ORIGINAL ARTICLE

Genetic variation and differentiation in populations of a threatened tree, *Magnolia stellata*: factors influencing the level of within-population genetic variation

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Genetic variation and differentiation in *Magnolia stellata* were studied in 20 populations distributed across most of the species' range using 10 microsatellite markers, and the factors influencing their levels of within-population genetic variation were examined. Generally, populations distributed intermittently from southern Gifu to central Aichi Prefecture showed substantially higher levels of genetic variation (exceptions included populations located at unusually high altitude sites or western and southern edges of the range) than more isolated populations on the Atsumi Peninsula of southern Aichi Prefecture and in northern Mie Prefecture. Significant isolation-by-distance patterns were detected in genetic differentiation among the studied populations, and a

neighbor-joining tree based on D_A distances among the populations reflected well the geographical positions of the populations. The level of within-population genetic variation was significantly influenced not only by the size of the populations (represented by the number of reproductive individuals) but also by their degree of isolation (represented by the number of populations within a radius of 0.5 km around them). Therefore, areas within radii of 0.5 km may encompass *M. stellata* metapopulations, in which gene flow may usually occur. We suggest that this area may be a suitable standard for constructing conservation units for the species.

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Introduction

Endangered species, whose populations are in many cases small and isolated, are susceptible to loss of within-population genetic variation by genetic drift, which results in random fluctuations of allele frequencies. Loss of genetic variation reduces the evolutionary potential of populations to adapt to changing environments and increases the likelihood of biparental inbreeding, which decreases the fitness of progenies (Ellstrand and Elam, 1993; Frankham, 2005; Leim *et al.*, 2006; Ouborg *et al.*, 2006). Consequently, affected populations tend to decline and can fall into an 'extinction vortex' (Gilpine and Soule, 1986). Thus, detailed information on within- and among-population genetic variation over species' ranges and the factors influencing their levels of within-population genetic variation are needed to conserve such populations of endangered species.

Two factors that are believed to strongly influence the level of genetic variation within populations are the effective population size and degree of population isolation (Cruzan, 2001; Silvertown and Charlesworth,

2001; Ouborg *et al.*, 2006). Thus, it is important to consider the effects of these variables in analyses of genetic variation, but choosing the best measures for them is not straightforward. Many authors have used the actual number of individuals within investigated populations as a measure of population size, assuming that it is correlated with their effective population size, and found stronger relationships between this variable and genetic variation (for example, Young *et al.*, 1999; Galeuchet *et al.*, 2005; Gao, 2005; Leim *et al.*, 2006). The degree of population isolation can be quantified by measuring positional relationships between a focal population and its surrounding populations. The simplest way of doing this is to measure the distance to the nearest population. This approach has been used by various authors (for example, Llorens *et al.*, 2004; Galeuchet *et al.*, 2005; Lu *et al.*, 2005), but few have found significant relationships between this parameter and genetic variation (Lu *et al.*, 2005), probably because it provides a too simplistic indication of isolation (Moilanen and Nieminen, 2002). The cited authors studied possible connectivity measures in spatial ecology and recommended use of a 'buffer measure' based on the number of populations within a given radius around the focal population rather than simply the distance to the nearest population.

The species studied here, *Magnolia stellata* (Sieb. et Zucc.) Maxim., is a threatened tree species endemic to the Tokai region of central Japan (Environment

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Agency of Japan, 2000). It occurs in swampy places such as small rivers and marshes (Ueda, 1988) and at 40–700 m (mainly 200–500 m) elevations (Japan Association for Shidekobushi Conservation, 1996), and forms hierarchical metapopulation structures with sets of adjacent local populations (Setsuko *et al.*, 2007). About 75% of these local populations are comprised of less than 100 individuals (Japan Association for Shidekobushi Conservation, 1996). Most populations are intermittently distributed from southern Gifu to central Aichi Prefectures, but a small number of populations are isolated on the Atsumi Peninsula of southern Aichi Prefecture and in northern Mie Prefecture (Figure 1; Japan Association for Shidekobushi Conservation 1996). Since habitat destruction and vegetation succession have decreased both the numbers of populations and numbers of individuals within each population, the species is now listed as 'vulnerable' in the Japanese Red Data Book (Environment Agency of Japan, 2000). However, only a few populations are currently protected, including

several designated as natural monuments by the national government, or authorities in Prefectures or cities.

The genetic variation and differentiation in *M. stellata* populations have been investigated in three previous studies. First, Kawahara and Yoshimaru (1995) investigated nine populations using 15 allozyme markers, and found that the level of within-population genetic variation was lower in the isolated populations, while there was a high level of genetic differentiation among all populations ($G_{ST}=0.254$). In the cited study, however, few populations were sampled from the central part of the species' range. Second, Nakashima and Sakai (2003) investigated 14 populations sampled solely from the Tono region (southeastern Gifu Prefecture) using 10 allozyme markers, but did not identify the factors influencing the level of within-population genetic variation. Third, Ueno *et al.* (2005) investigated 11 populations, including the nine examined by Kawahara and Yoshimaru (1995), using four microsatellite markers developed in *M. obovata*, and also failed to identify

Figure 1 Locations of the 20 sampled *Magnolia stellata* populations (black dots) and the species' range (grey areas). Populations are represented by the codes listed in Table 1.

factors influencing the level of within-population genetic variation, although they did detect some regional genetic structure. Contributory reasons for the failure of these three studies, and various studies of other species, to detect the factors influencing the level of within-population genetic variation were probably the regionally biased sampling of small numbers of populations and low resolution of the genetic markers used (allozymes and small numbers of microsatellites). Microsatellite markers, especially those developed in target species, generally have far larger numbers of alleles, including many rare alleles, than allozyme markers. Since the number of alleles is susceptible to random genetic drift, microsatellite markers are also suitable for detecting reductions in within-population genetic variation associated with random genetic drift. Thirty microsatellite markers were recently developed in *M. stellata* (Setsuko *et al.*, 2005). We hypothesized that use of these markers to investigate a large number of populations extensively sampled from the species' range would allow us to identify factors influencing their levels of within-population genetic variation, which could not be detected in the previous studies.

The objectives of this study were (i) to estimate within- and among-population genetic variation in 20 *M. stellata* populations distributed across most of the species' range using 10 microsatellite markers, nine of which were developed in *M. stellata* (Setsuko *et al.*, 2005) and one developed in the related species *M. obovata* (Isagi *et al.*, 1999); (ii) to identify the factors influencing the level of within-population genetic variation, focusing on the effects of variations in the number of reproductive individuals within each population and the degree of population isolation represented by the number of surrounding populations and (iii) to consider possible conservation strategies for this species based on the findings.

Materials and methods

Study species and population sampling

M. stellata is a deciduous broad-leaved tree that grows up to 10 m in height and 20 cm in diameter at breast height. It reproduces both by seeds and by clonal growth (sprouts and layering) (Gotoh *et al.*, 1998; Setsuko *et al.*, 2004). Flowers are bisexual, protogynous and pollinated by insects, and mixed mating occurs (Hirayama *et al.*, 2005). Fruits are aggregated, with at most two red seeds per follicle that may be dispersed by birds (Callaway, 1994).

The natural range of *M. stellata* was roughly divided into three regions: southern Gifu to central Aichi Prefectures (region A); the Atsumi Peninsula in southern Aichi Prefecture (region B) and northern Mie Prefecture (region C) (Figure 1). Twenty populations were randomly selected across its natural range as study populations (Figure 1 and Table 1). A population was defined as an aggregation of individuals along streams or in marshes. We counted the number of reproductive individuals in flowering seasons in each population and defined this parameter as the population size. For DNA analysis, leaf samples were collected from 27 to 77 reproductive individuals in each population during 2004–2006 and stored at -30°C until DNA extraction.

DNA extraction, PCR amplification and microsatellite analysis

Genomic DNA was extracted from the sampled leaves using the hexadecyltrimethylammonium bromide method (Murray and Thompson, 1980) with minor modifications. Genotypes were determined using 10 microsatellite markers: nine (*stm0148*, *stm0184*, *stm0191*, *stm0222*, *stm0223*, *stm0251*, *stm0334*, *stm0353* and *stm0423*) developed for *M. stellata* (Setsuko *et al.*, 2005),

Table 1 Location, sample size and population size of the 20 investigated *Magnolia stellata* populations

Population name	Population code	Latitude (N)	Longitude (E)	Sample size	Population size
<i>Region A</i>					
Hirukawa	Hir	35°31'43"	137°24'41"	29	101
Hoshigami	Hos	35°29'30"	137°27'52"	30	257
Iji	Iji	35°29'39"	137°17'35"	30	56
Izumi	Izu	35°23'20"	137°10'23"	29	70
Kakishita	Kak	35°23'44"	137°05'39"	31	54
Kokeizan	Kok	35°20'50"	137°07'35"	30	339
Obora	Obo	35°27'13"	136°51'53"	27	42
Okute	Oku	35°25'37"	137°17'13"	67	69
Shizuno	Shi	35°31'00"	136°56'17"	32	61
Sue	Sue	35°26'23"	136°53'46"	30	156
Chikusui-ike	Chi	35°18'07"	137°03'09"	34	108
Kotohira	Kot	35°02'05"	137°11'05"	30	79
Togokusan	Tog	35°15'08"	137°03'13"	34	40
Yatogawa	Yat	35°11'25"	137°06'55"	77	77
<i>Region B</i>					
Kurogawa	Kur	34°38'44"	137°14'30"	29	29
Toshichibara	Tos	34°40'39"	137°14'46"	30	130
Ikawazu	Ika	34°37'11"	137°08'56"	27	30
Nagusa	Nag	34°37'12"	137°08'38"	29	53
<i>Region C</i>					
Asahidani	Asa	34°58'37"	136°31'39"	29	29
Tabika	Tab	35°04'41"	136°30'43"	31	51

Population sizes are the numbers of reproductive individuals in the populations.

and one (*M6D8*) developed for *M. obovata* (Isagi *et al.*, 1999). PCR fragments were separated using a 3100 Genetic Analyzer and GeneScan software (Applied Biosystems) (see Setsuko *et al.*, 2005, for details).

Data analysis

The following parameters were calculated at each locus and over all loci in each population using FSTAT ver. 2.9.3.2 (Goudet, 2001): allelic richness (AR), calculated with a fixed sample size of 27 (54 gene copies) (El Mousadik and Petit, 1996), gene diversity (H_E ; Nei, 1987) and inbreeding coefficient ($F_{IS} = 1 - H_O/H_E$). Departures from Hardy–Weinberg equilibrium (HWE) at each locus and linkage disequilibrium between loci were tested in each population by an exact test using the Markov chain method implemented in GENEPOP ver. 3.4 (Raymond and Rousset, 1995), with sequential Bonferroni corrections (Rice, 1989). Assuming that the microsatellite loci used in this study were randomly sampled from an infinite number of loci, bootstrap resamplings among loci (20 000 samples) were used to create 95% bootstrap confidence intervals for AR, H_E and F_{IS} for each population. Deviations of F_{IS} from zero over all loci in each population were tested using bootstrap confidence intervals of various percentages generated by the same bootstrap resampling method, with sequential Bonferroni corrections (Rice, 1989).

The total number of alleles detected (A), allelic richness (AR), observed heterozygosity (H_O), average gene diversity within populations (H_S), gene diversity in the total populations (H_T), inbreeding coefficient (F_{IS}) and the coefficient of genetic differentiation among populations defined under the infinite allele model (F_{ST} ; Weir and Cockerham, 1984) and the stepwise mutation model (R_{ST} ; Slatkin, 1995; Rousset, 1996) were calculated across all populations at each locus and over all loci using FSTAT. Overall tests for departures from HWE across all over populations at each locus and over all loci were performed by Fisher's procedure (Fisher, 1970). The significance of population differentiation at each locus was tested by the log-likelihood (G)-based exact test (Goudet *et al.*, 1996), with sequential Bonferroni corrections (Rice, 1989), using the Markov chain method implemented in GENEPOP, and its significance over all loci was tested by Fisher's procedure (Fisher, 1970). To test the presence of isolation-by-distance patterns in population differentiation, the Mantel test between population-pairwise geographic distances and genetic distances (D_A distances; Nei *et al.*, 1983) was applied. A dendrogram was constructed to interpret the relationships among populations by the neighbor-joining method (Saitou and Nei, 1987) based on D_A distances between populations using DISPAN (Ota, 1993). The node significances of the dendrogram were evaluated using bootstrap probabilities based on 10 000 replicates.

Linear mixed-effect models were used to investigate the relationships between the level of genetic variation within each population and both its population size and degree of population isolation. We defined the size of each population as the number of reproductive individuals (s) within it, and its degree of population isolation as the number of populations within radii of 0.5–10.0 km with 0.5 km intervals (20 distance classes; $n_{0.5} - n_{10.0}$). We set the distance interval to 0.5 km since this was the

minimum distance that significantly exceeded the measurement errors regarding the positions of populations. The number of surrounding populations was obtained from detailed distributional information of *M. stellata* populations published by the Japan Association for Shidekobushi Conservation (1996) together with our field observations. The population size and degree of population isolation were treated as fixed effects and the differences between loci as random effects. Two different response variables were used in the models: allelic richness (AR) and gene diversity (H_E). Data for H_E were arcsine-transformed to obtain closer approximations to normality. Forty-two candidate models with 1–3 fixed explanatory variables were constructed for each response variable. All models had fixed and random intercepts (the latter arose from the differences between loci). We estimated parameters in each model by the maximum likelihood method using R 2.3.1 (R Development Core Team, 2006).

To evaluate the candidate models, we calculated Akaike's information criterion (AIC; Akaike, 1973) values for each of them using the equation, $-2 \times (\log\text{-likelihood}) + 2 \times (\text{the number of estimated parameters})$. Models with low AIC values have higher likelihoods and contain smaller number of variables than models with higher values. A substantial advantage of using AIC, for the purposes of this study, is that it is valid for comparing non-nested models (Burnham and Anderson, 2002; Johnson and Omland, 2004). The differences between the AIC values obtained for each of the models and the minimum AIC value (ΔAIC) were calculated, and each model with a ΔAIC value ≤ 2 was considered to be one of the most parsimonious, and thus selected as one of the best models (Burnham and Anderson, 2002). We also calculated Akaike weights, from $\exp[-0.5 \times (\Delta\text{AIC of the focal model})] / \sum \exp[-0.5 \times (\Delta\text{AIC})]$, to assess the relative support for each model (Burnham and Anderson, 2002). Akaike weights range from zero to one and are normalized so that they sum to one over all candidate models. Akaike weights are convenient for comparing the relative likelihoods of models in a set of candidate models.

Results

Within-population genetic variation

Two hundred tests for deviations from HWE at each locus in each population were applied. Significant deviations were detected by 16 of these tests. There were no significant deviations at any locus across more than 3 populations, and 6 of the 16 significant deviations detected were at 6 loci in the Asahidani population. In addition, 900 tests for linkage disequilibrium between pairs of loci in each population were applied, 20 of which detected significant deviation from equilibrium, but the deviation was not significant for any pairs of loci across more than 3 populations. The loci used in this study exhibited high polymorphism (Table 2). The total number of alleles detected (A) and average gene diversity within populations (H_S) at each locus across all populations were, on average, 22.1 and 0.719, respectively. F_{IS} values across all populations were positive at nine loci and over all loci, and departures from HWE were significant at the same nine loci and

Table 2 Within- and among-population genetic variation in the 20 investigated *Magnolia stellata* populations

Locus	A	AR	H_O	H_S	H_T	F_{IS}	Tests for HWE ^a	F_{ST}	R_{ST}	Tests for differentiation ^b
stm0148	26	8.61	0.690	0.747	0.883	0.076	***	0.166	0.207	***
stm0184	18	6.21	0.629	0.648	0.827	0.029	*	0.224	0.251	***
stm0191	19	8.46	0.725	0.780	0.894	0.071	**	0.130	0.241	***
stm0222	25	7.57	0.653	0.739	0.882	0.116	***	0.159	0.184	***
stm0223	14	5.16	0.557	0.554	0.810	−0.005		0.291	0.316	***
stm0251	17	7.06	0.663	0.733	0.874	0.095	***	0.167	0.365	***
stm0334	27	9.27	0.711	0.789	0.927	0.099	**	0.151	0.209	***
stm0353	21	7.69	0.696	0.751	0.897	0.073	*	0.163	0.300	***
stm0423	31	8.13	0.689	0.749	0.937	0.080	**	0.201	0.292	***
M6D8	23	6.35	0.649	0.698	0.887	0.070	**	0.211	0.377	***
Mean	22.1	7.45	0.666	0.719	0.882	0.074	***	0.185	0.274	***

Abbreviations: A, the total number of alleles detected; AR, allelic richness; H_O , observed heterozygosity; H_S , average gene diversity within populations; HWE, Hardy–Weinberg equilibrium; H_T , gene diversity in the total populations; F_{IS} , inbreeding coefficient; F_{ST} and R_{ST} , the coefficients of genetic differentiation among populations defined under the infinite allele model and the stepwise mutation model, respectively.

Sequential Bonferroni correction (Rice, 1989) was used to determine significance in the multiple tests.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

^aDepartures from HWE at each locus and over all loci were tested by Fisher's procedure (Fisher, 1970), which combined probabilities from exact tests for departures from HWE in each population.

^bThe significance of population differentiation at each locus was tested by the log-likelihood (G)-based exact test (Goudet et al., 1996), and that over all loci was tested by Fisher's procedure (Fisher, 1970).

over all loci. The allelic richness (AR) and gene diversity (H_E) of the populations ranged from 2.99 to 11.64 and from 0.517 to 0.863, with mean values of 7.45 and 0.719, respectively (Figures 2a and b). Levels of within-population genetic variation were generally higher in populations in region A (although they were substantially lower in three populations in this region: Iji, Obora and Kotohira) than in populations in regions B and C, except for the Tabika population. Unexpectedly, the Tabika population showed the highest genetic variation ($H_E = 0.863$ and $AR = 11.64$) of all the populations, even though it was highly isolated from other populations (Figure 1) and its population size was modest (Table 1). F_{IS} values of the populations ranged from −0.126 to 0.297, and averaged 0.073 over all the populations (Figure 2c). Significant positive deviations of F_{IS} values from zero were detected in three populations (Chikusui-ike, Kotohira and Asahidani).

Among-population genetic variation

All the loci used in this study showed significant population differentiation ($P < 0.001$) and the values of F_{ST} and R_{ST} over all loci were 0.185 and 0.274, respectively (Table 2). There was also a significant correlation between pairwise-population geographic distances and D_A distances ($R^2 = 0.579$, $P < 0.001$, Mantel test; Figure 3). The neighbor-joining tree based on D_A distances between populations seems to reflect the populations' geographical distribution (Figures 1 and 4). Four nodes of this tree were supported with high bootstrap probabilities (more than 88%). These nodes correspond to geographical locations at the edge of the eastern Gifu Prefecture (98%), the root of the Atsumi Peninsula (90%) and two within the peninsula (88 and 100%).

Factors influencing the level of within-population genetic variation

All regression coefficients of fixed explanatory variables (c , s , $n_{0.5}$ – $n_{10.0}$) against the two response variables

Figure 2 Allelic richness (a), gene diversity (b) and inbreeding coefficient (c) in the 20 investigated *Magnolia stellata* populations. Dots and bars show values calculated using the original data set and their 95% bootstrap confidence intervals were estimated by 20 000 bootstrap resamplings among loci, respectively. Deviations of F_{IS} from zero at each population were tested using bootstrap confidence intervals of various percentages generated by the same bootstrap resampling method, with sequential Bonferroni corrections (Rice, 1989). ** $P < 0.01$; *** $P < 0.001$. Populations are represented by the codes listed in Table 1.

(AR and H_E) estimated by the maximum likelihood method were positive. The models, providing the best explanations for AR and H_E , had the same parameters (s and $n0.5$) with $\Delta AIC \leq 2$ (Table 3). Akaike weights of these models were 0.611 and 0.237 for AR and H_E , respectively. The models had two fixed explanatory

Figure 3 D_A distances between the 20 investigated populations of *Magnolia stellata* versus geographical distances between them.

Figure 4 Neighbor-joining tree constructed using D_A distances between the 20 investigated *Magnolia stellata* populations. The numbers shown are bootstrap probabilities exceeding 50% based on 10 000 replicates. The length of the bar is equal to the genetic distance of 0.1. Populations are represented by the codes listed in Table 1.

variables: the population size (regression coefficients $\pm 95\%$ confidence intervals: 0.0106 ± 0.0047 for AR; 0.0004 ± 0.0003 for H_E) and the number of surrounding populations within a radius of 0.5 km around the focal population (0.3657 ± 0.2080 for AR; 0.0230 ± 0.0143 for H_E) (Table 4).

Discussion

Within- and among-population genetic variation

The level of within-population genetic variation tended to be higher for populations in region A than for populations in regions B and C, in accordance with the results of previous studies (Kawahara and Yoshimaru, 1995; Ueno *et al.*, 2005). This trend may be partly due to region A being central to the species' range, while regions B and C are marginal, and partly due to the populations in regions B and C being more isolated from one another than those in region A. A finding that was not observed in the previous studies was that some populations in region A (Iji, Obora and Kotohira)

Table 3 The best models (in bold), and four next best models, explaining the levels of within-population genetic variation of the 20 investigated *Magnolia stellata* populations, selected according to Akaike's information criterion (AIC)

Model rank	AR			Arcsine (H_E)		
	Model	ΔAIC	Akaike weight	Model	ΔAIC	Akaike weight
1	$c+s+n0.5$	0.000	0.611	$c+s+n0.5$	0.000	0.237
2	$c+s+n1.5$	4.824	0.055	$c+s+n1.5$	2.980	0.053
3	$c+s+n1.0$	5.329	0.043	$c+s+n2.0$	3.071	0.051
4	$c+s+n2.0$	5.500	0.039	$c+s+n3.0$	3.186	0.048
5	$c+s+n2.5$	6.559	0.023	$c+s+n3.5$	3.382	0.044

Abbreviations: AR, allelic richness; H_E , gene diversity; ΔAIC , the difference in AIC between the model considered and the most parsimonious one; Akaike weight, the relative likelihood of the model; c , intercept; s , population size; $n0.5$, $n1.0$, $n1.5$, $n2.0$, $n2.5$, $n3.0$ and $n3.5$, the number of surrounding populations within a radius of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 km around the focal population, respectively. All regression coefficients of fixed explanatory variables were positive.

Table 4 Fixed-effect explanatory variables of the linear mixed-effect models (random effect: locus) that best explained the level of within-population genetic variation of the 20 investigated *Magnolia stellata* populations, selected according to Akaike's information criterion (AIC)

Response variables	Explanatory variables	Regression coefficients	s.e.	df	t	P
AR	c	5.8251	0.4865	188	11.974	0.000
	s	0.0106	0.0024	188	4.398	0.000
	$n0.5$	0.3657	0.1061	188	3.448	0.001
Arcsine (H_E)	c	0.9385	0.0332	188	28.275	0.000
	s	0.0004	0.0002	188	2.601	0.010
	$n0.5$	0.0230	0.0073	188	3.159	0.002

Abbreviations: AR, allelic richness; c , intercept; df, degrees of freedom; H_E , gene diversity; $n0.5$, the number of surrounding populations within a radius of 0.5 km around the focal population; s , population size.

showed substantially lower variation than the others in this region, even lower in some respects than those in regions B and C (Figure 2). One of these populations is located at an unusually high altitude (the Iji population, located 700 m above sea level, while most populations are located 200–500 m above sea level) and the other two (Obora and Kotohira) at the edges of the species' range. Thus, these populations are in isolated environments, like those in regions B and C, and this may account in large part for their low levels of variation. We discuss the influence of isolation on the level of within-population genetic variation in the following section.

Interestingly, although the Tabika population was highly isolated and did not have a large population size, it showed the highest within-population genetic variation. High genetic variation in this population has also been revealed by nuclear microsatellite analyses in a previous study (in which it was called the Komono population; Ueno *et al.*, 2005). Ueno *et al.* (2005) also examined cpDNA variation in 11 populations, and found that the level of variation was unusually high in the Tabika population. The unusually high genetic variation in the population could not be explained by contemporary gene flow due to its isolated distribution and its modest current size. Therefore, the reasons for it may be related to historical dynamics of the population and surrounding populations in region C.

Significant positive values of F_{IS} estimated across all populations at nine loci (as indicated by their significant departures from HWE), and those over all loci in three populations, indicate an excess of homozygotes compared to expectations under HWE, main causes of which include inbreeding, population substructure and the presence of null alleles. *M. stellata* produces protogynous, hermaphrodite flowers that prevent autogamous selfing, but its asynchronous flowering within individuals leads to geitonogamous selfing (Hirayama *et al.*, 2005). Selfing rates of 26.1–38.8% at the mature seed stage (Hirayama *et al.*, 2007) and 5.7% at the germinated seedling stage (Setsuko *et al.*, 2007) have been reported. In addition, the Asahidani population showed significant deviations from HWE at six of the loci used in this study and the highest value of F_{IS} among the studied populations. These features were probably not due to locus characteristics such as the presence of null alleles, but to population characteristics such as inbreeding and population substructure. However, genotype frequencies at the locus *stm0353* significantly deviated from HWE in three adjacent populations (Sue, Shizuno and Chikusui-ike), suggesting the presence of null alleles at the locus that are regionally distributed over the three populations.

Population differentiation among the 20 populations was significant ($F_{ST}=0.185$ and $R_{ST}=0.274$), and a significant isolation-by-distance pattern was also detected. The value of F_{ST} obtained in this study is very similar to that obtained by Ueno *et al.* (2005) using nuclear microsatellites (Φ_{SC} , an analog to $F_{ST}=0.18$), but lower than that found by Kawahara and Yoshimaru (1995) using allozymes (G_{ST} , an analog to $F_{ST}=0.254$). Values of F_{ST} and G_{ST} cannot be simply compared between loci if the loci to be compared exhibit different levels of within-population genetic variation (Hedrick, 2005), as in the material examined here, since there were considerably higher values of H_S at microsatellite loci

(0.719, this study; 0.58, Ueno *et al.*, 2005) than at allozyme loci (0.092, Kawahara and Yoshimaru, 1995). Hedrick (2005) addressed this problem, and proposed a standardized measure (G'_{ST}), which allows more appropriate comparisons between loci that have different levels of genetic variation. Using the microsatellite data in this study and the previous allozyme data (Kawahara and Yoshimaru, 1995), G'_{ST} values were estimated to be 0.683 and 0.283, respectively. Genetic differentiation for microsatellite loci was found to be more than twice that for allozyme loci.

The separation of populations in region B (the Atsumi Peninsula) from those in the other regions was supported by a bootstrap probability of 90%, indicating that the differentiation of populations in region B from the populations in the other regions is due to their geographical isolation. Furthermore, there was significant support, with a bootstrap probability of 100%, for the separation of populations (Kurogawa and Toshichibara) at the root of the Atsumi Peninsula from even the populations (Ikawazu and Nagusa) at the tip of the same peninsula, indicating that there was little gene flow between populations at the tip and root of the peninsula, although they were located close to each other (about 5 km apart).

Factors influencing the level of within-population genetic variation

This study demonstrated significant relationships of genetic variation with population size and the degree of population isolation in *M. stellata* populations using linear mixed models. The model with population size and degree of population isolation (the number of surrounding populations in a radius of 0.5 km around the focal population) as fixed explanatory variables was selected as the best for explaining the level of within-population genetic variation in both response variables, AR and H_E . The regression coefficients of the best models are all positive, implying that populations with large sizes and high numbers of surrounding populations within a radius of 0.5 km should have high levels of genetic variation. The best models predict that a decrease in population size, s (which ranged from 29 to 339 individuals among the studied populations), of 100 individuals corresponds to reductions in AR and H_E of 1.060 and 0.039, respectively, and a decrease in the number of populations within a radius of 0.5 km, $n_{0.5}$ (which ranges from 0 to 7 among the populations), of five populations corresponds to reductions in AR and H_E of 1.829 and 0.113, respectively.

Many studies have reported significant relationships between population size and genetic variation (for example, Young *et al.*, 1999; Galeuchet *et al.*, 2005; Gao, 2005; Leim *et al.*, 2006). However, a previous study on *M. stellata* published by Nakashima and Sakai (2003) did not detect such a relationship, possibly because the measure of population size they used was not the number of reproductive individuals but the number of individuals within populations. In contrast to the multitude of reported relationships between population size and genetic variation, few studies have detected significant relationships between the degree of population isolation and genetic variation (Cruzan, 2001; Lu *et al.*, 2005). Since it is more difficult to measure the

degree of population isolation than population size, good measures for quantifying the degree of population isolation have not yet been established. However, we detected significant relationships between the degree of population isolation and genetic variation, using the number of surrounding populations as the measure of isolation, whereas the distance from the nearest population was used in many previous studies (for example, Llorens *et al.*, 2004; Galeuchet *et al.*, 2005; Lu *et al.*, 2005). Thus, the detection of a significant relationship in this study, and the failure of many of the cited studies to detect such relationship, supports the assertion by Moilanen and Nieminen (2002) that the number of surrounding populations is a much better indicator of isolation than the distance from the nearest population. In addition, Cruzan (2001) reported significant relationships between the degree of population isolation and genetic variation, using the number of surrounding populations. Important components affecting the degree of population isolation are likely to be the number and distribution of population in proximity to the focal population. However, the most important of these components appears to be the number of populations in the proximity of the focal population, especially for species that form hierarchical metapopulation structure, such as *M. stellata*.

The area encompassed by a radius of 0.5 km around each population, selected in the best model in this study, may cover the range of metapopulations in *M. stellata* because the number of populations within the area had significant effects on within-population genetic variation. This is consistent with observations published by Setsuko *et al.* (2007), who found the maximum distance that pollen was directly transferred across local populations within a metapopulation of the species to be ca. 420 m, although long-distance gene flow may occur over 500 m, at least through seed dispersal by birds. However, gene flow between populations within radii of 0.5 km in metapopulations may principally influence the level of genetic variation within populations in the species.

Conservation implications

The populations distributed in region A have been shown to contain higher genetic variation than the populations distributed in regions B and C, but even in region A populations located at an unusually high altitude (Iji) or at the western and southern edges of the range (Obora and Kotohira) showed similar levels of genetic variation to those in the populations distributed in regions B and C. These populations with low genetic variation (especially the Obora population) have small population sizes and high degrees of population isolation, and may be highly prone to further reductions in genetic variation through random genetic drift (Frankham *et al.*, 2002; Primack, 2002; Hartl and Clark, 2006). Therefore, further reductions in the numbers of individuals in these populations should be avoided to prevent their extinction.

The neighbor-joining tree indicates significant differentiation between populations at the edge of the eastern Gifu Prefecture and the other regions, the Atsumi Peninsula and the other regions, and between the tip and root in the peninsula. If trees are to be transplanted from other populations to these populations to counter

population decline in the future, use of trees from populations outside the regions should be avoided. In addition, even within the Atsumi peninsula, use of trees from neighboring populations would be most appropriate for transplanting.

In attempts to conserve rare species, only specific, large populations are often designated as conservation targets, although the surrounding populations may be important as sources of pollens and seeds with different genetic variations (Setsuko *et al.*, 2007). We have found that not only the number of reproductive individuals within the focal population but also the number of surrounding populations within a radius of 0.5 km around it influences its level of within-population genetic variation. These findings support the notion that the metapopulation structure of *M. stellata* should be retained in attempts to conserve the species, and suggest that an appropriate conservation strategy could include the designation of areas encompassed by circles with a radius of 0.5 km as conservation units. Certain *M. stellata* populations have been designated as natural monuments for conservation purposes, which we support, but such measures only protect specific local populations that may not be sustainable in isolation. Thus, to ensure the long-term conservation of populations of *M. stellata*, the importance of surrounding populations around them must be considered.

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