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Patterns of Allozyme Variation in Relation to Population Size of the Threatened Plant *Megaleranthis saniculifolia* (Ranunculaceae) in Korea

Chin-Sung Chang[1], Do Yol Choi[1], Hui Kim[2]*, Tae Yoon Park[3], and Yong-Sik Kim[4]

[1]Department of Forest Sciences and the Arboretum, Seoul National University, Seoul 151-921, Korea
[2]Department of Medicinal Plants Resources, Mokpo National University, Muan-gun 534-729, Korea
[3]Graduate School of Education, Yonsei University, Seoul 120-749, Korea
[4]Faculty of Natural Resources, Yeungnam University, Gyongsan 712-749, Korea

Patterns of variation at 27 allozyme loci were investigated in the endangered endemic plant *Megaleranthis saniculifolia*. Levels of allozyme variation (A = 1.47, P = 40%, He = 0.088) were also compared with other endemic plant species. Genetic divergence between populations was very high (G_{ST} = 0.271), with moderate to high interpopulation differentiation, which probably arose through historical bottlenecks in a landscape of habitat fragmentation and/or human influence. The percentage of polymorphic loci, heterozygosity, and mean number of alleles per locus were positively related to population size, probably due to the stochastic loss of rare alleles in the smaller populations. Individuals in the small and marginal populations (TB, KD, and CJ) showed higher proportions of fixed loci. These ecologically marginal populations were typically more distant from the nearest neighboring population and were more genetically distinct from one another. The genetic structure of the current population of *M. saniculifolia* is probably the result of local extinctions of intervening populations. This, in turn, is due to the Pleistocene climatic change and increased habitat destruction. A positive association appears to exist between genetic diversity and population size. Although these small population sizes are more sensitive to stochastic events, securing a certain number of individuals from the three larger populations (SB, JB, and TG) could be accomplished as part of a conservation strategy. In addition, it is important to prioritize populations in different regions in order to limit population declines caused by large-scale environmental catastrophes.

Keywords: allozyme, conservation, endangered species, endemic plant, genetic structure, *Megaleranthis saniculifolia*

The major causes of plant endangerment are habitat loss, degradation, and subsequent fragmentation of once-continuous habitats (Holsinger and Gottlieb, 1991; Falk, 1992; Neel and Ellstrand, 2001). In particular, the genetic consequences of recent habitat loss are generally related to the effects of reduced population size and increased geographical isolation among populations (Coates and Hamley, 1989; Barrett and Kohn, 1991; Young et al., 1996). This geographical or ecological isolation can cause peripheral populations to diverge genetically from the major populations, as a result of genetic drift and/or natural selection (Lesica and Allendorf, 1995). Such populations are often more remote and smaller than the central populations. Many studies (Lynch, 1996; Frankham et al., 2002; Lu et al., 2005) have also demonstrated that the former type tends to accumulate a deleterious mutational load and lose quantitative genetic variation via drift, possibly threatening that population's persistence. A better understanding of the interactions between population size and genetic variation would benefit conservation biologists seeking to preserve genetic diversity in rare species (Fahrig, 2003).

Megaleranthis saniculifolia Ohwi (Ranunculaceae) is an annual species found at mesic sites in the deciduous forest. It is considered endangered (ER) according to the IUCN Red List categorization (Chang et al., 2001). Although it is treated as an endemic species (a monotypic genus) in Korea, its taxonomic status has been a matter of dispute, often being classified instead as a species in the genus *Trollius* (Lee, 1990).

According to Takhtajan (1986), this species is a glacial plant relic of the northeastern Chinese flora (=Manchurian Province flora). Naturally fragmented and disjunct population systems are a feature of such Chinese flora in Korea. A significant proportion of this species is likely a relic that probably had wider, more

*Corresponding author; fax +82-61-450-6443
e-mail huikim@mokpo.ac.kr

continuous distributions during the Pleistocene Age. Climatic instability following glaciation was a major factor in the localized extinction and fragmentation of many plant species in this region (Chang et al., 2003). The reduction or absence of gene flow due to the level of geographical separation among populations, plus fluctuations in population size, probably led to significant interpopulation divergence.

M. saniculifolia, found in only seven regions (eight populations) of South Korea, has a significantly isolated distribution. Its current population structure may have been caused by recent fragmentation of a more continuous system of populations, but, more likely, has resulted from the historical fragmentation associated with Pleistocene change. The lack of information about *M. saniculifolia* has prompted concern about its status and future management in Korea. Therefore, knowledge of its breeding system, reproductive strategies, and patterns and levels of genetic variation are required to establish its current status.

The effective population size, N_e, is one of the most important parameters in conservation research. In particular, the genetic consequences of a small population depend on its effective size, rather than on the absolute number of individuals. Real populations deviate in structure from the assumptions of an idealized population in terms of unequal sex ratios, high variation in family sizes, and fluctuating numbers in successive generations (Hedrick, 1985; Frankham et al., 2002; Lu et al., 2005). Because endangered species have small or declining populations, they are expected to have lower genetic diversity than non-endangered species with larger population sizes (Frankham et al., 2002).

The aims of our study were to 1) estimate the genetic variation and structure of all present populations of *M. saniculifolia* using allozyme electrophoresis, 2) evaluate the species' population history, 3) assess genetic diversity and levels of inbreeding based on population size, and 4) discuss the genetic effects of fragmentation on populations. The resulting data would then help provide information in guiding conservation decision-making for management of this taxon in Korea.

MATERIALS AND METHODS

Collection Sites and Species Biology

M. saniculifolia is primarily restricted to the major mountain chain (>800 m elevation) in eastern Korea

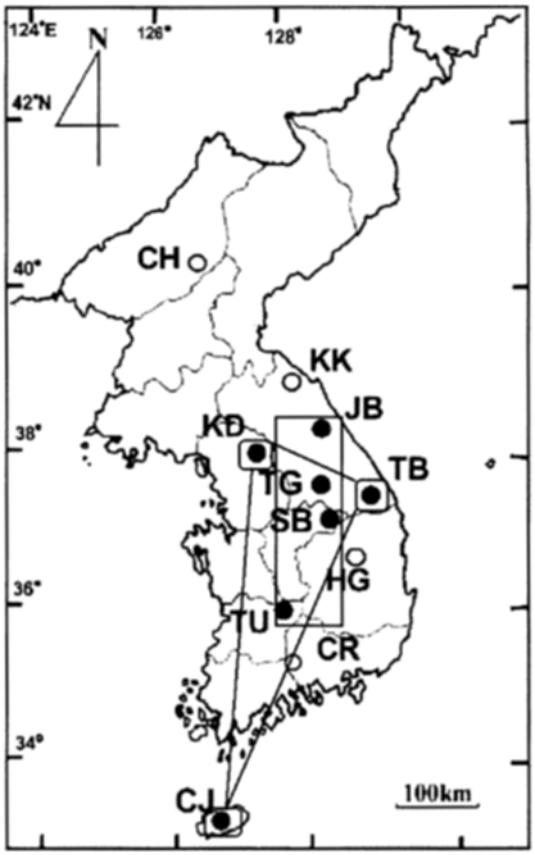

Figure 1. Distribution of collection sites (●) and historic populations (○) in Korea. Population acronyms are shown in Table 2.

(Fig. 1) (Kim and Lee, 1987; Im, 1996; Yoo et al., 1999). Populations that occur in seven regions (except SB-1 and SB-2) are ca. 130 km apart (Fig. 1), and range in size from 100 to 4500 individuals each.

The dissected leaves of *M. saniculifolia* emerge in the spring and become dry by summer's end, at which time the stems remain dormant above ground. Most flowering occurs from mid-April to mid-May. This species, a low-growing perennial herb, reproduces sexually. Successful pollination results in an achene fruit containing many seeds (Lee, 1980). When mature, the fruit splits and seeds drop to the ground, exhibiting no obvious long-distance dispersal mechanism. Although the mating system was not specifically examined here, some general inferences can be made from the related genus *Trollius* (Pellmyr, 1989, 1992), i.e., that it is an obligate and insect-mediated outcrosser. A variety of flies and bees (*Bombylius major* and species of *Anthomyiid* and *Tachinid*), dominated by the families Bombyliidae, Anthomyiidae, and Tachinidae, are its primary pollinators (Choi, 2002).

Table 1. Genetic variability at 30 loci in six populations of *M. saniculifolia*. *P*, percentage polymorphic loci; *N*, sample size; *A*, number of alleles per locus; *Ae*, effective number of alleles per locus; *Ho*, observed genetic diversity; *He*, expected genetic diversity; *Np*, estimated population size.

Population	*N*	*A*	*Ae*	*P*	*Ho* (SE)	*He* (SE)	*Np*
JB	50	1.27	1.11	26.7	0.085 (0.041)	0.069 (0.027)	ca. 1,300
TG	50	1.37	1.36	30.0	0.061 (0.032)	0.075 (0.024)	ca. 1,300
SB-1	50	1.23	1.09	23.3	0.065 (0.032)	0.054 (0.023)	ca. 4,500
SB-2	50	1.23	1.12	23.3	0.055 (0.022)	0.075 (0.027)	ca. 2,500
TU	50	1.17	1.06	16.7	0.024 (0.019)	0.039 (0.021)	ca. 600
TB	50	1.20	1.06	20.0	0.042 (0.025)	0.056 (0.025)	ca. 1,000
KD	50	1.10	1.07	10.0	0.024 (0.016)	0.036 (0.023)	ca. 400
CJ	20	1.0	1.0	0	0	0	ca. 100
Mean	50	1.25	1.12	23.33	0.045	0.057	1,463
Species level		1.47	1.35	40.0	0.068	0.088	

In the wild, *M. saniculifolia* usually has two dormancy stages (summer and winter). Under natural conditions, the rate of seed germination increases by about 68% after two low-temperature periods, i.e., 15°C in the summer and 4°C in the winter (Lee et al., 2003). Two types of pre-chilling are required to break seed dormancy and enhance germination. Therefore, it is apparent that temperature is a major and limiting factor in determining the success of seed-established plants.

Sampling

Our sampling was undertaken on a variety of scales to ensure the representation of inter- and intra-population genetic diversity. In the spring of 1999, 2000, and 2002, leaves were collected from eight populations found in the species' natural range of South Korea: Mt. Jeombong-san (JB), Mt. Taegi-san (TG), Mt. Sobaek-san (SB-1 and SB-2), Mt. Dukyou-san (TU), Mt. Taebek-san (TB), Mt. Gwangdeok-san (KD), and Mt. Halla-san on the island Jeju-do (CJ) Population codes and the estimated number of individuals from all populations are provided in Figure 1 and Table 1.

The populations found at JB, TG, and SB were quite large, comprising thousands of individuals. We generally attempted to collect 50 per site (at JB, TG, and SB, which are 3 m apart), from these large populations. In contrast, the existing populations at KD, TB, TU, and CJ were very small, such that the number of plants sampled from those sites was limited to less than 50 individuals each.

The magnitude of genetic differentiation within a population was characterized for the SB-1 site in 1999. Because our research strategy was intended to sample a smaller portion of the population on a finer scale, two 1 × 1 m square plots were subdivided into a 10 × 10 cm lattice.

Allozyme Electrophoresis

Leaves were transported on ice to the laboratory, where they were refrigerated at 4°C. Enzymes were extracted by grinding approximately 3 cm^2 of leaves in extracting buffer (Wendel and Weeden, 1989) with ceramic mortars and pestles. The homogenized extracts were absorbed into filter paper (Whatman 3MM, 4 × 10 mm) and kept frozen at -70°C. Samples were then electrophoresed for 4 to 6 h on four gel (12% starch) and buffer systems (Conkle et al., 1982; Wendel and Weeden, 1989). The following enzyme systems were assayed in all individuals: acid phosphatase (ACP), alcohol dehydrogenase (ADH), aldolase (ALD), catalase (CAT), glutamate oxaloacetate transaminase (GOT), glucose-6-phosphate dehydrogenase (G6PD), glyeraldehyde-3-phosphate dehydroge nase (G3PD), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), leucine aminopeptidase (LAP), menadione reductase (MNR), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGD), and shikimate dehydrogenase (SKD). Enzymes were visualized using the staining methods detailed by Wendel and Weeden (1989). Most staining recipes were modifications of widely used assays presented earlier (Conkle et al., 1982; Soltis et al., 1983). Loci were considered putative because no previous genetic analyses had been performed on this species. The patterns observed, however, were typical of the same enzyme systems studied in other species, and were consistent with the

expected banding patterns of those species for which formal analyses had been carried out previously (Gottlieb, 1981; Wendel and Weeden, 1989; Kephart, 1990). The number of loci scored for each enzyme system included: ACP (3), ADH (2), ALD (1), CAT (1), G3DP (1), G6DP (1), GOT (2), IDH (2), LAP (1), MDH (2), MNR (2), PGI (2), PGM (2), 6PGD (3), and SKDH (2). Allele frequencies for each locus in each population and the genetic structure of the entire set of populations were analyzed using BIOSYS-1 (Swofford, 1981).

To help verify genotypes for the within-population genetic structure, we used data from the allelic constitution of five polymorphic loci -- 6PG-2, ACP-2, ACP-3, SKDH-2, and MNR-1 – that were obtained from individuals out of seven populations known to be heterozygous.

Statistical Analyses

We calculated several genetic parameters, including the number of alleles per locus (A), the percent of polymorphic loci (P), the observed (H_o) and expected (H_e) heterozygosity, and a fixation index (F). The effective number of alleles (A_{es}) was determined for each locus and averaged over all loci (Hamrick and Godt, 1990). A goodness-of-fit test of genotypic frequencies to the Hardy-Weinberg equilibrium was then performed on each variable locus. Population structure was analyzed using Wright's (1965) F-statistics, where F_{IT} represented the overall inbreeding coefficient; F_{IS}, the levels of inbreeding due to non-random mating within populations; and F_{ST}, due to population subdivision. To test the effects of fragmentation (Tomimatsu and Ohara, 2003), we applied least-square linear regressions to explore the relationships between population genetics parameters and the log of population size, using the REG procedure of SAS (SAS Institute, 1997).

We estimated interpopulation gene flow using two different methods, first by the formula for G_{ST}: $N_m = (1\text{-}G_{ST})/4\ G_{ST}$ (Wright, 1951). A dendrogram based on genetic similarity was created using the UPGMA (unweighted pair group method). Genetic divergence among populations was estimated by calculating Nei's genetic identity (I) for all pairs of populations (Nei, 1978). Nei's genetic identity measure ranges from '1' for population with identical allele frequencies to '0' for populations with no alleles in common to unity for populations with identical allele frequencies. Analyses of correlation (Spearman rank correlation coefficients, r_s; Siegel, 1959) were used to examine the relationships between population size and genetic diversity.

RESULTS

Genetic Diversity

Ten (P_s =37.0%) out of 27 loci were polymorphic in at least one population of *M. saniculifolia*, and 16.2% (P_p) were polymorphic, on average, at the population level. Only three loci were scored for the single population: GOT-1 for TC, G6P-1 for KD, and PGM-2 for the TB populations, whereas no polymorphic locus was found in CJ. Six loci (6PG-2, ACP-2, ACP-3, MDH-2, SKD-2, and MNR-1) had relatively uniform allele frequencies across all populations, but three (6PG-2, ACP-3, and SKD-2) showed significant allele frequency differences between the large populations (JB, TG, SB-1, and SB-2) and small populations (TU, TB, KD, and CJ) (Table 2). Populations TU, TB, and KD, with the fewest number of estimated plants, had only four or five polymorphic loci, whereas the larger populations (JB, TG, and SB) had five or six polymorphic loci. At the species level, the mean number of alleles per polymorphic loci was 2.11, and the total genetic diversity (H_{es}) was 0.088. Including the 18 monomorphic loci, the average number of alleles per locus (A_s) was 1.30 at the species level and 1.17 at the population level (A_p).

Values for P_p ranged from 0% (CJ) to 25.9% (TG). The mean number of alleles per polymorphic locus within populations was 1.77, and varied little among populations. Genetic diversity within populations (H_{ep}) also varied little, ranging from 0 (CJ) to 0.071 (TG). Average expected heterozygosity was 0.045, similar to values found in the local populations of other endemic plant species (Table 1; Hamrick and Godt, 1989). Significant deviations from Hardy-Weinberg expectations were found for 19 out of 34 fixation tests (Table 3; Wright, 1965). This demonstrated both heterozygote excesses and deficiencies for this species (Table 4).

The TG population with the highest allelic diversity, having two unique or "private" alleles, possessed 75% of the total allelic diversity represented in our sample: (36-27) × 100/(39-27). If we combined the three poorest and most peripheral populations (KD, TB, and CJ), we could encompass only 42% of the allelic diversity. These three populations had low values for all genetic diversity measures (Table 1).

Table 2. Allele frequency data for polymorphic loci from seven populations of *M. saniculifolia*. JB, Mt. Jeombong-san; TG, Mt. Taegi-san; SB, Mt. Sobaek-san; TU, Mt. Dukyou-san; TB, Mt. Taebek-san; KD, Mt. Gwangdeok-san; CJ, Mt. Halla-san on island of Jeju-do.

Locus	Allele	Population							
		JB	TG	SB-1	SB-2	TU	TB	KD	CJ
GOT-1	a	0.000	0.110	0.000	0.000	0.000	0.000	0.000	0.000
	b	1.000	0.890	1.000	1.000	1.000	1.000	1.000	1.000
6PG-2	a	0.574	0.000	0.280	0.234	0.041	0.000	0.000	0.000
	b	0.426	1.000	0.720	0.766	0.959	1.000	1.000	1.000
G6P-1	a	0.000	0.060	0.000	0.000	0.000	0.000	0.000	0.000
	b	1.000	0.920	1.000	1.000	1.000	1.000	1.000	1.000
	c	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000
ACP-2	a	0.100	0.190	0.140	0.277	0.150	0.240	0.592	0.000
	b	0.900	0.810	0.860	0.723	0.850	0.760	0.408	1.000
ACP-3	a	0.960	0.860	0.970	0.900	1.000	1.000	1.000	1.000
	b	0.040	0.140	0.030	0.100	0.000	0.000	0.000	0.000
PGM-2	a	1.000	1.000	1.000	1.000	1.000	0.940	1.000	1.000
	b	0.000	0.000	0.000	0.000	0.000	0.060	0.000	0.000
MDH-2	a	0.224	0.150	0.000	0.000	0.000	0.040	0.040	0.000
	b	0.776	0.850	1.000	1.000	1.000	0.960	0.960	1.000
SKD-2	a	0.000	0.080	0.000	0.000	0.000	0.000	0.000	0.000
	b	0.100	0.860	0.870	0.806	0.194	0.920	1.000	1.000
	c	0.900	0.070	0.130	0.194	0.806	0.080	0.000	0.000
CAT-1	a	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000
	b	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000
MNR-1	a	0.510	0.440	0.610	0.620	0.592	0.360	1.000	0.000
	b	0.490	0.560	0.390	0.380	0.408	0.640	0.000	1.000

Table 3. Fixation indices (*F*) for seven loci that were polymorphic at the population level.

Locus	JB	TG	SB-1	SB-2	TU	TB	KD	CJ
GOT-1	----[a]	0.898**	----	----	----	----	----	----
6PG-2	-0.654**	----	-0.389**	-0.306*	-0.043	----	----	----
G6P-1	----	1.000**	----	----	----	----	----	----
ACP-2	-0.111	0.155	-0.163	0.362**	0.922**	0.013	0.071	----
ACP-3	-0.042	0.169	0.656**	0.111	----	----	----	----
PGM-2	----	----	----	----	----	1.000**	----	----
MDH-2	-0.055	-0.176	----	----	----	1.000**	-0.042	----
SKD-2	0.333*	0.521**	0.381**	0.021	0.804**	0.185	----	----
CAT-1	----	----	----	----	----	----	----	----
MNR-1	-0.801**	-0.786**	-0.639**	0.321*	-0.183	-0.476**	----	----

Chi-square tests were used to determine if fixation indices were different from expected values ($F=0$). [a]Populations that were monomorphic for a particular locus are indicated with a dash; *<0.05; **<0.01.

Distribution of Genetic Variability among Populations

A UPGMA dendrogram illustrating the genetic relationships among populations (Fig. 2) reflected a straightforward spatial configuration. Mean genetic identity (*I*) among pairs of populations, which was usually low (mean = 0.958; sd = 0.027), suggested that many alleles contributed significantly to the among-population genetic variation. More than 45% of the genetic variation in our sample could be attributed to variation among populations (F_{ST} =0.451). The average F_{ST} among seven populations, excluding the CJ population (all monomorphic loci), was slightly

Table 4. Estimates of genetic diversity parameters for eight polymorphic loci surveyed for seven populations.

Locus	H_T	H_S	F_{IS}	F_{IT}	F_{ST}	G_{ST}	N_m
GOT-1	0.027	0.024	0.898	0.908	0.098	0.097	2.327
6PG-2	0.242	0.166	-0.443	-0.011	0.315	0.314	0.546
G6P-1	0.019	0.018	1.000	1.000	0.057	0.060	3.916
ACP-2	0.333	0.279	0.183	0.315	0.162	0.162	1.293
ACP-3	0.074	0.069	0.172	0.228	0.067	0.067	3.481
PGM-2	0.014	0.014	1.000	1.000	0.053	0.052	4.557
MDH-2	0.107	0.094	0.012	0.128	0.117	0.117	1.886
SKD-2	0.409	0.178	0.393	0.121	0.563	0.563	0.194
CAT-1	0.468	0	----	1.000	1.000	1.000	0
MNR-1	0.499	0.360	-0.443	-0.034	0.278	0.278	0.649
Mean	0.219	0.120	-0.034	0.433	0.451	0.271	0.673

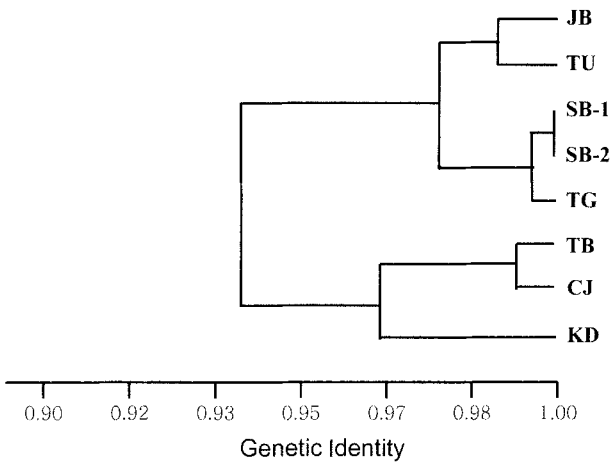

Figure 2. Phenogram showing genetic similarity among eight subpopulations, based on Nei's (1978) unbiased genetic identity.

lower, = 0.387, but not statistically significant (jackknifed interval was 0.262 < 0.451 < 0.639, at the 95% level). High G_{ST} values (0.271; Table 4) indicated significant genetic differentiation at the population level. As noted above, the genetic differentiation among populations was typically attributable to many aberrant alleles or genotypes, which were common or fixed within one population but uncommon or absent elsewhere.

We split the populations of *M. saniculifolia* into two groups, based their levels of genetic variation: 1) those characterized by high mean heterozygosity per locus (JB, TG, TU, and SB), and 2) those manifesting low values (TB, KD, and CJ). The inclusion of the TU population into the first group was surprising, because the population was much smaller at this site than at any other.

Regional Variation

We examined all patterns of genotypes within two square plots for the SB-1 population, where 16 and 18 genotypes were detected (Fig. 3, Table 5). The two 1 × 1 m plots contained 158 individuals and 27 genotypes, with seven genotypes shared between them (Fig. 4). The arrangements of major multilocus genotypes in the mapped plots were statistically tested by nearest neighbor analysis, which examined the distances between each point and the closest point to it, and then compared to expected values for a random sample of points from a complete spatial randomness (CSR) pattern (Fig. 3; Donnelly, 1978; Boots and Getis, 1988). Major genotypes in Plot A (genotypes K, H and I; Table 4) and Plot B (genotypes K and E) had negative Z-statistics (Donnelly, 1978), indicating that all genotypes were more clumped than found in complete spatial randomness. Genotype K in Plot A was significantly ($p<0.01$) clumped rather than showing CSR.

Relationships between Population Genetic Variation and Population Size

The percentage of polymorphic loci (*P*), the mean number of alleles per locus (*A*), and heterozygosity (*He* and *Ho*) all had significant relationships with population size (as a logarithm scale; Fig. 5), indicating that genetic variation was greatly reduced in the small populations. Three populations (CJ, KD, and TU) of small size (i.e., < 1000 flowering plants each) exhibited low polymorphism (P=0% for CJ, P=10.0% for KD, and P=16.7% for TU) as well as low allelic

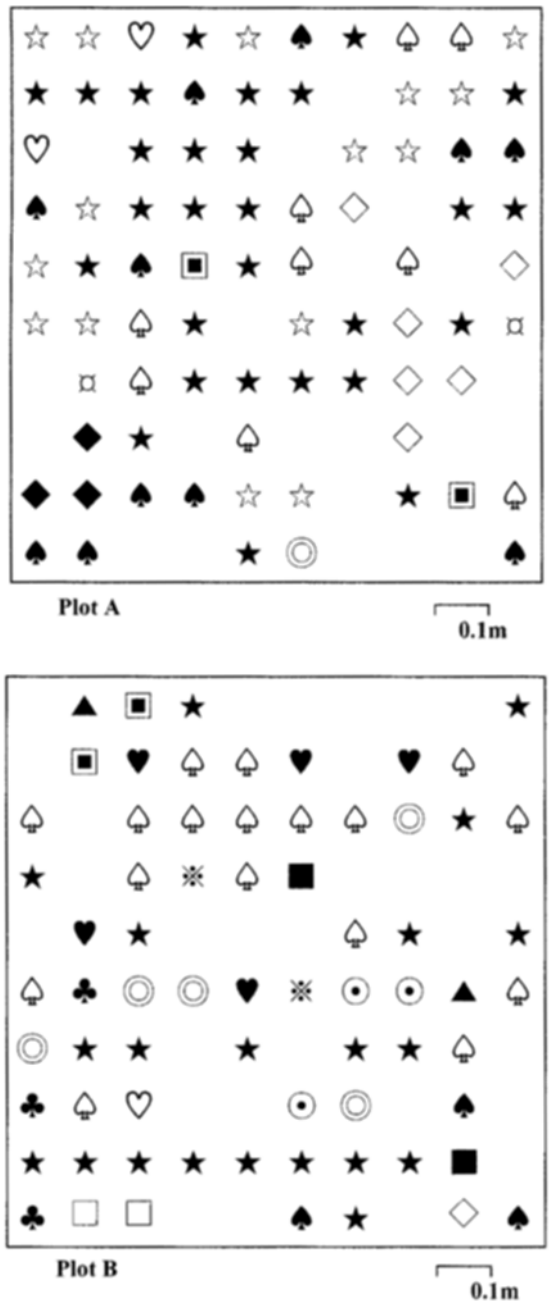

Figure 3. Spatial distribution of multilocus genotypes in two 1 x 1 m plots from the regional population, SB-1. Each symbol indicates identical individuals carrying same genotype, which was detected at five loci (Table 5). The same symbol does not necessarily represent a clone.

Table 5. Number of individuals with the same genotype at two plots from the SB-1 population.

Allozyme order: 6PG-2, ACP-2, ACP-3, SKDH-2, MNR-1	Symbols	Plot A	Plot B
AA-AA-AA-AB-AB	A(□)		2
AA-BB-AA-AA-AB	B(▣)	2	2
AA-BB-AA-AB-AB	C(♣)		3
AA-BB-AA-BB-AA	D(■)		2
AA-BB-AA-BB-AB	E(♤)	9	17
AA-BB-BB-BB-AB	F(◆)	3	
AB-AA-AA-BB-AB	G(♡)	2	1
AB-BB-AA-AA-AB	H(☆)	15	
AB-BB-AA-AB-AB	I(♠)	11	3
AB-BB-AA-BB-AA	J(◇)	6	1
AB-BB-AA-BB-AB	K(★)	28	21
BB-AA-AA-BB-AB	L(※)		2
BB-BB-AA-AA-AB	M(♥)		5
BB-BB-AA-AB-AB	N(▲)		2
BB-BB-AA-BB-AA	O(◉)		3
BB-BB-AA-BB-AB	P(◎)	1	5
BB-BB-BB-BB-AB	Q(¤)	2	
Unique genotypes at each plot		6	4
Total sample number		85	73
Total genotypes		16	18
Similarity index		0.412	

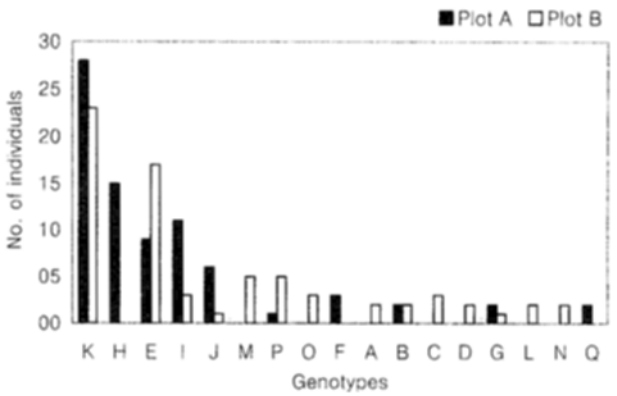

Figure 4. Frequency distribution of genotypes in two plots from SB-1 population. Note that genotypes K and E were common to both plots. Seven out of 17 genotypes (41.2%) as common genotypes were found in both plots.

richness (A=1.0 for CJ, A=1.10 for KD, and A=1.17 for TU). In contrast, the large populations at JB, TG, and SB had relatively more multilocus genotypes, more polymorphic loci, and higher levels of observed heterozygosity than the other populations (Table 1).

DISCUSSION

Overall Levels of Genetic Variation

Several of the life history traits in *M. saniculifolia* are only slightly associated with genetic diversity, includ-

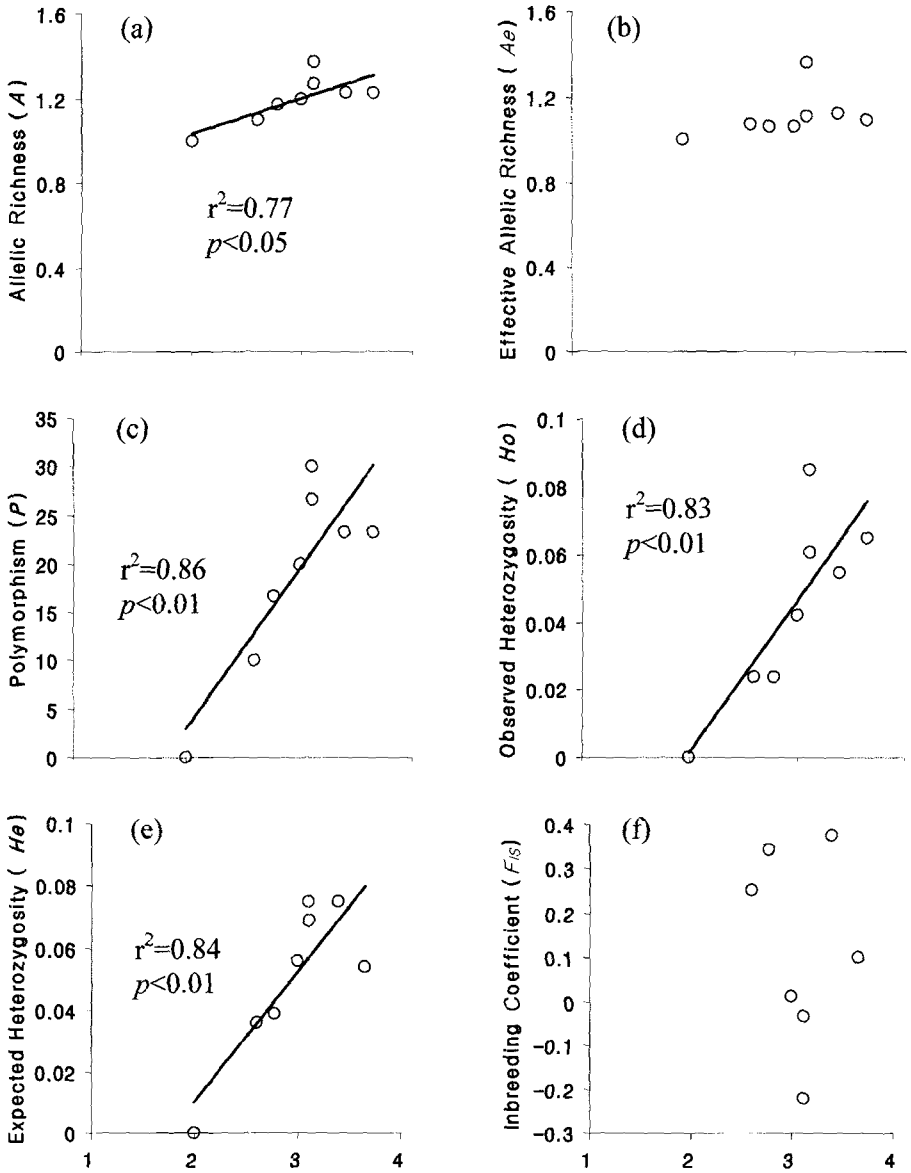

Figure 5. Relationships among log of population size (estimated number of flowering plants) and various population genetics parameters: (a) mean number of alleles per locus, (b) mean number of effective alleles per locus, (c) percent polymorphic loci, (d) observed heterozygosity, (e) expected heterozygosity, and (f) inbreeding coefficient. Least-square linear regressions are indicated in those cases where slope of regression line differs significantly from zero ($p<0.05$).

ing outcrossing, insect pollination, and gravity-dispersed seeds, as well as rarity and endemics (Hamrick et al., 1991). Our analysis revealed levels of variation that were lower than average for species grouped on the basis of each of these traits. Although most of the results obtained in recent analyses corroborate earlier findings (Hamrick and Godt, 1989; Linhart and Premoli, 1993), *M. saniculifolia* provides an apparent exception to this general rule. One possible explanation for this discrepancy was that our populations were small and lacked gene flow into populations. Another reason was that this species is in evolutionary equilibrium, such that its low levels of variation were reflected long after fragmentation, when the populations were extensively isolated.

Predictions concerning genetic diversity are often based on the present-day size and vigor, despite the fact that current status may not reflect a population's demographic history (Hamrick and Godt, 1996). From theoretical predictions and a number of empirical studies, the smaller populations might be expected to show reduced levels of polymorphism and allelic

richness (Frankham et al., 2002). For *M. saniculifolia*, comparisons between size and diversity showed that the decreased population was strongly associated with a reduction in the measure of genetic variation.

It is possible that the initial decline in population size was great enough to cause a detectable loss in genetic variation. In addition, the proportions of fixed loci for *M. saniculifolia* were somewhat higher in given sites with small population sizes than those that were larger. Small populations that are geographically distant are genetically more closely related to one another than to more adjacent populations. This may be due to the effects of drift, which is to be expected in small populations that occupy unstable, repeatedly disturbed montane habitats (Chang et al., 2003).

Our analysis of genetic differentiation showed that 45% of the variation was found among populations. That is, the genetic differentiation among populations was marked within *M. saniculifolia*. Heterogeneities of allele frequencies were significant for most loci. Gene flow also appeared to be very low between populations, based on indirect population genetic statistics (Nm=0.673). However, one mechanism of gene flow -- seed migration -- was very unlikely given the extensive space separating neighboring populations, even within the larger SB population. The limited potential for dispersal likely contributed to those low rates of gene flow between populations. Pollen dispersal by bees clearly was the most probable mechanism for gene flow within, but not between, populations. Based on the mating system of this species, the low level of genetic diversity may not be consistent with habitual inbreeding and/or population fluctuations.

Genetic composition (e.g., the presence of many heterozygotes) may argue that seeds produced by successful pollinations are mainly recruited into the population. Our within-population analysis showed that very few multilocus genotypes were shared between plots and populations, indicating that these populations were founded sexually. They may have been established by many individuals from diverse sources, resulting in populations with high levels of genotypic diversity. Perhaps the most important role of seeds, then, was to maintain the genetic diversity within populations.

At least ca. 75% of multilocus genotypes within two plots were unique genotypes (at least 56% of the genotypes within a plot were unique). However, 26% of the non-unique genotypes for two plots were shared by ca. 90 individuals (ca. 55% of the total number of individuals investigated; Fig. 3, 4). It is likely that *M. saniculifolia* primarily reproduced sexually through outcrossed mating, although a relatively large proportion of the individuals within samples shared multilocus genotypes. Sufficient time may have passed since fragmentation for genetic drift and increased isolation to be effective in reducing the genetic variation of this species.

Relationships between Population Genetic Variation and Population Size

Levels and patterns of diversity documented here also allowed us to make inferences about some aspects of the demography of this species. Its small percentage of polymorphic loci and number of alleles per locus indicated that *M. saniculifolia* has had a history of sufficiently severe or long-lasting population bottlenecks to cause a loss of genetic diversity. The major differentiation among populations suggested that either gene flow was limited among populations or fragmented populations have been isolated long enough for genetic drift (or selection) to have caused population differentiation (Ellstrand and Elam, 1993).

Fragmentation forces populations to face a stochastic loss of rare alleles first, because only a portion of the original genetic diversity remains after breakup (Barrett and Kohn, 1991). Our small, fragmented *M. saniculifolia* populations had lost rare alleles that might initially have been present at low frequencies (Fig. 5). In addition, a decrease in allelic richness in the small populations was attributable to genetic bottlenecks during fragmentation (Tomimatsu and Ohara, 2003).

Although we observed no effect of population size on inbreeding coefficients, those coefficients were relatively high in two small populations, CJ and KD (Fig. 5). Fragmentation, coupled with localized pollinator movements and seed dispersal, may have resulted in significant bi-parental inbreeding, thereby causing heterozygote deficiencies for the small populations, even though F_{IS} values for the large populations did not differ significantly from the Hardy-Weinberg expectation. Therefore, fragmentation of the original population provides the most probable explanation for the high inbreeding coefficient found in the small populations, rather than this species' inherent mating system.

When we considered the large difference in variation detected in populations of the mountain chain and marginal populations, the potential negative impacts of smaller size became apparent. In general, individuals in the small and marginal populations (TB,

KD, and CJ) showed higher proportions of fixed loci. These ecologically marginal populations were typically more distant from the nearest neighboring population and were more genetically distinct from one another. Links between population size and genetic variation suggest variation was lost in the small populations through inbreeding and drift. Based on the F_{IS} value and heterozygote excess, however, no evidence was found for the inevitable mating between relatives in small populations.

Although many plant species have declined rapidly during the past few hundred years because of human activity, many others owe their population disjunctions to climatic changes during the quaternary (Sage and Wolff, 1986). The long-term effects of Pleistocene climatic change and increased habitat fragmentation on the main mountain chain in South Korea suggest that the current population genetic structure of *M. saniculifolia* is the result of local extinction of intervening populations; range contraction was particularly evident in a number of species occurring in those areas (Chang et al., 2004). The small patches of forest in which *M. saniculifolia* exists are apparently remnants of a once continuous forest that began to break up after glaciation. The distribution of genetic variation was consistent with an ancient separation of forest patches, limiting the dispersal of plants specific to forest habitats. Their high levels of genetic divergence indicate that the two population groups, large and small, have been historically isolated for an extended period of time.

Serious loss of variation can accumulate with modest population restrictions. The major significance of a small size to genetic diversity is not usually single-generation bottlenecks, but the insidious loss of genetic diversity over many generations. If size is constant in each generation, we can obtain an expression for the effects of sustained population restrictions in heterozygosity $\{H_t/H_0=[1-1/(2N_e)]^t\}$ (Frankham et al., 2002). For example, it might require 3500 generations for the major large populations (e.g., JB, TG, and SB) to lose about 50% of their initial heterozygosity with respect to genetic diversity and become current small populations (KD and TB).

Although we have no clear idea as to when the CJ population migrated from the main population in the peninsula, Im (1992) has indicated that Jeju (Cheju) Island was connected to the peninsula during an ice age at least 32000 years ago. Based on the above calculations, it would have taken 10000 generations for the peripheral population to be the CJ population; 45000 generations, for the main population. The greater extent of differentiation calculated in *M. saniculifolia* is a reflection of the exact age of the demographic history of this species.

Fluctuations in population size and local extinctions over a relatively long period of time were all likely contributing factors. These patterns reflect the influences of historical and contemporary influences in shaping the genetic structure of this species. It is possible that many generations have already passed with the erosion of genetic variation in these sites. Therefore, the concomitant reduction in fitness and decreased genetic variation is just one of a suite of potential negative consequences of a small population size.

Conservation Implications

Regardless of the evolutionary interpretation, information about the genetic composition of a local population provides an important tool for the conservation of endangered species (Holsinger and Gottlieb, 1991).

Our genetic data supports the view that the large population group should be recognized only as a distinct unit for conservation. Individual populations containing as much as ca. 26% and as little as 0% polymorphic loci were recorded in our genetic sample. Likewise, a group characterized by high mean heterozygosity per locus was found in three large populations, while a group characterized by low values was recorded in another three. The probability of extinction in the small populations is high because they are close to trails and vulnerable to human disturbances. Patterns of genetic diversity and habitat quality suggest that many large populations are not considered threatened because they are well protected within the National Park. Protection of such a large population, with > 1000 individuals, would ensure the preservation of about 90% of its initial heterozygosity even after 500 generations. In contrast, the heterozygosity of a small population size would be dramatically decreased after only a few hundred generations.

Using the current genetic data, we can estimate the long-term effective population size for *M. saniculifolia* from the formula - $\{H_t/H_0=[1-1/(2N_e)]^t\}$ (Frankham et al., 2002). If we wish to retain 95% of the current heterozygosity over 100 generations, we must maintain at least 975 individuals as the N_e. These data demonstrate why population size at certain sites is important for the conservation strategy.

Conservation organizations must frequently choose

which populations to acquire and protect. The preservation of genetically diverse populations should be a priority. Although these small population sizes can become more sensitive to stochastic events, securing a certain number of individuals from three large populations (e.g., SB, JB, and TG) would be necessary with respect to conservation strategy. In addition, it is important to prioritize populations in different regions in order to limit population declines caused by large-scale environmental catastrophes.

The data presented here provide a valuable baseline for future comparisons of genetic diversity to evaluate the effectiveness of protected areas and ex *situ* conservation for long-term management. The correlations among population sizes, genetic diversity, and high genetic differentiation also highlight the need to conserve large population sizes with a certain number of individuals.

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