District, Tohoku District, and Kanto District).

Recent effective population size reductions were assessed using BOTTLENECK 1.2.02 (Piry et al. 1999). The sign test and the Wilcoxon signed-rank test were employed to test the significance of heterozygote excess under the infinite allele model (IAM) and the stepwise mutation model (SMM).

To infer the population demographic dynamics of *C. umbellata* in Japan, we employed approximate Bayesian computation (ABC) implemented in DIYABC 2.1.0 (Bertorelle et al. 2010; Cornuet et al. 2010). Each of the three genetic groups based on the STRUCTURE results above (genetic groups 1–3) were treated as a separate population. The following five scenarios were tested for the three populations; scenario 1 assumes that group 3 (the southernmost group) first diverged from the ancestor of groups 1 and 2 at time  $t_2$  and that groups 1 and 2 split at time  $t_1$  (Fig. 2a). Scenarios 2 and 4 assumed that either group 2 or group 1, respectively, first diverged from the ancestor of the other two groups at time  $t_2$ , and then the remaining two groups split at time  $t_1$  as in scenario 1 (Fig. 2b and 2d). In scenario 3, all three groups diverged at the same time  $t_1$  (Fig. 2c). Scenario 5 assumes that group 2 (the 12 midway populations) was created by gene flow between the remaining two groups (the southern and northern group) at time  $t_1$  (Fig. 2e). In all of the five scenarios above, population size changes were allowed for each population and admixture by migration was allowed in scenario 5.

One million simulations were run using the following summary statistics implemented in DIYABC 2.1.0: single and two samples of mean gene diversity across loci (Nei, 1987),  $F_{ST}$  between two samples (Weir and Cockerham, 1984), and  $(\delta\mu)^2$  distance between two samples (Goldstein et al., 1995). After the pilot run, the prior distributions of each parameter were set as shown in Online Resource 1. The scenarios were compared using both a direct approach and a logistic approach (Cornuet et al. 2010).

The absolute time of the population divergence can be calculated by multiplying the estimated number of generations above and the generation time of *C. umbellata*. Unfortunately we did not have any data on the time from germination to the first flowering of *C. umbellata*; however, considering that the span was estimated to be 4–6 years in *Erica lustranica* (Mather and Williams 1990), a dwarf shrub species of Ericaceae to which *C. umbellata* also belongs, we set the generation time to four, five and six years.

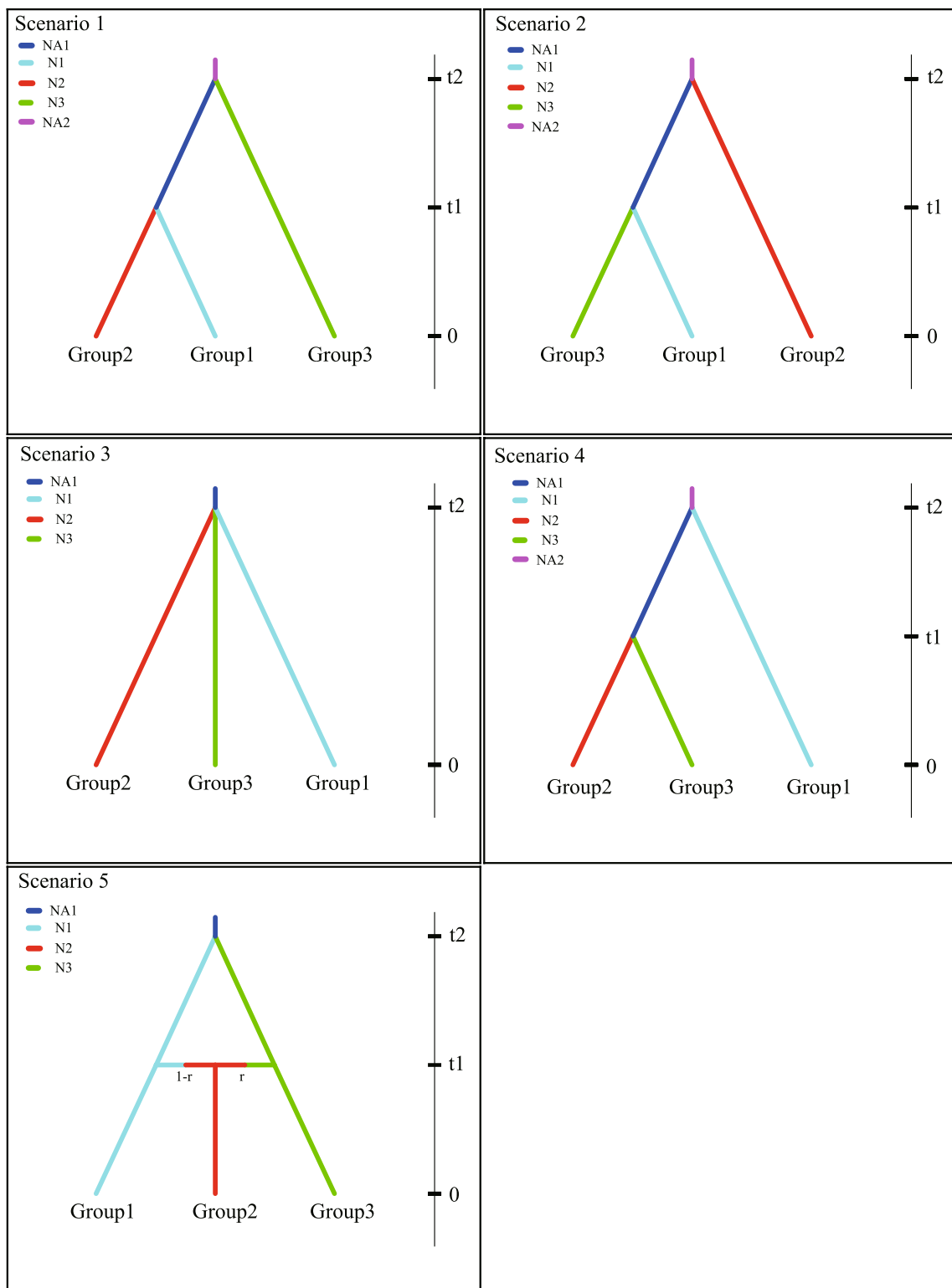
To examine population prioritization based on gene diversity and allelic diversity, METAPOP 2.1 (Pérez-Figueroa et al. 2009) was employed. This program assesses the impact of the removing a given population on the whole allelic diversity of the remaining populations and estimates the relative contribution of each population on species-level genetic diversity. Based on the results, we can infer the relative importance of each population in terms of the conservation of the genetic diversity at the species level.

## Results

### Genetic variation

For the 16 nSSR loci examined, a total of 71 alleles were found among 215 individuals (Online Resource 2 and 3). Null alleles were detected in very limited cases and there was no locus in which null alleles were estimated consistently across the populations examined (data not shown). The number of alleles per locus ranged from 2 to 10, with an average of 4.4, and inbreeding coefficients ( $F_{IS}$ ) at each locus ranged from -0.188 to 0.378, and no values significantly departed from the Hardy–Weinberg equilibrium. Linkage disequilibrium among the nSSR loci was not statistically significant after the sequential Bonferroni correction.

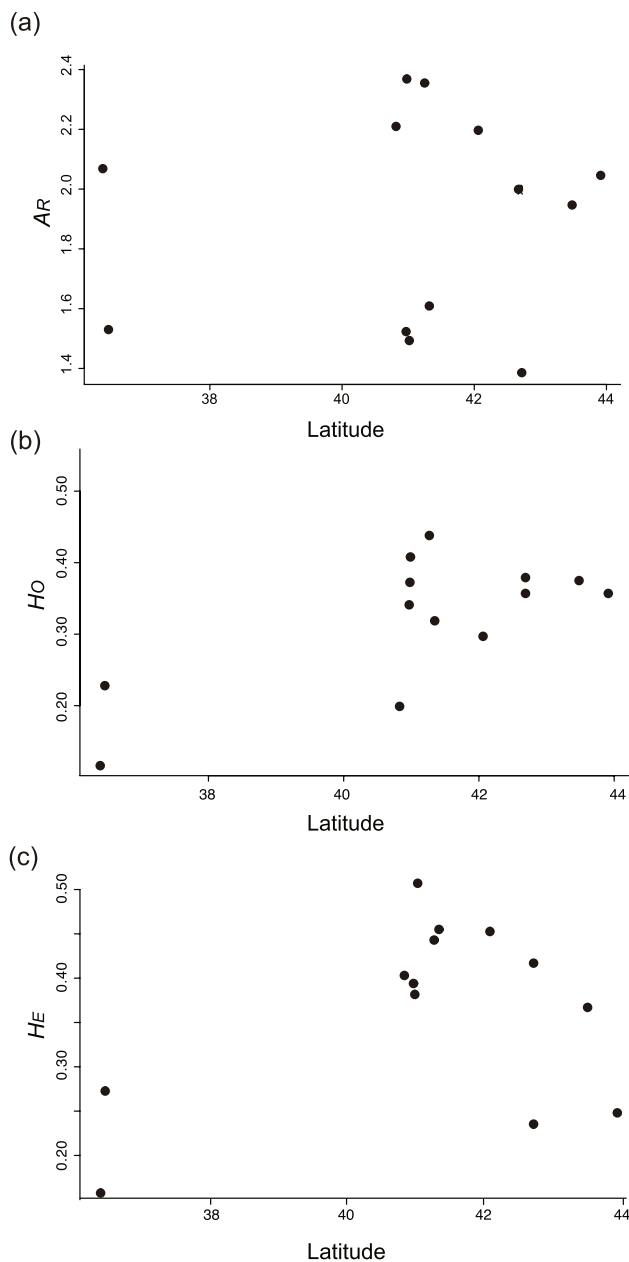
The population genetic parameters for each population examined are shown in Table 1. The BIE population in central Hokkaido had the largest number of private alleles, and four populations (NOU, SAR, FUK, and MSI) in the northeastern part of the Tohoku District had 1–3 private alleles. Allelic richness ( $A_R$ ) ranged from 1.386 to 2.375, and the values did not correlate with the latitudes of the population localities (Fig. 3a; Kendall's rank correlation test:  $\tau = 0.219$ ,  $P = 0.328$ ). The observed ( $H_O$ )



**Fig. 2** Five scenarios on the divergences of the three genetic groups examined by DIYABC

and expected ( $H_E$ ) heterozygosity ranged from 0.119 to 0.542 and from 0.160 to 0.507, respectively. The observed heterozygosity ( $H_O$ ) was marginally correlated with the

latitude of the population locality (Fig. 3b; Kendall's rank correlation test:  $\tau = 0.374$ ,  $P = 0.087$ ), although the correlation between  $H_E$  and latitude was not statistically



**Fig. 3** Correlation between the latitude of population locality and genetic diversity values. **a** Allelic richness ( $A_R$ ); **b** Observed heterozygosity ( $H_O$ ); **c** Expected heterozygosity ( $H_E$ )

significant (Fig. 3c; Kendall's rank correlation test:  $\tau = 0.271$ ,  $P = 0.221$ ). The populations in Hokkaido and Tohoku had larger overall values of  $A_R$ ,  $H_O$ , and  $H_E$  than the two populations in the Kanto, at the southern edge of the species distribution in Japan. This trend was obvious at the genetic group level (Table 1). Significant heterozygote deficiency was found only in two populations (KOM and MIS), suggesting that most of the populations crossed randomly (Table 1).

### Population genetic structure

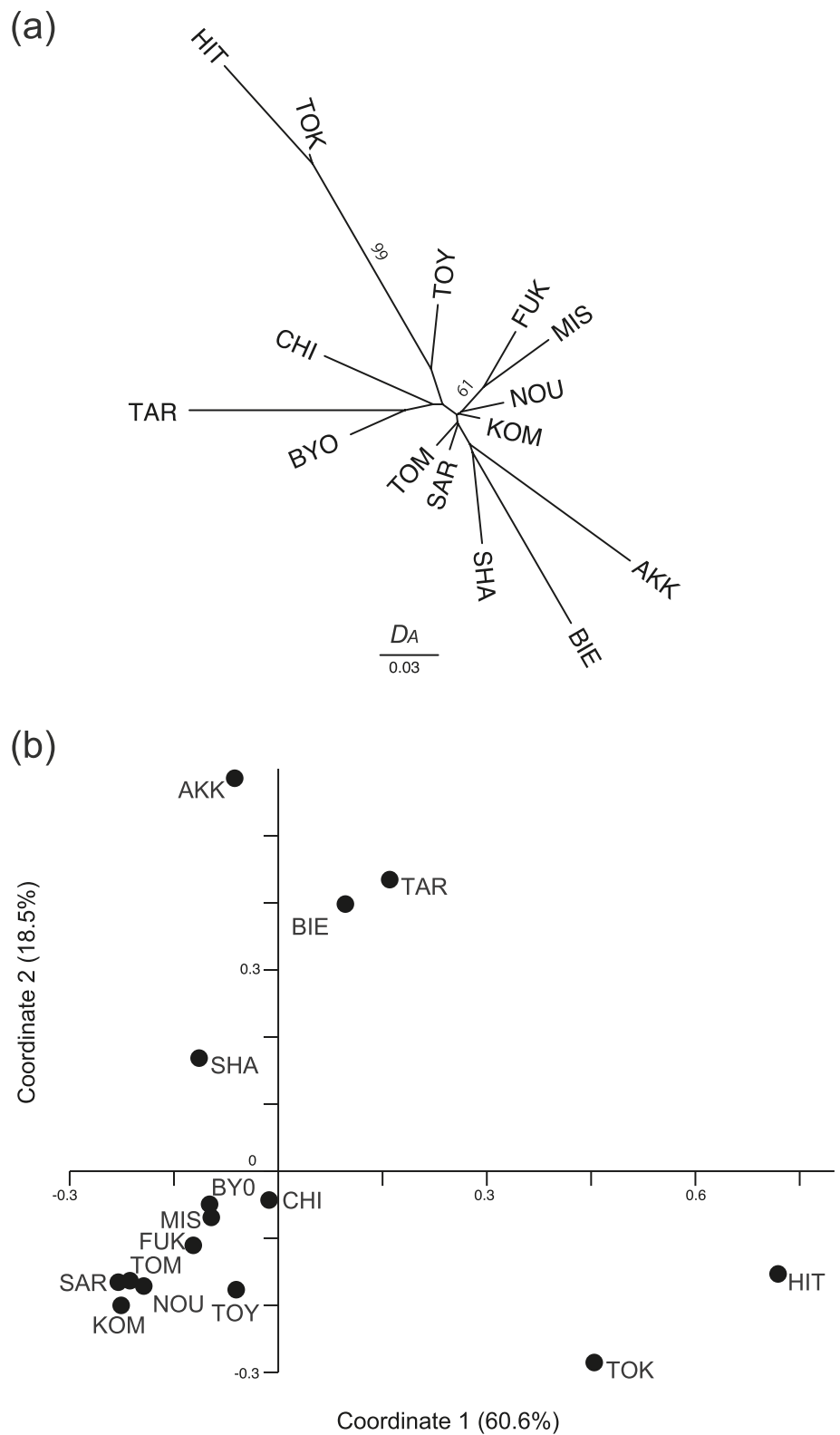
The pairwise comparisons of the  $F_{ST}$  values among the populations examined are summarized in Online Resource 4. The lowest value (0.014) was found within Hokkaido (KOM vs. SAR) while the largest value (0.408) was found between the two remote populations (TAR in Hokkaido vs. HIT in Kanto). The  $F_{ST}$  values were positively correlated with the geographic distance between the populations (Mantel-test,  $r^2 = 0.355$ ,  $P < 0.01$  using GenAIEx 6.501).

In the Bayesian cluster analysis using STRUCTURE, the  $\Delta K$  and  $L(K)$  values indicated that the optimal number of clusters was  $K = 4$  (Online Resource 5). Figure 1A shows the assignment of clusters for each individual examined. Almost all of the individuals in the two populations in the Kanto District (TOK and HIT) were assigned to a unique cluster (shown in gray in Fig. 1) that rarely occurred in the other populations. Similarly, most of the individuals in the population in central Hokkaido (BIE) were assigned to a unique cluster (shown in green in Fig. 1) that was rarely found in other populations, suggesting that this population was genetically differentiated from the others. The remaining 13 populations were admixed between two clusters (shown in blue and orange in Fig. 1), although their proportions varied widely among populations. Because we could not assign these populations to a distinct genetic cluster, we treated these 13 populations as one genetic group for the AMOVA and ABC analyses described below. The neighbor-joining tree (Fig. 4A) and the result of the PCoA (Fig. 4b) also revealed genetic differentiation between the two populations in the Kanto District (TOK and HIT) and the population in central Hokkaido (BIE) from the others. However, the PCoA also showed potential genetic differentiation in the southmost population in the Tohoku District (AKK; Fig. 4b). Since these two results on the genetic similarity among the populations are based on the data that included populations with a small number of genets, they may have to be viewed with some caution.

For the grouping based on the STRUCTURE results shown above, the AMOVA revealed that the percentage of variation among groups was 19.03%, and that within populations was 73.27%, showing that most of the total genetic variation was maintained within populations (Table 2). Only 7.70% of genetic differentiation existed among the populations within the groups. In the case of grouping by district, the percentages of genetic variation maintained among districts, among populations within a district, and within populations were 9.05%, 10.71%, and 80.24%, respectively. Most of the total genetic variation was attributed to differences within populations (Table 2).



**Fig. 4** Genetic similarity among the populations. **a** Neighbor joining trees; values above branches denote bootstrap probabilities (%); **b** Principal coordinate analysis



**Demographic analyses**

Table 3 summarizes the results of the BOTTLENECK test. Only a few cases, such as the TAR and FUK populations,

clearly showed recent declines in population size, suggesting that most of the populations have remained stable in size, at least in recent times.

**Table 2** Analysis of molecular variance (AMOVA) in the populations examined. Two populations with only three genets, i.e., populations CHI and TAR, were excluded from the analysis

Source	d.f	Sum of squares	Percentage of variation
(A) Grouping based on the STRUCTURE results			
Among groups	2	156.96	19.03***
Among populations within groups	12	142.85	7.70***
Within populations	417	1347.08	73.27***
(B) Grouping by district			
Among districts	2	128.63	9.05***
Among populations within districts	12	171.17	10.71***
Within populations	417	1347.08	80.24***

\*\*\* $P < 0.001$ **Table 3** Bottleneck tests using the sign test and Wilcoxon's signed-rank test under the infinite allele model (IAM) and strict stepwise mutation model (SMM) in the populations examined

Population	Sign		Wilcoxon's Signed-rank	
	IAM	SMM	IAM	SMM
SHA	0.017*	0.249	0.015*	0.151
BIE	0.455	0.297	0.280	0.135
TOM	0.286	0.349	0.188	0.851
KOM	0.129	0.383	0.126	0.647
BYO	0.022*	0.583	0.011*	0.473
TOY	0.136	0.498	0.104	0.533
NOU	0.130	0.523	0.006**	0.490
SAR	0.236	0.440	0.180	0.738
FUK	0.011**	0.085	0.000***	0.033**
MIS	0.100	0.407	0.029**	0.213
AKK	0.227	0.376	0.160	0.861
TOK	0.031*	0.214	0.017*	0.170
HIT	0.069	0.231	0.010**	0.125

Two populations with only three genets, i.e., populations CHI and TAR, were excluded from the analysis. Asterisks denote the significance levels of heterozygote excesses (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ )

The ABC analysis indicated that scenario 3, in which the three genetic groups diverged at the same time, is the most likely scenario. However, the posterior predictive error was 0.559 and 0.560 using direct and logistic approaches, respectively, indicating relatively limited confidence even in the best choice scenario 3. The posterior means and 95% quantiles for N1, N2, N3, and NA2 were  $6.13 \times 10^2$  ( $1.64 \times 10^2 - 1.98 \times 10^3$ ),  $2.61 \times 10^2$  ( $9.82 \times 10^1 - 5.23 \times 10^2$ ),  $3.76 \times 10^3$  ( $6.77 \times 10^2 - 9.28 \times 10^3$ ), and  $2.36 \times 10^2$  ( $2.13 \times 10^1 - 6.63 \times 10^2$ ), respectively, suggesting that genetic group 3 (southern populations) had significantly smaller

population sizes than genetic group 2 (northern populations except BIE). Assuming the generation time as four, five and six years, the scaled divergence times of the three genetic groups were 2260 (95% HPD: 699–3736), 2825 (95% HPD: 874–4670), and 3390 (95% HPD: 1048–5604), indicating the recent divergence of the genetic groups.

### Population prioritization for conservation

The contribution of each population to total population-level genetic diversity and allelic diversity is summarized in Fig. 5. Although the FUK population positively contributed to both the genetic diversity and allelic diversity of the whole population, a few populations (KOM, SAR, and TOK) showed conflicting circumstances regarding population prioritization because the populations positively contributed to total population genetic diversity but contributed negatively to allelic diversity, or vice versa. The remaining populations mainly contributed only to one of the two diversity indices, or had almost no influence on either diversity index.

## Discussion

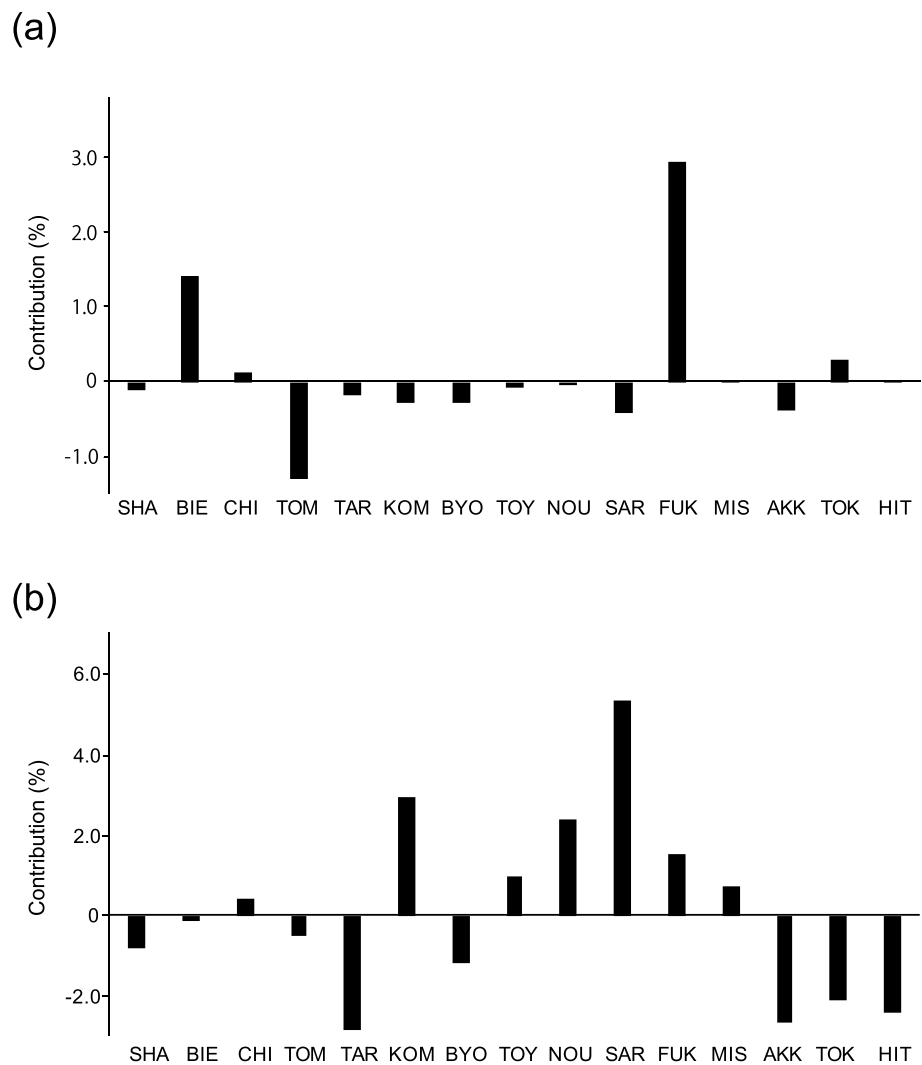
### Genetic diversity in the southern edge and other populations

The correlation between genetic diversity and the latitude of the population locality was equivocal in this study; marginally significant correlations between the observed heterozygosity ( $H_O$ ) and the latitude of the population locality were observed, whereas the expected heterozygosity ( $H_E$ ) or the allelic richness ( $A_R$ ) and the latitude were not significantly correlated. However, the population genetic parameters such as the number of alleles per locus ( $A$ ), number of private alleles ( $P_{R_i}$ ), allelic richness ( $A_{R_i}$ ), observed heterozygosity ( $H_{O_i}$ ) and expected heterozygosity ( $H_{E_i}$ ) were lower in the southern peripheral populations than in other populations (Table 1), supporting the central-marginal hypothesis.

The ABC analysis suggested that the size of the southern population (genetic group 3) was significantly smaller than that of the Tohoku and Hokkaido populations (genetic group 2) excluding the genetic group 1 (the population BIE). In addition, the analysis suggested that the size of the inland population in Hokkaido (genetic group 1) was larger than that of the southern population, although this difference was not statistically significant. Therefore, a reduction in size of the southern peripheral populations during the post-glacial period may be one of the reasons for the smaller genetic variation.

The mating system of plant species is known to considerably affect intrapopulation genetic variation (Loveless and Hamrick 1984). Generally selfing or mixed mating

**Fig. 5** The contributions of each population to total genetic diversity estimated using METAPOPOP 2.1. **a** Gene diversity; **b** Allelic diversity



tends to promote genetic differentiation among populations and reduce genetic diversity within a population. The 16 nSSR loci did not significantly deviate from Hardy–Weinberg equilibrium across the populations examined (Online Resource 3). At the population level, significant heterozygote deficiency was observed in only two populations (Populations KOM and MIS; Table 1). These results suggest that *C. umbellata* mainly outcrosses and mates randomly. In fact, bees such as *Bombus* frequently visited *C. umbellata* during the flowering season (M. Maki personal observation). This reproductive system of *C. umbellata* may contribute to the maintenance of genetic diversity within populations.

**Climate-driven range dynamics**

The results of the ABC analysis showed that the three genetic groups of *C. umbellata* in the Japanese archipelago split simultaneously and their divergence times were estimated to be less than 3000 yrs, although the scenario does

not provide full confidence. If this value was not underestimated, then the range of *C. umbellata* was fragmented very recently. Considering the relatively small population size estimated for the ancient population before the range fragmentation (NA2) and the weak sign of recent genetic bottlenecks, the genetic variation of *C. umbellata* in the Japanese archipelago may have already been smaller before its range was fragmented. After the range split, it is possible that the southern populations maintained small genetic variations while the remaining populations have recovered their genetic variability along with population size growth.

**Genetic differentiation among the populations**

Based on the STRUCTURE results, the populations of *C. umbellata* in Japan were categorized into three genetic groups based on their clusters. The AMOVA results suggested that these groups were slightly but significantly isolated. Based on the results of the neighbor-joining tree and

PCoA, the Kanto group was highly differentiated from the other groups. Considering the distance between the populations of the Kanto group and the southernmost population of the Tohoku group (AKK), the Kanto group is strongly isolated at present.

The STRUCTURE results, neighbor-joining tree, and PCoA results implied that the AKK population, the southernmost population in the Tohoku District, is somewhat differentiated from the other populations in Tohoku. This is probably because this population is located on the southern periphery of the species distribution in this district. Following the central-marginal hypothesis, such populations tend to be isolated from other populations and are strongly influenced by genetic drift (Eckert et al. 2008). In addition, because the AKK population lies inland, the population is highly isolated from other populations located in coastal regions is not likely to occur.

Similarly, the BIE population in central Hokkaido lies inland and is isolated from other Hokkaido populations. As a result of fragmentation of the past distribution and the resultant isolation from other populations, BIE has become genetically differentiated from other populations because it might be highly isolated from other populations. In fact the number of private alleles found was largest in the BIE population.

Each population in the eastern part of the Tohoku District (NOU, SAR, FUK, and MIS) had at least one private allele, although these populations are located more closely together than populations in other regions, and are genetically similar. This suggests that these populations are somewhat genetically isolated from each other. Although relatively there are many populations of *C. umbellata* exist in the eastern part of Tohoku District, the populations occur sporadically, possibly impeding gene flow among populations. However, the pairwise  $F_{ST}$  values among these populations were not large, suggesting that small but at least certain gene flow exists among them.

### Implications for conservation

Global climate change is considered to strongly impact species distributions and even cause population extinctions (Parmesan et al. 1999; Thomas et al. 2004; Parmesan 2006). In particular, populations at the southern edge of arctic species distributions, and those at lower altitudes of alpine species, will be severely influenced by global climate warming (Thuiller et al. 2005; Lenoir et al. 2008; Stubbs et al. 2018).

If current global climate warming continues, several local populations of *C. umbellata*, particularly at the southern margin of their distribution, will become extinct because there are no favorable habitats nearby to which the populations will be able to escape. Consequently, the implementation of ex situ conservation should be taken into consideration in the near future. Since *C. umbellata* probably requires

symbiosis with mycobionts during seed germination (Eriksson and Kainulainen 2011) even though adult plants appear to be autotrophic (Hynson et al. 2012; Johansson et al. 2015), the technical development of *C. umbellata* cultivation and further investigation into its ecology, will be necessary.

Assessing the relative importance of populations to conserve genetic diversity and, based on the results, determining which populations should be prioritized, are primary goals in conservation genetics. Based on the METAPOPOP analysis, the FUK population showed the largest contribution to the gene diversity in the total population, and the BIE population also contributed considerably to the total genetic diversity. In contrast, the SAR population contributed most to the allelic diversity in the total population, and the KOM, NOU, and FUK populations also made large contributions to the total allelic diversity.

Since FUK is the only population that contributed considerably to both genetic and allelic diversity, this population should be ranked highest in terms of conservation priorities to maximize the genetic diversity pool of the whole population in Japan. However, there are other populations that ranked high for either gene or allelic diversity, contributing to one but not both. These populations should be ranked second with respect to conservation priority to ensure genetic variation of the species in Japan. The SAR population positively contributed to the total allelic diversity but negatively to the total genetic diversity. The conservation priority of such populations showing conflicting patterns should be treated with caution. In such cases, ecological and phenotypic variations may need to be considered to determine conservation prioritization.

Although the two populations in the Kanto District were genetically differentiated from the populations in other regions, the METAPOPOP results did not show high conservation priority for these populations. This probably resulted from the genetic similarity of the populations because METAPOPOP evaluates the contribution of each population to total gene or allelic variation by removing the given population. Considering the genetic distinctness of the populations, their priority for genetic conservation is incontrovertible.

At present, *C. umbellata* is not protected statutorily in Japan, although some of the populations examined in this study can be found within the National Parks (populations TAR and KOM), the restricted area in the Hitachi Seaside Park (Population HIT), and the Nuclear Science Research Institute of Japan Atomic Energy Agency (Population TOK). Considering this situation, the populations located in unprotected areas should also be given high conservation priority by local governments.

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**Author contribution** AK and MM collected samples, AK performed molecular experiment, AK and RK performed statistical analyzes, AK and MM conceived the study and wrote the manuscript. All authors approved the final manuscript.

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**Data availability** Gene frequency data of SSR used in the study were given in Online Resource 2.

**Declaration**

**Conflict of interest** There is no conflict of interest.

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