Microsatellite genetic variation in small and isolated populations of *Magnolia sieboldii* ssp. *japonica*

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Magnolia sieboldii ssp. *japonica*, distributed mainly in western Japan, is restricted to high elevation areas (1000–2000 m above sea level) and usually forms small isolated populations. Four microsatellite loci were assayed for 19 populations from six regions spanning the range of distribution, and the levels and distribution of genetic variation were estimated. All four loci were variable, with a total of 39 alleles, but the overall level of microsatellite genetic variation was low, especially compared with a related species, *M. obovata*. Genetic structure in *M. sieboldii* was characterised by low intrapopulational genetic variation ($A = 3.74$ and $H_o = 0.366$ on average) and high genetic differentiation even among regional populations. Highly significant isolation-by-distance (IBD) models at the short distance were detected. Genetic drift and limited gene flow was considered to be important

in determining the genetic structure within regions. Total genetic differentiation was remarkably high ($F_{ST} = 0.488$ and $R_{ST} = 0.538$), suggesting genetic barriers among regions. Neighbour-joining dendrograms relating the 19 populations, and further analysis on the IBD models, revealed that a step-wise mutation model was more suited than an infinite allele model to explain the genetic differentiation among regions. It is suggested that mutation at microsatellite loci might be influential in generating the genetic differentiation among regions. These results showed the potential of hypervariable microsatellite loci to evaluate the effects of genetic drift and population isolation within regions, and to detect genetic distinctiveness, in spite of the loss of overall genetic variation in *M. sieboldii*.

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Introduction

Within a species, the genetic structures of plant populations are determined by factors such as mutation, migration, selection and genetic drift. The effects of genetic drift are important when populations are small or isolated. Species consisting of such populations usually have small within-population genetic variation and large among-population differentiation (Loveless and Hamrick, 1984). Genetic information can be useful in the conservation of rare plant species, which usually consist of small and/or isolated populations. For example, the risk of extinction of a population or a species can be assessed, and the steps and priorities necessary to prevent extinction can be determined by estimating the level of genetic diversity, the effect of genetic drift, and the extent of genetic differentiation and gene flow, from genetic data (Ellstrand and Elam, 1993). However, the genetic variability of isolated or local endemic populations sometimes collapses because of severe genetic drift or historical bottlenecks; in such cases little or no genetic variation is detected in the population using genetic markers, such

as allozymes or RAPDs (eg, Ledig and Conkle, 1983; Bauert *et al.*, 1998; Vogel *et al.*, 1999).

Recently, hypervariable microsatellite loci have become a popular genetic tool in population and conservation genetics. Microsatellites have such strong discriminating power that they have been utilised for parentage analysis (Dow and Ashley, 1996; Isagi *et al.*, 2000) and detection of fine-scale genetic differentiation (van Oppen *et al.*, 1997; Streiff *et al.*, 1998). We would expect to find variability in severely bottlenecked or isolated populations where genetic markers, such as allozymes and RAPDs, detect little or no variation (Hughes and Queller, 1993). Microsatellites are also considered to be appropriate for surveying phylogenetic relationships among populations, although problems of size homoplasy, due to convergent mutations, remain (Valdes *et al.*, 1993). Studies have shown the high efficiency of microsatellites in distinguishing populations or groups of populations (eg, Rowe *et al.*, 1998; Widmer and Schmid-Hempel, 1999). These applications make microsatellites suitable tools for the conservation and population genetics of rare and/or isolated species. In spite of this potential, there is still a paucity of data of microsatellite variation in plant populations.

In this study, microsatellite variation in a rare and patchily distributed plant, *Magnolia sieboldii* C. Koch ssp. *japonica* Ueda, was surveyed across its distributional range. *M. sieboldii* ssp. *japonica* is a deciduous shrub that

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reaches a height of 3 m. It is distributed mainly in western Japan, extending from Yakushima Island to northern Kanto. Shrubs of *M. sieboldii* in Japan grow at high elevations in mountainous regions, 1000–2000 m above sea level. They live on restricted areas such as narrow stony ridges, among rocks, or on the edges or in openings of upper cool-temperate-deciduous to subalpine-coniferous forests; and they can be found in limestone or serpentine areas (Ueda, 1980). Shrubs of *M. sieboldii* usually form small isolated populations.

The aim of this study was to survey microsatellite variation within and among populations of *M. sieboldii* and to assess the usefulness of microsatellites as a tool in the conservation of small and isolated populations of *M. sieboldii*. The focus of the study was on the level of microsatellite variability within populations, the extent of hierarchical genetic differentiation at intrapopulation, regional, and whole-species levels, and the comparison of genetic relationships with geographical relationships among populations.

Materials and methods

Sampling sites

Nineteen populations were sampled across six regions (Mts Hodaka, Akasawa Recreational Forest, Mts Ohmine, Mts Ishizuchi, Mt Kanmuri, and Mt Kunimi) throughout the distribution range of *M. sieboldii* (Figure 1). The populations of *M. sieboldii* were usually small and isolated, and consisted typically of only tens of shrubs. An exception was the Hakkyougatake population (OMN1), which consisted of hundreds of shrubs. For each population leaves were sampled from 11 to 56 shrubs. In all, 658 samples

were collected (Table 1). Intensive sampling was carried out within the Ishizuchi and Ohmine mountain ranges; seven populations were sampled in each of these regions. Additionally, two populations were sampled in the Hodaka mountain range. Populations within regions were 0.3–14 km apart.

DNA isolation and microsatellite analysis

DNA was extracted from leaf materials using a CTAB method (Milligan, 1992). Four microsatellite loci (M6D3, M6D4, M10D8, M17D5) were analysed in this study, selected from 11 loci identified by Isagi *et al* (1999) in *M. obovata*. These primer pairs had been found applicable to most of the other *Magnolia* species in Japan (Isagi *et al*, 1999). PCR reactions were carried out as described in Isagi *et al* (1999). The PCR products were electrophoresed on the ABI 377 instrument, and the sizes were determined using Genescan™ analysis software.

Data analysis

For each population, genetic polymorphism was measured as the mean number of alleles per locus (A), observed heterozygosity (H_o) and heterozygosity expected from Hardy-Weinberg assumptions (H_e). Weir and Cockerham's F_{IS} (Weir and Cockerham, 1984) was calculated for each population by using the FSTAT program (Goudet, 1995). The significance of F_{IS} was tested based on randomisation. P -values, which gave the probability that the null hypothesis ($F_{IS} = 0$) was true, were computed through 1000 random permutations.

The interpopulational genetic differentiation was estimated by calculating fixation indices based on an infinite allele model (IAM) and a stepwise mutation model (SMM). For the former, Weir and Cockerham's F_{ST} values were calculated with the TFGA program (Miller, 1997). For the latter, the R_{ST} CALC program (Goodman, 1997) was used to calculate Slatkin's R_{ST} (Slatkin, 1995). One thousand bootstraps for all loci gave the 95% confidence intervals to F_{ST} and R_{ST} . In addition, population pairwise F_{ST} and R_{ST} values were estimated using the programs FSTAT and Microsat (Dr E Minch, Department of Genetics, Stanford University, USA, <http://lotka.stanford.edu/distance.html>).

To evaluate spatial genetic structure among populations, mean pairwise F_{ST} and R_{ST} values between populations within mountain ranges, and between populations belonging to different regions, were calculated separately. An isolation-by-distance (IBD) model was tested by regressing the population pairwise F_{ST} and R_{ST} against the $\log_{10}$ value of the geographical distances between populations.

Unrooted dendrograms of populations were constructed using the neighbour-joining method, as implemented in the program PHYLIP (Felsenstein, 1989). Nei's standard distance D (Nei, 1978) and Goldstein's delta-mu square ($\delta\mu^2$) (Goldstein *et al*, 1995), based on an SMM, were calculated as genetic distances, using Microsat.

A spatial autocorrelation analysis was performed for the largest population, OMN1, to determine intrapopulation spatial genetic structure. All of 52 sampled shrubs, covering most part of this population (about 0.4 ha), were mapped, and a distance matrix among the shrubs was constructed. Distance classes were set for 5 m, and then

Figure 1 Distribution of the sample populations of *Magnolia sieboldii*, with population identification codes in parentheses, according to Table 1.

Table 1 Locations and identification codes of the 19 sampled populations of *Magnolia sieboldii*

Locality	Code	Longitude (°N)	Latitude (°E)	Altitude (m)	No. of samples
Akasawa Recreational Forest, Chubu District	AKS	35.7	137.6	1140	31
Mts Hodaka, Chubu District					
Hidarimata Path	HDK1	36.3	137.6	1300	35
Migimata Path	HDK2	36.3	137.6	1520	45
Mts Ohmine, Kansai District					
Mt Hakkyougatake	OMN1	34.2	135.9	1900	52
Mt Misen	OMN2	34.2	135.9	1880	13
Mt Misen	OMN3	34.2	135.9	1800	41
Mt Misen	OMN4	34.2	135.9	1740	56
Mt Misen	OMN5	34.2	135.9	1600	41
Mt Daihugen	OMN6	34.2	135.9	1770	28
Mt Sanjougatake	OMN7	34.2	136.0	1530	14
Mts Ishizuchi, Shikoku District					
Mt Ishizuchi	ISZ1	33.8	133.1	1960	21
Mt Iwaguro	ISZ2	33.8	133.2	1740	11
Mt Tebako	ISZ3	33.7	133.2	1800	30
Mt Tebako	ISZ4	33.7	133.2	1800	51
Mt Kamegamori	ISZ5	33.8	133.2	1700	31
Mt Kamegamori	ISZ6	33.8	133.2	1890	51
Mt Kanpusan	ISZ7	33.8	133.3	1700	24
Mt Kanmuri, Chugoku District	KMR	34.5	132.1	1320	29
Mt Kunimi, Kyushu District	KNM	32.5	131.0	1730	54
				Total	658

Moran's index I' was calculated for each class at each locus (Sokal and Oden, 1978).

Results

In total, 12, 8, 17 and 12 alleles were detected at loci M6D3, M6D4, M10D8 and M17D5, respectively (see Appendix). At the intrapopulation level, numbers of alleles per locus ranged from 2.00 to 6.00, with an average of 3.74. The observed and expected heterozygosities ranged from 0.196 to 0.642 and from 0.254 to 0.652, with averages of 0.366 and 0.430, respectively (Table 2). Of the 19 populations, 17 had inbreeding coefficients greater than zero, and nine of those had significant P -values ($P < 0.05$). Two populations with negative F_{IS} values, KNM and OMN6, had one and two loci fixed, respectively.

Population genetic differentiation was unusually high for both F_{ST} (=0.488, 95% CI 0.461–0.519) and R_{ST} (=0.538, 95% CI 0.522–0.571). The mean values of pairwise genetic differentiation were 0.31 for F_{ST} and 0.26 for R_{ST} among populations within a mountain range, whereas pairwise F_{ST} and R_{ST} were 0.52 and 0.57 when averaged among populations belonging to different mountain ranges. Significant isolation-by-distance was revealed for both F_{ST} (Figure 2a; $r = 0.676$, $P < 0.001$) and R_{ST} (Figure 2b; $r = 0.665$, $P < 0.001$).

We examined relationships between pairwise values of genetic differentiation and population genetic diversity averaged for each of the population pairs (Figure 3). Pairwise values of genetic differentiation were correlated with averaged A (F_{ST} : $r = -0.419$, $P < 0.0001$; R_{ST} : $r = -0.291$, $P = 0.0001$), H_o (F_{ST} : $r = -0.593$, $P < 0.0001$; R_{ST} : $r = -0.462$, $P < 0.0001$) and H_e (F_{ST} : $r = -0.631$, $P < 0.0001$; R_{ST} : $r = -0.440$, $P < 0.0001$). Pairs of populations both coming from the Ohmine region depicted patterns different from other population pairs (Figure 3). Correlation

was emphasised when the population pairs from the Ohmine region were removed from analysis; pairwise F_{ST} and R_{ST} were correlated with mean A (F_{ST} : $r = -0.756$, $P < 0.0001$; R_{ST} : $r = -0.556$, $P < 0.0001$), H_o (F_{ST} : $r = -0.794$, $P < 0.0001$; R_{ST} : $r = -0.599$, $P < 0.0001$) and H_e (F_{ST} : $r = -0.884$, $P < 0.0001$; R_{ST} : $r = -0.598$, $P < 0.0001$).

On both of the neighbour-joining trees (Figure 4), the one based on $(\partial\mu)^2$ and the other on Nei's D , populations coming from Ishizuchi, Ohmine, and the Chubu District, formed a group in each of these regions. However, they did not show any monophyletic clusters in each of the regions, and bootstrap values of the nodes supporting these groups were not so high. On the neighbour-joining tree based on $(\partial\mu)^2$, the KNM population (in the Kyushu District) and the KMR population (in the Chugoku District) stemmed from the Ishizuchi group, and formed a cluster together as populations from southwestern Japan, while the populations from the Chubu District stemmed from the Ohmine group (Figure 4a). The genetic relationships among regions agreed well with their geographic origins on $(\partial\mu)^2$ (Figure 1). On the other hand, for Nei's D , genetic and geographic relationships among the regions did not agree (Figure 4b).

Spatial autocorrelation analysis for the OMN1 population revealed no apparent deviation of Moran's I' values from zero until they fluctuated at the longest distance class (85–90 m), which contained only two pairs of individuals; alleles were randomly distributed within the OMN1 population.

Discussion

A direct comparison of microsatellite variation with other studies is problematic because different microsatellite loci may generate different levels of variation. However, a comparison with the results from a related species, *M.*

Table 2 Number of alleles per locus (A), average observed (H_o) and expected (H_e) heterozygosities, and fixation indices (F_{IS}), with standard errors in parenthesis, in 19 populations of *Magnolia sieboldii*

Population	A (s.e.)	H_o (s.e.)	H_e (s.e.)	F_{IS}
AKS	3.75 (0.95)	0.242 (0.088)	0.344 (0.117)	0.311**
HDK1	2.50 (0.29)	0.193 (0.099)	0.309 (0.119)	0.388**
HDK2	4.50 (0.96)	0.196 (0.062)	0.352 (0.143)	0.453**
Hodaka mean	3.50	0.194	0.330	0.421
OMN1	3.50 (0.65)	0.490 (0.112)	0.524 (0.109)	0.073
OMN2	2.50 (0.29)	0.308 (0.140)	0.385 (0.109)	0.238*
OMN3	3.50 (0.50)	0.368 (0.085)	0.420 (0.095)	0.098
OMN4	2.75 (0.48)	0.313 (0.121)	0.328 (0.135)	0.055
OMN5	2.50 (0.50)	0.280 (0.132)	0.303 (0.145)	0.148
OMN6	2.00 (0.71)	0.268 (0.155)	0.254 (0.147)	-0.038
OMN7	3.00 (0.41)	0.232 (0.084)	0.406 (0.114)	0.457**
Omine mean	2.82	0.323	0.374	0.147
ISZ1	5.25 (0.65)	0.492 (0.068)	0.645 (0.070)	0.256**
ISZ2	3.75 (0.48)	0.409 (0.059)	0.499 (0.091)	0.119
ISZ3	4.50 (0.50)	0.463 (0.109)	0.555 (0.108)	0.149*
ISZ4	6.00 (1.44)	0.642 (0.063)	0.652 (0.042)	0.021
ISZ5	4.25 (0.75)	0.331 (0.094)	0.378 (0.117)	0.138*
ISZ6	6.00 (0.91)	0.597 (0.072)	0.634 (0.069)	0.105**
ISZ7	2.75 (0.25)	0.292 (0.109)	0.308 (0.122)	0.074
Isizuchi mean	4.64	0.461	0.524	0.123
KMR	4.75 (1.03)	0.560 (0.080)	0.615 (0.061)	0.107
KNM	3.25 (1.44)	0.273 (0.177)	0.265 (0.163)	-0.020
mean	3.73	0.360	0.427	0.174

Levels of significance for $F_{IS} > 0$ were determined after 1000 permutations: * $P < 0.05$; ** $P < 0.005$.

Figure 2 Correlation of genetic differentiation with geographic distance between populations of *Magnolia sieboldii*. Population differentiation was measured as pairwise F_{ST} (a) and R_{ST} (b).

obovata, that includes the same four loci, will provide important evidence for evaluating the level of genetic diversity in *M. sieboldii*. Isagi *et al* (2000) detected an average of 24 alleles and a mean observed heterozygosity of $H_o = 0.90$ for the same four loci in 83 adult trees within a plot. The level of allelic diversity in *M. obovata* not only far exceeded the level of population allelic richness in *M. sieboldii*, but also was higher than the total number of alleles found in this study, covering the nearly entire distribution of *M. sieboldii*.

The difference in total genetic variation between *M. sieboldii* and *M. obovata* could be attributed to biological factors and molecular properties. For the former, *M. sieboldii* might have suffered from historical bottlenecks at the species' level because of its restricted distribution. *M. sieboldii* populations are restricted to narrow ranges of habitat at the high elevation and are narrowly distributed along the ridges and rivers in backbones of western Japan. *M. sieboldii* is considered to be a relict (Ueda, 1980), and might have been bottlenecked severely through climate fluctuations.

For the latter, mutation pressure or ascertainment bias might lead to the lower levels of microsatellite variability in *M. sieboldii*. We had examined amplification of 11 microsatellite loci characterized for *M. obovata* in advance, and eight had produced polymorphic bands for *M. sieboldii*. They had been assayed for the OMN1 population, and the loci other than the four in this study had shown similar levels of polymorphism, with the number of alleles ranging from two to five. Four loci assayed in this study were neither the ones of lower variability in *M. sieboldii* nor the ones of higher variability in *M. obovata* in Isagi *et al* (2000). Thus, consistently lower variability

Figure 3 Relationships between population pairwise genetic differentiation and averaged genetic diversity in *Magnolia sieboldii*. Solid circles, open circles, and crosses indicate population pairs within the Ohmine region, within the Ishizuchi region, and pairs of populations belonging to different regions, respectively.

detected in *M. sieboldii* could not be attributed to ascertainment bias. It would be due to structural or other properties of microsatellite that differ between the two species. This agrees with the general trends in plants that amplification and polymorphism decrease with increasing evolutionary distance from the species from which microsatellite loci were isolated (eg, Dayanandan *et al*, 1997; Butcher *et al*, 2000). Allele size ranges in *M. sieboldii* were narrower at all of the four loci, and shifted to smaller size ranges at the loci M6D3 and M6D4, than those of *M. obovata*; PCR product size ranged from 270 bp to 304 bp at M10D8, from 116 bp to 138 bp at M6D3, from 292 bp to 314 bp at M17D5 and from 144 bp to 158 bp at M6D4 for *M. sieboldii*, while it ranged from 257 bp to 313 bp at M10D8, from 106 bp to 198 bp at M6D3, from 289 bp to 317 bp at M17D5 and from 146 bp to 235 bp at M6D4 for *M. obovata*. Smaller allele size in *M. sieboldii* suggests that fewer repeats at microsatellite motifs might contribute to lower mutation rate at microsatellite loci in *M. sieboldii*. Lower mutation rate could be also caused by interruption of repeat motifs in some of the loci. It is necessary for the microsatellite loci to be sequenced for *M. sieboldii*.

It is difficult to evaluate the relative importance of the biological and molecular factors in determining the overall level of genetic variation in *M. sieboldii* in Japan from the present data. Potential microsatellite variability in *M. sieboldii* spp. *japonica* may be estimated by surveying the same loci for *M. sieboldii* spp. *sieboldii*, which is a common

species in the Korean Peninsula and thus more unlikely to have suffered from severe historical bottlenecks. Assuming that these subspecies are similar in the molecular properties at these microsatellite loci, and if the lower genetic variability in spp. *japonica* in this study resulted from historical bottlenecks with no or little mutation pressure at these loci, overall level of genetic variation at the same loci in spp. *sieboldii*, probably less bottlenecked historically, will be much more abundant than that in spp. *japonica* and nearer to the level in *M. obovata*.

At the intrapopulation level, genetic variation of *M. sieboldii* was low (Table 2). Although the problem of comparing other studies using different microsatellite loci remains as stated above, studies using microsatellites usually detect higher levels of intrapopulation genetic variation. For example, Streiff *et al* (1998) detected averages of 19.8 and 18.5 alleles per locus in two *Quercus* species, whilst Dow and Ashley (1996) detected an average of 18.25 alleles per locus in a third *Quercus* species. Population genetic variation in *Swietenia humilis* ($A = 7.05$, $H_o = 0.482$; White *et al*, 1999) and the orchid, *Gymnadenia conopsea* ($A = 6.97$ and $H_o = 0.667$; Gustafsson, 2000), were slightly higher than the results in this study. *Argyroxiphium sandwicense*, a narrow endemic and severely bottlenecked species, had lower intrapopulation genetic variation than the result of this study ($A = 2.75$, $H_o = 0.157$; Friar *et al*, 2000).

The inbreeding coefficient (F_{IS}) was positive in most populations (Table 2). Genetic substructuring within a

Figure 4 Neighbour-joining dendrograms relating 19 *Magnolia sieboldii* populations based on: (a) Nei's D and (b) Goldstein's $(\delta\mu)^2$. Population identification codes were according to Table 1. Bootstrap values (percentages) were computed over 35 replications by resampling loci.

population might lead to positive F_{IS} (Wahlund effect), however, this probability can be excluded because the autocorrelation analysis of the OMN1 population revealed no evidence of spatial clustering of alleles. Thus the positive F_{IS} in most populations suggested that excessive inbreeding was common in *M. sieboldii*. Although knowledge on reproductive traits of *M. sieboldii* was absent, it is reported that *Magnolia* flowers are generally protogynous (Thien, 1974) and that related *Magnolia* species are self-compatible (Ishida and Nagasaka, 1994; Ishida, 1996). Considering this, inbreeding is thought to be possible in *M. sieboldii* through geitonogamy, but not through autogamy. It was not clear whether inbreeding led to positive F_{IS} , since the extent of inbreeding depression is not known for *M. sieboldii*. Estimates of inbreeding depression was high in related species, *M. obovata* (Ishida and Nakamura, 1997), although genetic load might be purged in *M. sieboldii* because of its small population size. Positive F_{IS} are probably due to an excess of geitonogamy over allogamy or accumulated inbreeding because of small population size.

Mean pairwise F_{ST} and R_{ST} within regions (0.31 and 0.26), with a maximum distance of 14 km between populations, indicated that the level of local population differentiation could be regarded as high. Although microsatellite data on a comparable spatial scale for plants are limited, much lower differentiation is observed in *S. humilis* ($R_{ST} = 0.032$; White *et al.*, 1999) and *G. conopsea* ($F_{ST} = 0.06$, $R_{ST} = 0.02$; Gustafsson, 2000).

The mean values of pairwise differentiation among regions ($F_{ST} = 0.52$, $R_{ST} = 0.57$) was similar to, though slightly higher than, the total genetic differentiation ($F_{ST} = 0.49$, $R_{ST} = 0.54$). Similar levels of microsatellite differentiation were obtained across the entire worldwide range of some plant species, eg *Zostera marina* ($R_{ST} = 0.384$; Reusch *et al.*, 2000) and *Avicennia marina* ($F_{ST} = 0.410$, $R_{ST} = 0.710$; Maguire *et al.*, 2000). Thus the values of genetic differentiation detected in this study were considered to be notably high as the spatial scale among regions (up to 737 km), suggesting the existence of severe genetic barriers among regions. For animals, a maximum R_{ST} of 0.568 was reported for natterjack toads among isolated regions on the spatial scale comparable to this study (Rowe *et al.*, 1998).

Thus genetic structure in *M. sieboldii* was characterised by low genetic variation within populations, high genetic subdivision among regional populations and remarkable genetic differentiation among regions. Since populations of *M. sieboldii* were typically small and isolated, the low levels of population genetic variation and the high genetic differentiation among regional populations were probably due to stochastic loss of genetic variation by small population size, such as genetic drift and to some extent inbreeding, and limited gene flow (Ellstrand and Elam, 1993).

Some evidence suggests that the effect of genetic drift and limited gene flow were important. First, significant negative correlation between pairwise genetic differentiation and genetic variation averaged for the population pairs (Figure 3) indicated that populations suffering from the loss of genetic variation differentiated from other populations. It suggested that differentiating and lowering effects on population genetic variation, such as genetic drift, were very influential in determining the genetic structure.

Second, pairwise analysis on genetic and spatial distance revealed significant IBD models (Figure 2). The IBD models were still significant when only the population pairs within each region were analysed (F_{ST} : $r = 0.619$, $P < 0.0001$; R_{ST} : $r = 0.475$, $P = 0.013$; Figure 5). The significant IBD models at the regional scale suggested that genetic differentiation were counterbalanced by distance-dependant gene flow and populations had reached near gene flow-drift equilibrium (Slatkin, 1993).

Figure 5 Relationships between pairwise genetic differentiation and spatial distance in *Magnolia sieboldii*. IBD models were tested separately, within regions (<14 km) and among regions. To test IBD models among regions, one population in each region, with the most variable loci and most unlikely to be suffered from genetic drift (OMN1 in the Ohmine, ISZ6 in the Ishizuchi and HDK2 in the Hodaka) was selected to represent regional genetic variation and the rests were removed from analysis. Open and shaded circles indicates the data points for pairwise F_{ST} and R_{ST} , respectively. Regression lines are expressed as dotted for F_{ST} and solid for R_{ST} .

Third, those trends in genetic differentiation, the negative correlation between pairwise genetic distance and averaged genetic variation, and the IBD at the regional scales, were both clearer for F_{ST} than for R_{ST} (Figures 3 and 5). They could be attributed to the mutation model they assumed. Because the SMM, on which R_{ST} was based, assumes contiguous allelic states at mutation-drift equilibrium (Slatkin, 1995), discontinuity in the distribution of allele length generated by random genetic drift might perturb R_{ST} values when genetic drift were more important than mutation (Gaggiotti *et al.*, 1999; Balloux *et al.*, 2000). Clearer IBD models for F_{ST} based on the IAM may suggest relative importance of random genetic drift rather than mutation in generating this differentiation among regional populations. These evidences above suggested genetic drift and limited gene flow were important in determining genetic structure of *M. sieboldii*, at least on the regional spatial scales.

Microsatellite variation in *M. sieboldii* showed a remarkable hierarchical genetic structure; significant genetic differentiation among regional populations, further differentiation among regions and significant IBD models, although intrapopulation genetic structure was absent. This hierarchical structure would contribute to the regional genetic groups in neighbour-joining dendrograms, both using an IAM and an SMM. However, genetic relationships between regions were different among the mutation models (Figure 4). The neighbour-joining tree based on an SMM agreed better with the geographical origins of the populations than that based on an IAM. Since Goldstein's $(\partial\mu)^2$ is based on the lengths of microsatellites alleles, similarity in allele sizes across nearby regions might cause the neighbour-joining tree based on $(\partial\mu)^2$ to match their geographical relationships among regions.

This leads to an idea that a SMM was more appropriate to differentiate populations among regions in *M. sieboldii* than an IAM. We further tested IBD models among regions, by selecting one population in each region into analysis. In each of the three regions where more than one population were sampled (ie, the Ishizuchi, Ohmine and Hodaka regions), a population with the highest genetic diversity and thus most unlikely to have suffered from genetic drift (ie, the OMN1, the ISZ6 and the HDK2, respectively), was selected to represent regional genetic variation. The rest were removed from analysis, in order to reduce the effects of random genetic divergence among the regional populations on the IBD patterns. Pairwise R_{ST} values were highly correlated with geographic distance ($r = 0.697$, $P = 0.0028$), while pairwise F_{ST} values were not significantly correlated ($r = 0.403$, $P = 0.0829$) (Figure 5). The SMM might be more suited to explain the genetic structure among regions for *M. sieboldii*. R_{ST} is a sensitive measure to estimate genetic differentiation among populations if microsatellite variation follows the SMM, but may be confused when the contiguous allele distribution is disturbed by random genetic drift. It is considered that mutation in microsatellite loci was more important in determining the genetic differentiation among regions for *M. sieboldii* than random genetic drift and gene flow.

Population genetic variation was relatively low in the Ohmine mountain range, especially compared with that in the Ishizuchi mountain range (Table 2). Even the largest known OMN1 population had smaller genetic vari-

ation than that of most Ishizuchi populations. Total allele richness and allele length variation were higher in the Ishizuchi mountain range than in the Ohmine range (Appendix). Moreover, lowered population genetic variation in the Ohmine populations was shown by the correlation between pairwise genetic differentiation and averaged genetic variation (Figure 3). These differences in the level of genetic variation among regions might be caused by some biological factors. For example, populations in a region may have experienced recent demographic changes. Gene flow might be more limited in the Ohmine region, by a difference in geographical isolation among regional populations, within-species variation in biological interactions such as pollination and seed dispersal, or other factors. Another possibility is that historical bottlenecks or founding events may have limited the allele richness in the Ohmine mountain range. In addition, the lower level of population genetic variation in the Ohmine might be due to mutation pressure at some microsatellite loci. However, the real factors that influenced the difference in the level of genetic variation among regions were unclear in this study.

Conclusions

Implications for conservation

The results reported here showed that microsatellite variation in small and isolated populations of *M. sieboldii* was relatively small. But microsatellite variation in *M. sieboldii* was thought to be still more informative than allozymes, for example in assessing the effect of genetic drift, because it was much higher than the mean level of allozyme genetic variation in long-lived plants (Loveless and Hamrick, 1984). The results also detected local genetic differentiation and geographical structures among regions, in spite of severe random genetic drift.

The data in this study suggested limited interpopulation gene flow, even among close populations; recolonization by a population is probably difficult once it disappears. It also suggested the existence of genetic barriers among regions. Regional populations with genetic remoteness may contain evolutionary distinctiveness, though neither morphological nor ecological variation in *M. sieboldii* has been reported. Although *M. sieboldii* is not included on the Ministry of the Environment's Red List, it is rare, and recently, even the largest Hakuyougatake (OMN1) populations have been severely damaged by herbivores. Both the conservation of existing populations and the conservation of the genetic distinctiveness of regions should be made priorities.

Especially, the importance of Ishizuchi populations, where higher genetic variation was found in this study, should be noticed for the conservation of genetic resource. In addition, understanding the factors lowering the level of genetic variation in the Ohmine region will be important in managing populations of *M. sieboldii*. For instance, if differences in the level of genetic diversity among regions reflected differences in current interpopulation gene flow among regional populations, then conservation of the process that maintained the higher levels of gene flow among the Ishizuchi populations, will be necessary to maintain populations' chances of survival or recolonization. The factors that might change the level of genetic variation of a population and a region remain to be solved.

These results showed the potential of hypervariable microsatellite loci to evaluate the effects of genetic drift and population isolation within regions, and to detect genetic distinctiveness, in spite of the loss of overall genetic variation in *M. sieboldii*.

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Appendix

Allele frequencies at the 19 populations in *M. sieboldii*. The names of alleles were expressed as the PCR product size. Population identifications were according to Table 1.

locus	allele	AKS	HDK1	HDK2	OMN1	OMN2	OMN3	OMN4	OMN5	OMN6	OMN7	ISZ1	ISZ2	ISZ3	ISZ4	ISZ5	ISZ6	ISZ7	KNM	KMR	
M10D8	270	-	-	-	-	-	-	-	-	-	-	0.381	-	-	0.010	-	0.049	-	-	-	
	272	-	-	-	-	-	-	-	-	-	-	0.045	-	-	-	-	-	-	-	-	
	274	-	-	-	-	-	-	-	-	-	0.119	0.136	-	-	0.029	-	-	-	-	-	
	276	-	-	-	-	-	-	-	-	-	0.119	0.119	-	-	-	0.613	0.069	0.021	-	-	
	278	-	-	-	-	-	-	-	-	-	0.143	-	-	0.283	0.431	0.048	0.078	0.333	-	-	
	280	-	0.457	0.034	-	-	-	-	-	-	0.119	-	-	0.400	0.167	0.048	0.529	0.646	1.000	-	
	282	0.565	0.243	0.023	0.163	0.077	0.024	-	0.071	0.232	0.536	-	-	-	0.039	-	-	-	-	0.121	
	284	-	-	-	-	-	-	-	-	-	-	-	-	-	0.010	-	-	-	-	-	
	286	0.016	0.300	-	-	-	-	-	-	0.625	0.107	0.071	0.045	-	-	-	-	-	-	-	
	288	0.387	-	-	0.192	-	0.049	0.027	0.049	0.071	0.286	-	0.455	0.067	-	0.258	0.059	-	-	0.017	
	290	0.032	-	-	0.644	0.923	0.915	0.973	0.951	0.071	-	-	-	0.200	-	0.016	0.108	-	-	0.276	
	292	-	-	-	-	-	0.012	-	-	-	-	-	-	0.050	-	-	-	-	-	0.293	
	294	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	296	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.017	
298	-	-	-	-	-	-	-	-	-	-	-	0.318	-	-	-	-	-	-	0.259		
300	-	-	-	-	-	-	-	-	-	-	-	-	-	0.029	0.032	0.039	-	-	-		
302	-	-	-	-	-	-	-	-	-	-	-	-	-	0.127	0.016	0.069	-	-	0.017		
304	-	-	-	-	-	-	-	-	-	-	-	-	-	0.078	-	-	-	-	-		
M6D3	116	-	-	-	-	-	-	-	-	-	0.286	-	-	-	-	-	-	-	-	-	
	118	0.016	-	-	0.019	-	0.134	0.071	0.012	-	-	-	-	-	-	-	-	-	-	-	
	120	0.710	0.014	-	0.462	0.538	0.744	0.920	0.988	-	-	-	-	-	-	-	-	-	-	-	
	122	0.242	0.871	0.033	-	0.154	-	-	-	0.375	0.143	-	-	-	0.029	-	0.186	-	-	-	
	124	0.016	0.114	0.944	0.269	-	0.049	0.009	-	0.625	0.571	0.300	0.364	0.367	0.441	0.871	0.392	-	0.111	0.190	
	126	-	-	0.022	-	-	-	-	-	-	-	0.050	-	0.500	0.343	0.048	0.196	-	0.111	0.190	
	128	0.016	-	-	0.250	0.308	0.073	-	-	-	-	-	-	0.050	0.157	-	0.069	0.021	0.056	0.483	
	130	-	-	-	-	-	-	-	-	-	-	0.150	0.591	0.017	0.010	-	-	0.938	0.028	0.328	
	132	-	-	-	-	-	-	-	-	-	-	0.500	0.045	0.067	0.010	-	0.127	0.042	0.009	-	
	134	-	-	-	-	-	-	-	-	-	-	-	-	-	0.010	0.081	0.029	-	0.009	-	
	136	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.029	-	0.352	-	
	138	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	M17D5	292	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.028	0.707
		294	-	0.012	-	-	-	-	-	-	-	-	0.238	-	0.483	0.147	0.935	0.735	0.333	-	-
296		-	0.037	-	-	-	-	-	-	-	0.024	0.024	0.045	0.200	0.402	0.032	0.049	0.104	-	0.121	
298		-	-	-	-	-	-	-	-	-	-	0.024	0.545	-	-	0.032	-	-	-	0.172	
300		-	-	0.012	-	-	-	-	-	-	0.357	0.357	0.364	0.033	-	-	0.059	-	0.213	-	
302		-	-	-	-	-	-	0.563	0.402	0.071	-	-	-	-	-	-	-	-	-	-	
304		-	-	-	0.125	0.846	0.427	0.438	0.598	0.929	-	-	-	-	-	-	-	-	0.750	-	
306		-	-	-	0.875	0.154	0.573	-	0.402	0.071	-	-	-	-	-	-	-	0.009	-	-	
308		-	0.829	0.171	-	-	-	-	0.598	0.929	-	-	-	-	-	-	-	-	-	-	
310		1.000	0.171	0.659	-	-	-	-	-	-	-	-	-	0.033	-	-	0.029	-	-	-	
312		-	-	0.061	-	-	-	-	-	-	0.286	-	-	-	0.059	-	-	0.563	-	-	
314		-	-	0.049	-	-	-	-	-	-	0.071	0.071	0.045	0.250	0.392	-	0.127	-	-	-	
M6D4		144	-	-	-	-	-	-	-	-	-	-	-	-	-	0.059	-	0.412	-	-	0.034
		146	0.016	-	0.044	0.404	0.231	0.183	0.152	0.171	-	-	0.119	0.045	-	-	0.097	0.010	-	1.000	-
	148	-	0.043	0.022	0.125	0.615	0.232	0.313	0.305	0.821	0.714	0.864	-	0.867	0.627	0.097	0.108	0.958	-	-	
	150	0.048	-	-	0.125	-	-	0.313	0.305	0.143	0.048	-	-	0.033	0.118	0.016	0.049	-	-	0.017	
	152	0.081	-	-	0.308	-	0.549	0.509	0.512	-	0.024	-	-	0.100	0.196	0.016	0.010	0.042	-	0.086	
	154	-	-	0.300	0.154	0.154	0.037	0.018	0.012	0.036	0.024	0.091	-	-	-	0.016	0.355	0.042	-	0.017	
	156	0.758	0.957	0.167	0.010	-	-	0.018	0.012	-	0.095	-	-	-	-	0.516	0.392	-	-	0.483	
	158	0.097	-	0.467	-	-	-	-	-	-	-	-	-	-	-	-	0.020	-	-	-	