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A Preliminary Population Genetic Study of an Overlooked Endemic ash, *Fraxinus chiisanensis* in Korea Using Allozyme Variation

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Abstracts : We used enzyme electrophoresis to evaluate genetic diversity in five populations of endemic ash, *Fraxinus chiisanensis* in Korea. Of 15 putative allozyme loci examined 26.7% were polymorphic and expected heterozygosity for the species was low (0.082). Within the range, population were highly differentiated (F_{ST}=0.356) and little genetic variation was explained by geography. The pattern of distribution of variation showed low genetic variation within populations and pronounced divergence among populations, which was consistent with the prediction for the effects of limited gene flow and local genetic erosion. Although the frequencies of male plants were dominant ranging from 79.3% to 89.4%, most mating events seems to be inevitable mating between relatives in small populations based on heterozygote deficiency of this species. Small effective population size and the limited dispersal contributed to the low rates of gene flow within as well as between populations.

Key words **:** *androdioecy, endemic species, forest fragmentation,* Fraxinus chiisanensis, *genetic diversity, isolation*

Introduction

Fraxinus chiisanensis Nakai (Oleaceae) is an endangered and endemic tree species, found in deciduous forest areas of southwestern Korea (Figure 1). This species is categorized as endangered in the IUCN redlisting study in Korea (unpublished data) due to limited number of populations and restricted areas (Table 1). Morphologically, *F. chiisanensis* is clearly distinguished from related taxa by the presence of panicle from leafless lateral bud of previous year, apetalous flower, persistent calyx, and brownish naked bud (Min *et al.* 2001; Chang *et al.*, 2002). Based on the results of nuclear ribosomal DNA ITS (Wallender and Albert, 2000), foliar flavonoids (Min *et al.*, 2001; Chang *et al.*, 2002), and morphology (Chang *et al.*, 2002), *F. chiisanensis* is included into subgen. *Fraxinus*, sect. *Melioides*, which is mainly distributed in North America. This species seems to be a highly primitive species within the section as indicated by no petal and androdioecious flower with calyx. *F. chiisanensis* has no related extant species within the section in eastern Asia (Chang *et al.*, 2002).

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F. chiisanensis is morphologically androdioecious, but it remains unknown whether it is functionally androdioecious. This species is presumed to be pollinated by wind, because it is inferred that most apetalous taxa of *Fraxinus* are wind pollinated based on the results of other studies (Wallender, 2001; Dommee *et al.*, 1999; Ishida and Hiura, 1998; Ishida and Hiura, 2002). The germination percent of seeds is ca. 50-60% and decreases rapidly to 15% after two years under laboratory conditions (unpublished data). In general, germination percents of *F. rhynchophylla* and *F. mandshurica* range from 20 to 50% (Oh *et al.*, 1991; Chung *et al.*, 1984). Therefore, it is assumed that germinations occur at similar rates within the genus.

Decline in genetic variation due to recent population bottlenecks in wind pollinated species is of great concern to conservation biologists (Hedrick and Miller, 1992; Lacy, 1997; Matocq and Villablanca, 2001), because it is expected to have destructive effects since such reductions are generally accompanied by inbreeding (Lacy, 1997). In addition, many narrowly endemic taxa persist for long periods in small, naturally isolated populations (Ellstrand and Elam, 1993; Holsinger, 1993; Wolf *et al.*, 2000). Especially genetic impoverishment is expected to increase the risk of local extinction in these small pop-

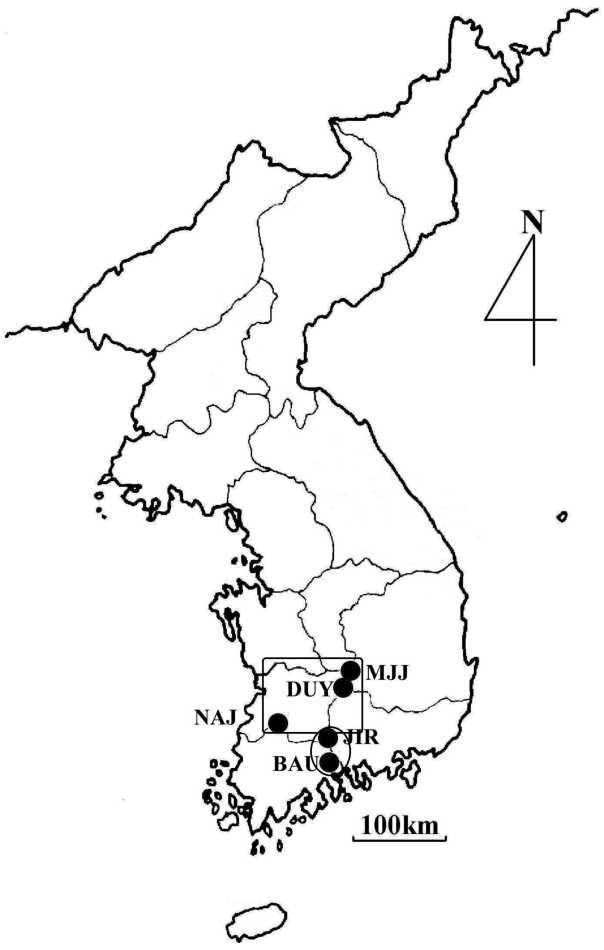

Figure 1. Locations of five populations of *F. chiisanensis* in Korea from which samples were collected for electrophoretic analysis.

ulations (Hanski and Gilpin, 1991). Diminished genetic variation between populations reduces the opportunity for adaptive responses to geographically varying local conditions. The utility of the variation depends upon its likely importance for current and future adaptation, and therefore its contribution to the viability of populations (Sherwin and Moritz, 2000).

This study was conducted to elucidate population genetic structure underlying its reproductive and genetic diversity, which helped provide insight into conservation of this taxon. We used protein electrophoresis to address several questions about *Fraxinus chiisanensis* populations: 1) What proportion of the species' genetic variation was preserved within local populations? 2) How were genetic relationships among populations affected by androdioecy on breeding pattern in natural population?

Materials and Methods

1. Field sampling

Distribution of the species is reasonably well known as five restricted localities in Korea (Figure 1), with populations ranging in size from <100 individuals in Mt. Nae-jang-san to > 10,000 individuals in the largest populations, Mts. Ji-ri-san and Min-Ju-Ji-san. Because all populations are located in mountain stream side as one of dominant species in the forest, we could easily estimate population size, and male frequency (Table 1).

For the allozyme analyses, branch samples with buds and young leaves randomly were collected from five known populations, Mt. Ji-ri-san (JIR, 50), Mt. Duk-yu-san (DUY, 50), Mt. Min-ju-ji-san (MJJ, 50), Mt. Baek-un-san (BAU, 50), and Mt. Nae-jang-san (NAJ, 29) in mid April of 2001 and 2002 (Figure 1). Sampled trees were widely spaced more than 50 m in order to avoid colleting closely related individuals.

2. Laboratory analyses

Leaves were transported on ice to the laboratory, where they were refrigerated at 4°C. For the extraction of enzymes, leaves were cut into 3 cm^2 pieces and ground with extracting buffer (Wendel and Weeden, 1989) using ceramic mortars and pestles. The homogenized extracts were absorbed into filter paper (Whatman 3 mm, 4 mm×10 mm), frozen at -70°C, and electrophoresed for 4-6 h on four gel and buffer systems using 12% starch gels (Conkle *et al.*, 1982; Wendel and Weeden, 1989). The following enzyme systems were assayed in all individuals in all populations: aspartate aminotransferase (AAP), acid phosphatase (ACP), catalase (CAT), glutamine dehydrogenase (GDH), isocitrate dehydrogenase (IDH), leucine amino peptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), mena-

Table 1. Occupation size, population size, and male frequency in five populations of *F. chiisanensis*.

Populations	Occupation size	No of Individuals	Male tree/ha	Hermaphrodite	Male frequency
Mt. Ji-ri-san (JIR)	> 100 ha	> 10,000	42	5	89.4%
Mt. Baek-un-san (BAU)	< 1 ha	< 500	-----[a]	-----	-----
Mt. Min-ju-ji-san (MJJ)	< 10 ha	< 5,000	34	6	85.0%
Mt. Nae-jang-san (NAJ)	< 1 ha	< 100	-----	-----	-----
Mt. Duk-yu-san (DUY)	> 30 ha	> 5,000	23	6	79.3%

[a]All individuals are juvenile.

dione reductase (MNR), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PGD), and shikimate dehydrogenase (SKDH). Enzymes were visualized using staining methods detailed by Wendel and Weeden (1989). Most staining recipes were modifications of the widely used assays presented in earlier manual (Conkle *et al.*, 1982; Soltis *et al.*, 1983). Loci were considered putative because no genetic analyses have 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 have been carried out (Wendel and Weeden, 1989; Kepart, 1990).

3. Statistical analyses

Allele frequencies for each locus in each populations and genetic structure of the entire set of populations were analyzed using BIOSYS-1 (Swofford and Selander, 1981). The following standard measures of genetic variation were calculated at the species level: number of alleles per locus (A), percent of polymorphic loci (P), and observed (H_o) and expected (H_e) heterozygosity. A goodness-of-fit test of genotypic frequencies to Hardy-Weinberg equilibrium was performed on each variable locus. Population structure was analyzed using Weir and Cockerham's (1984) F-statistics by Genepop (Raymond and Rousset, 1995), where F_{IT} represents the overall inbreeding coefficient, and F_{IS} and F_{ST}, the levels of inbreeding due to nonrandom mating within populations and population subdivision, respectively. Interpopulation gene flow was estimated by the equation, $Nm=(1-F_{ST})/4F_{ST}$ (Wright, 1951). A dendrogram based on the genetic similarity was created using UPGMA (unweighted pair group method). Genetic divergence among populations was estimated by calculating Nei's genetic distance (D) for all pairs of populations (Nei, 1973). Nei's genetic distance measure ranges from 0 for population with identical allele frequencies, infinity for populations with no alleles in common, to unity for populations with identical allele frequencies. Analyses of covariance and correlation were performed to examine the relationship between population and occupation sizes, and the various measures of genetic variation.

Results

Genetic diversity: The 13 enzyme systems examined resulted in the interpretation of 15 putative loci, of which, four (P_s = 26.7%) were polymorphic across the range of *F. chiisanensis*. At the population level, polymorphism was 22.7% (P_p) on average (Table 2). Values of P_p ranged from 13.3% (DUY) to 26.7% (BAU and JIR). Only four loci (ACP-1, LAP-1, MNR-2 and CAT-1) were polymorphic in at least one population. The mean numbers of alleles per polymorphic locus were 2.50 and 2.00 at the species and population levels, respectively. Including the 11 monomorphic loci, the average numbers of alleles per locus were 1.33 and 1.27 at the species (A_s) and population levels (A_p), respectively. One of the 20 alleles (MNR-2c), which occurred at a low frequency (< 0.02), was unique to a single population (=NAJ, private alleles; Table 3). Expected heterozygosity (=H_e) for the species was somewhat low

Table 3. Allele frequencies of polymorphic loci in *F. chiisanensis*.

	Population				
Locus	JIR	BAU	MJJ	NAJ	DUY
ACP-1					
1	0.210	0.070	0.060	0.000	0.000
2	0.430	0.480	0.780	0.621	0.870
3	0.360	0.470	0.160	0.379	0.130
LAP-1					
1	0.440	0.420	1.000	1.000	1.000
2	0.560	0.580	0	0	0
MNR-2					
1	0.010	0.060	0.260	0.724	1.000
2	0.990	0.940	0.740	0.259	0
3	0	0	0	0.017	0
CAT-1					
1	0.440	0.170	0.140	0.328	0.390
2	0.560	0.830	0.860	0.672	0.610

Table 2. Genetic variability at four polymorphic loci and mean identity values for five populations of *F. chiisanensis*. Abbreviations: *N*, number of individuals sampled; *P*, percent polymorphic loci; *A*, mean number of allele per locus; H_0, observed heterozygosity; H_e, expected heterozygosity.

Populations	N	A	P	H_0	H_e
Mt. Ji-ri-san (JIR)	50	1.3	26.7	0.650	0.111
Mt. Baek-un-san (BAU),	50	1.3	26.7	0.510	0.097
Mt. Min-ju-ji-san (MJJ)	50	1.3	20.0	0.045	0.067
Mt. Nae-jang-san (NAJ)	29	1.3	20.0	0.041	0.090
Mt. Duk-yu-san (DUY)	50	1.1	13.3	0.019	0.047
Mean	45.8	1.26	21.3	0.253	0.0.82

(0.082) (Table 2). When compared with other sites, levels of genetic diversity (*A, P,* and H_e) were high in JIR and BAU.

Population size and sex ratio: Among the extent populations, the frequency of flowering on males and hermaphrodites from three populations in 2004 were observed and summarized in Table 1. Only less than 20% of hermaphrodites were observed whereas the frequencies of male plants were dominant ranging from 79.3% to 89.4%. This data indicated a significant deviation from 1:1 in the frequencies of the male vs. hermaphrodite.

Distribution of genetic variability among populations: More than 31.4% of the genetic variation in our sample could be attributed to variation among populations (F_{ST} =0.356). F_{IT}, which reflected inbreeding within populations (for five populations) and population structuring, indicated an overall deficiency of heterozygotes at all four polymorphic loci. The inbreeding coefficient, F_{IS} (0.457), for four polymorphic loci were positive (Table 4), reflecting that observed levels of heterozygosity within populations (H_S) were lower than would have been expected if each plant were the product of random sexual reproduction (Wright, 1965). This, of course, is unlikely, given the known ability of this species, due to the outcrossing system. The values of F_{IS} are indicative of inbreeding within populations. Although all estimates were not significant at 95% confidence level, the high values of F_{IT} and F_{ST} (Table 4) indicate low levels of gene flow among populations (Hamrick and Godt, 1989). Indirect estimates of gene flow indicated low levels of migration among *F. chiisanensis* populations. Application of Wright's (1951) model gave an estimate of N_m=0.57. This estimates of N_m<1 suggested that gene flow between populations was inadequate to counter the effects of genetic drift in local populations.

A UPGMA dendrogram illustrating genetic relationships among populations (Figure 2) reflected straightforward spatial configuration of populations. Mean genetic identity (*I*) among pairs of populations, on the other hand, was generally high (mean=0.958; SD=0.027), suggesting that small number of alleles contributed significantly to the among-population genetic variation. Results revealed, among all pairs of populations, genetic distances between populations were not associated with geographic distances (r_s=0.147, p<0.001) (Figure 3, Table 5).

Two UPGMA clustering solutions were identified. The first group included population from southern populations (JIR and BAU). The second group comprised three populations, two from large and small populations (DUY, NAJ) and one from a large one (MJJ). Significant regional variation was found in two parameters examined. Populations in southern mountains had significantly more polymorphic loci (*P*=26.7%) than those in northern mountains (*P*=17.8%). Expected heterozygosity also exhibited regional variation. The more amount of genetic diversity was found in southern (0.104) followed by northern mountains (0.068).

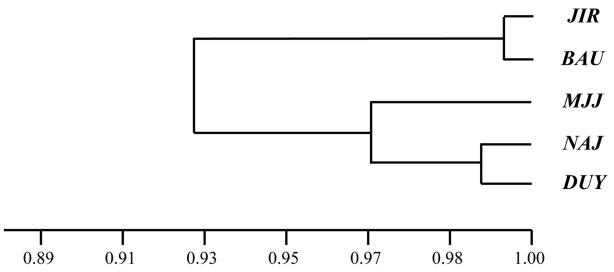

Figure 2. A UPGMA phenogram of Nei's genetic identity coefficient for five *F. chiisanensis* populations sampled.

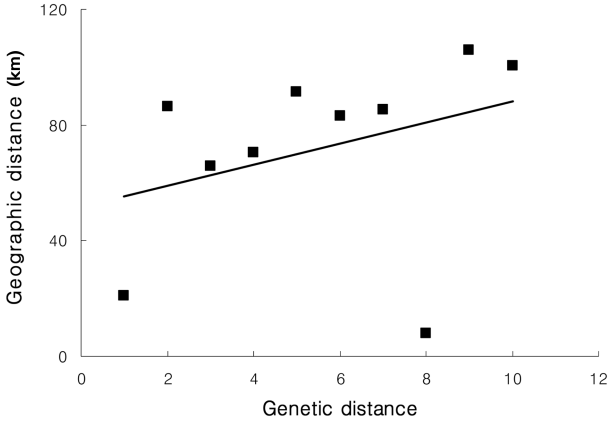

Figure 3. Plot of the genetic distance against geographic distance for all pairs of populations.

Table 5. Nei's (1978) genetic distance (lower triangle) and geographic distances (km, upper triangle) among five populations of *F. chiisanensis*.

Population	JIR	BAU	MJJ	DUY	NAJ
JIR	-----	20.8	86.3	65.9	70.4
BAU	.006	-----	91.3	83.3	85.4
MJJ	.040	.033	-----	7.9	106.0
DUY	.075	.081	.036	-----	100.4
NAJ	.123	.133	.085	.009	-----

Table 4. *F*-statistics were estimated as in Weir and Cockerham (1984) to calculate overall inbreeding coefficient, F_{IS} the levels of inbreeding due to nonrandom mating within populations, and F_{ST} due to population subdivision.

Locus	No. of alleles	F_{IS}	F_{IT}	F_{ST}
ACP-1	3	0.390	0.462	0.118
LAP-1	2	0.639	0.811	0.476
MNR-2	3	0.321	0.796	0.699
CAT-1	2	0.493	0.532	0.077
Mean	2.5	0.457	0.650	0.356

Discussion

Low genetic diversity and population differentiation: In general, endemic and rare taxa often have significantly lower genetic variability (Hamrick and Godt, 1989) and higher rates of self-pollination and inbreeding (Inoue and Kawahara, 1990; Linhart and Premoli, 1993). Given its endemism and history of fragmentation, *F. chiisanensis* supports the general rule of lower genetic variability in rare taxa. Species-level H_e for *F. chiisanensis* was lower than means reported by Hamrick and Godt (1989) in 81 endemic (0.096) and 101 narrowly distributed (0.137) taxa. The distribution pattern of allozyme variation in *F. chiisanensis* included low genetic variation within populations and pronounced divergence among populations, consistent with the prediction for the effects of limited gene flow among small populations that have been separated for a long time.

Genetic diversity and current population sizes indicated that size of JIR and DUY populations of *F. chiisanensis* was substantially larger than today. The low correlation between geographic and genetic distances might be explained through the result of genetic drift (Figure 3). It is possible that some of these cases of low genetic diversity are due to recent demographic crashes (Matocq and Villablanca, 2001) in some populations. In this case, small effective size of populations would have increased genetic drift and led to the loss of allelic diversity with random fixation of alleles (Gaudeul *et al,* 2000). Probably due to local extinctions and/or bottlenecks, this species might have been squeezed out of many areas and finally restricted to isolated stands at current five populations. Especially NAJ and BAU populations have been severely threatened. Until 70's timber harvest had been common on this area, but we have not assessed the extent of damage, if any, to the *F. chiisanensis*. Patterns of low genetic diversity on these two sites could be interpreted as reflecting a recent reduction in diversity, or a historical lack of diversity. Habitat destruction were obvious, reduced effective populations size also had caused detrimental effects on the demography, because we could not find any flowering adults but only juvenile suckers at these stands.

The results shown in Table 5 demonstrate that there is strong differentiation between the studied populations and their respective complements. Decreasing population size and stronger isolation from larger populations to marginal populations go along with loss of genetic variability (genetic drift) and increasing genetic distance. This would also agree with the results from Höltken *et al.* (2003) and Þvingila *et al.* (2005). This is corroborated by the summarizing analysis of F_{ST}, particularly in comparison with the results obtained from studies of Heuertz *et al.* (2001) on ash in Bulgaria (F_{ST}=0.087) and Höltken *et al.* (2003) on ash in South Finland (F_{ST}= 0.123). Our F_{ST} estimate (F_{ST}=0.356) tends to indicate higher degrees of average.

F. chiisanensis appeared to consist of two geographically isolated groups. The possible scenario for the present occurrence was that these two groups might have served as sources for the colonization of regions independently. Some populations may be old centers, and other could have been originated through dispersal.

Breeding system and high inbreeding coefficient: Breeding system has been shown to be one of the most important factors of life history in explaining the distribution of allozyme variation within and among populations (Hamrick and Godt, 1996). Androdioecy is a rare breeding system in which male plants coexist with hermaphrodite plants (Ishida and Hiura, 2002). All androdioecious species show variations in male frequency among populations, and self-compatibility (Akimoto *et al.*, 1999; Ishida and Hiura, 2002). It is generally known that the frequencies of males were consistently lower than those of hermaphrodite in most androdioecious populations, but a common feature in the androdioecious *Fraxinus* species was high male frequencies. For example, *F. ornus* and *F. longicuspis*, males were either as frequent as or more frequent than hermaphrodite (Pannell, 2002). With wind pollination, the concentration of pollen grains decreases rapidly with distance from the plant, making outcrossing unlikely (Allison, 1990).

Several previous studies on the mating system of *Fraxinus* (Dommée *et al.*, 1999; Ishida and Hiura, 2002) showed that outcrossing rate of *Fraxinus* increased with the number of male frequency and hermaphrodites produced viable pollen in dehiscent anthers and viable seeds as self-compatibility, although hermaphrodites function as females under natural conditions, and that populations function as a dioecious species (Charlesworth, 1984; Pannell, 2002). Partially self-fertilized hermaphroditic species under low male frequency is known as a facultative selfing phenomenon. Ishida and Hiura (2002)

Table 6. Fixation indices (*F*) for four polymorphic loci within the population level. Chi-square tests were used to determine if fixation indices were different from an expected values (*F*=0).

Locus	Population				
	JIR	BAU	MJJ	NAJ	DUY
ACP-1	0.376	0.324*	0.227	0.707**	0.381*
LAP-1	0.675**	0.589**	-----[a]	-----	-----
MNR-2	-0.010	0.291	0.272	0.409*	-----
CAT-1	0.188	0.646**	0.502*	0.452*	0.706**

[a]Populations that were monomorphic for a particular locus are indicated with a dash; *<0.05; **<0.01Legends of figures

insisted that these androdioecious species appeared to have a density-dependent outcrossing rate. In our study high male frequency data of *F. chiisanensis* showed asymmetric sex ratio in natural population (Table 1). In spite of high male frequency, however, *F. chiisanensis* showed high inbreeding coefficient. Decline in local abundances and positive fixation indices found in these populations suggested, in time, negative consequences of isolation and small size became apparent in the remnant populations (Table 6). Forest fragmentation have reduced the size and increased the spatial isolation of plant population. Therefore, this might result in a severe increase of unequal sex-ratio within the population.

Heterozygote deficiency: Heuertz *et al.* (2001)'s spatial genetic structure analyses showed that estimated neighborhood size of *F. excelsior* in Bulgaria ranged from 38 to 126 individual trees. That is, most mating events become sib-mating over time, unless significant gene flow by pollen or seeds increases local gene pools. Heterozygote deficiency of *F. chiisanensis* might be due to inevitable mating between relatives in small populations. The deficiency of heterozygotes led to partially higher estimates of F_{IS} for some populations, which could be explained by a high rate of inbreeding, restricted seed and moderate pollen dispersal (Heuretz *et al.*, 2003) or Wahlund effect due to fine scale genetic structuring (Hebel *et al.*, 2006). As a first reason for the excess of homozygotes, we can expect inbreeding, due to isolated nature of *F. chiisanensis* and the limited gene flow. The most current populations may have been characterized as small effective population size, and also limited potential for dispersal of *F. chiisanensis* was likely to contribute to the low rates of gene flow within as well as between populations. The relatively low genetic diversity and heterozygote deficiency documented for *F. chiisanensis* are most likely a result of habitat fragmentation (Young *et al.*, 1996). The forest areas necessary for the survival of this species have been destroyed and disturbed by anthropogenic activity during the past century. As a result, the numbers and sizes of extant *F. chiisanensis* populations have decreased greatly, which in turn has led to the loss of genetic diversity and alteration of population genetic structure (Ellstand and Elam, 1993). Overall, the low allozyme diversity documented for this species is likely a result of habitat fragmentation followed by genetic drift and limited gene flow among small populations.

Conclusion

To avoid the harmful effects of inbreeding, conservation management could aim at maintaining or re-establishing high tree density within large populations prior to severe fragmentation. We may have to manage population size instead of gene diversity (Sherwin and Moritz, 2000) for this species. In conclusion, because of limited flowering individuals at the shrinking populations, pollen transfer between related trees within the populations seemed to be common in *F. chiisanensis*. Further population genetic analysis combined with ongoing reproductive biology studies will be expected to provide valuable additional data to augment the present conclusions.

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